Characterization of the Membrane Binding and Fusion Events during Nuclear Envelope Assembly Using Purified Components

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Abstract. At the end of mitosis membrane vesicles are targeted to the surface of chromatin and fuse to form a continuous nuclear envelope. To investigate the molecular mechanisms underlying these steps in nuclear envelope assembly, we have developed a defined cell-free system in which the binding and fusion steps in nuclear envelope assembly can be examined separately. We have found that extensively boiled *Xenopus* egg extracts efficiently promote the decondensation of demembranated *Xenopus* sperm chromatin. When isolated membranes are added to this decondensed chromatin a specific subfraction of membrane vesicles (≈70 nM in diameter) bind to the chromatin, but these vesicles do not fuse to each other. Vesicle binding is independent of ATP and insensitive to *N*-ethylmalamide. Quantitative analysis of these sites by EM suggests that there is at least one vesicle binding site per 100 kb of chromosomal DNA. We show by tryptic digestion that vesicle–chromatin association requires proteins on both the vesicle and on the chromatin. In addition, we show that the vesicles bound under these conditions will fuse into an intact nuclear envelope when incubated with the soluble fraction of a *Xenopus* egg nuclear assembly extract. With respect to vesicle fusion, we have found that vesicles prebound to chromatin will fuse to each other when ATP and GTP are present in the boiled extract. These results indicate that nuclear envelope assembly is mediated by a subset of ≈70-nM-diam vesicles which bind to chromatin sites spaced 100 kb apart and that fusion of these vesicles is regulated by membrane-associated GTP-binding proteins.

At the beginning of mitosis in many eukaryotic cells the membrane component of the nuclear envelope vesicularizes and dissociates from the chromatin. At the end of mitosis these dissociated vesicles must bind back to the surface of the chromatin and then fuse with each other to reform an intact nuclear envelope composed of two membrane bilayers. Using a cell-free system made from *Xenopus* eggs which can assembly nuclei around added chromatin (Lohka and Masui, 1983; Newport, 1987; Sheehan et al., 1988) we have shown that targeting of vesicles back to the surface of chromatin during envelope assembly is mediated by a vesicle-bound receptor (Wilson and Newport, 1988; Pfaller et al., 1991). We have also shown that the association of vesicles with chromatin is regulated by phosphorylation (Pfaller et al., 1991). When an unknown membrane component is phosphorylated this inhibits association of vesicles with chromatin, and when dephosphorylated it allows vesicles to bind chromatin. Both the membrane receptor and the chromatin component recognized by the receptor have yet to be identified definitively. In mammalian cells it has been proposed that targeting and binding of membrane to chromatin during envelope assembly involves the interaction of chromatin-bound lamins A and C with membrane vesicle-bound lamin B (Gerace and Blobel, 1980; Burke and Gerace, 1986). In support of this it has been shown that lamins A and C will associate directly with the surface of condensed chromosomes (Burke, 1990; Glass and Gerace, 1990), and that depletion of lamins A and C from a CHO cell nuclear assembly cell-free system inhibits envelope assembly 60% (Burke and Gerace, 1986). However, the role of lamins in the assembly of the nuclear envelope in *Xenopus* extracts is somewhat less clear. To date, all studies indicate that *Xenopus* extracts contain a single lamin protein, L_{III}, which like mammalian lamins A and C is soluble rather than membrane associated (Krohne et al., 1981; Benavente et al., 1985; Krohne and Benavente, 1986; Stick, 1988). Furthermore, a quantitative depletion of soluble L_{III} from a *Xenopus* extract does not block formation of a nuclear envelope around added chromatin, although it does restrict the growth of the envelope which forms (Newport et al., 1990).

To further investigate the components involved in nuclear envelope assembly we have developed a system which allows us to study the interaction of membrane vesicles with chromatin under simple, well-controlled conditions. In particular, we have developed conditions in which isolated membrane vesicles bind in a specific and regulated manner to chromatin in the absence of almost all soluble proteins. Using this system we have; (*a*) characterized the nature and number of chromatin sites that bind to membrane vesicles, (*b*) characterized the size of the vesicles that bind to these

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sites, (*c*) determined the nucleotide requirements for binding, and (*d*) determined the nucleotide requirements for fusion of these bound vesicles to each other.

Materials and Methods

Demembranated *Xenopus* sperm chromatin was isolated from frog testis, and stored frozen until use as described by Wilson and Newport (1988). Membrane fractions and total soluble components were prepared from *Xenopus* extracts as described by Wilson and Newport (1988). Briefly, packed, dejellied, unfertilized *Xenopus* eggs were crushed by centrifugation for 10 min at 10,000 rpm in an HB-4 rotor. The cytosolic fraction was removed and centrifuged again for 2 h at 35,000 rpm in an SW50.1 rotor. The clear soluble fraction was removed with a pipetman centrifuged again for 20 min at 55,000 rpm in a TC100. To make a heat extract from this fraction the soluble material was heated for 5 min in a boiling water bath. After this, precipitated protein was removed by centrifugation for 10 min at 55,000 rpm in a TL100 centrifuge using a TLS55 rotor. Occasionally it was necessary to heat an extract for longer than 5 min in order to hydrolyze GTP and block fusion. The membrane fraction collected from the SW50.1 rotor spin represented a light fraction of membranes which is located just above the packed heavy membrane fraction at the lower end of the tube. This light membrane fraction was washed by resuspension in 30 vol of a buffer containing 0.5 M sucrose, 50 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, pH 7.5, 1 mM DTT, and 1 mM ATP. The resuspended membranes were then concentrated by pelleting them through a 0.5-ml cushion of 0.5 M sucrose in the same buffer for 20 min at 20,000 rpm. The final pellet was resuspended in a volume of 0.5 M sucrose buffer so that membranes were concentrated 10× compared to the initial crude extract. After this, 3–5-μl aliquots were frozen rapidly in liquid N_2 and stored until use at −80°C.

ATP Depletion

Heat extracts were depleted of ATP by dialyzing the extract for 12 h against 500 volumes of 50 mM Tris-HCl, pH 7.0, 1 mM MgCl, 50 mM KCl, and 1 mM DTT. Alternatively, the extract was depleted of ATP by preincubating it for 30 min at 23°C with 50 U/μl of hexokinase and 20 mM glucose.

Preparation of MPF

Partially purified MPF was prepared by ammonium sulfate fractionation of unfertilized eggs as described by Wu and Gerhart (1980) with the modifications described in Dunphy and Newport (1988). To convert the soluble component fraction of an extract to a mitotic state MPF was added to the soluble fraction at a 1:6 ratio. To observe dissociation of membrane from chromatin in mitotic extracts, decondensed sperm containing bound membranes was added at a concentration of 100 sperm/μl of mitotic extract (a 10-fold dilution of the sperm present in the heat extract). 3-μl samples were removed, added to 3 μl of buffer containing HOECHST and 3,3'-dihexyloxacarbocyanine (DECC), and then observed for membrane release by fluorescence microscopy.

Fusion of Prebound Membranes in the Soluble Fraction

To observe fusion of prebound vesicles in the soluble fraction, sperm chromatin (1,000/μl) was incubated in heat extract containing membranes for 60 min. The sperm vesicle substrate was then separated from unbound membrane vesicles by centrifuging the sperm through a cushion of 1 M sucrose for 5 min at 500 *g*. The pellet was resuspended directly into the total soluble fraction at a concentration such that the sperm were diluted to 50/μl of soluble extract. This combination of centrifugation fractionation and subsequent dilution reduced the unbound membrane concentration present in the soluble extract to <1% of the original membrane concentration present during the binding step. Fusion of the membranes was monitored by adding fluorescently labeled dextran (Newmeyer et al., 1986) to aliquots removed at different times, and then determining by visual observation with a fluorescent microscope whether the dextran was able to pass through the forming envelope, or whether the envelope was intact, as indicated by the observation that the added dextran was excluded from entering the nucleus. Occasionally exclusion of rhodamine labeled phycoerythrin was used instead of dextran to follow formation of an intact envelope. DNA replication of chromatin substrates added to soluble extracts was monitored by measuring incorporation of p^{32}-αdCTP. After this the reaction was diluted with 3 vol of 50 mM Hepes, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and then centrifuged for 5 min in a centrifuge (Eppendorf Inc., Fremont, CA). The DNA pellet was resuspended, treated with proteinase K, phenol extracted, and then loaded on a 1% Agarose gel as described in Newport (1987).

Trypsin Digestion

Trypsin digestion of either decondensed sperm or membranes was carried out as described by Wilson and Newport (1988).

EM

Samples were fixed in solution overnight in 3% glutaraldehyde, 100 mM Na phosphate, pH 7.0. They were then pelleted in a centrifuge (Eppendorf Inc.) for 5 min. The pellet was rinsed with 100 mM cacodylate (pH 7.4), stained for 1 h with 1% OsO_4 on ice, dehydrated, and embedded in Epon before sectioning. Sections were stained with uranyl acetate followed by lead citrate before observation.

Results

Extracts made from *Xenopus* eggs can be separated, by centrifugation, into several fractions including a fraction representing the soluble components present in the cytosol, and a fraction which contains the total membrane components initially present in the egg cytosol (Lohka and Masui, 1984; Newport, 1987). The membrane fraction can be further fractionated via sucrose block gradients to isolate a light membrane fraction (Wilson and Newport, 1988). When this light membrane fraction is combined with the soluble fraction, and a chromatin substrate such as demembranated sperm chromatin is added to the mixture, an intact nuclear envelope assembles around the chromatin substrate (Lohka and Masui, 1983; Forbes et al., 1983; Newport, 1987). If this same chromatin substrate is added to the soluble components in the absence of the membrane fraction the sperm chromatin decondenses but does not form a nuclear envelope (Lohka and Masui, 1984; Newport, 1987). Alternatively, we have found that when condensed chromatin is mixed directly with the purified membrane fraction, the sperm chromatin remains condensed and there is no direct contact between the chromatin and the membranes present in the mixture (results not shown).

The absence of an interaction between chromatin and purified membranes could be due to either the fact that; (*a*) such interactions require protein components present in the soluble fraction, or (*b*) that the chromatin sites that would interact with membrane are sterically blocked because of the highly condensed state of the DNA. To distinguish between these two possibilities, we developed conditions in which chromatin decondensation could be achieved in the absence of almost all of the proteins present in the soluble extract. When the soluble fraction of a *Xenopus* nuclear extract is heated at 100°C for 5 min almost all of the proteins precipitate (Earnshaw et al., 1980). The proteins that remain soluble after heat treatment include small amounts of proteins at 110, 55, and 48 kD and a large amount of the protein Nucleoplasmin at 33 kD (Laskey et al., 1978; Dilworth et al., 1987). Recently Laskey and co-workers have conclusively demonstrated that nucleoplasmin induces sperm chromatin to decondense (Philpott et al., 1991). Similarly, we

1. *Abbreviations used in this paper*: DECC, 3,3'-dihexyloxacarbocyanine; MPF, maturation-promoting factor; NEM, *N*-ethylmalamide.

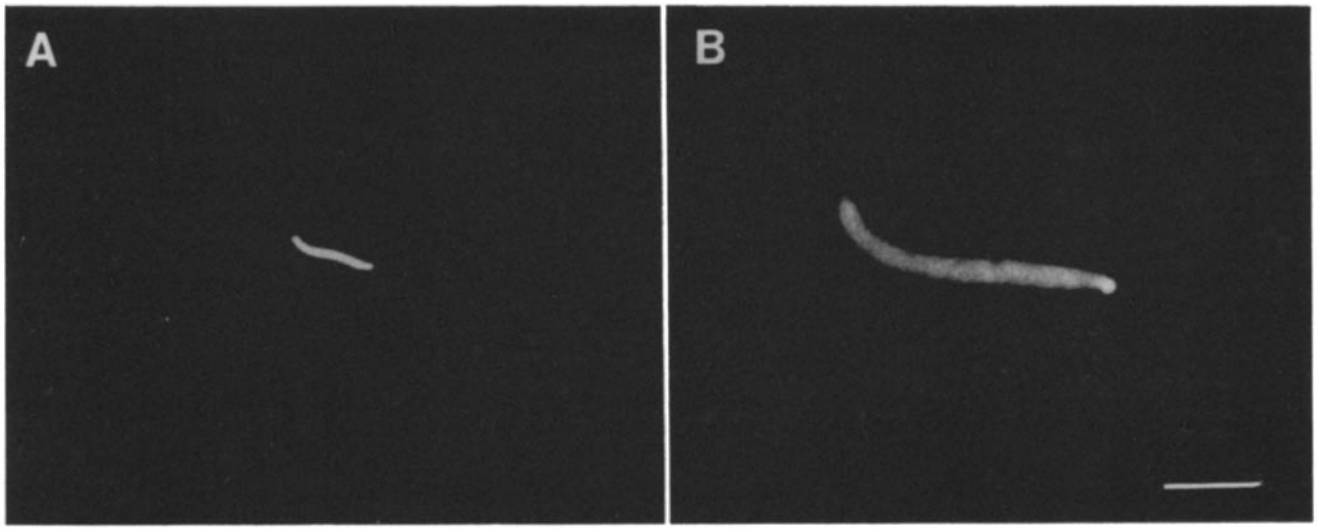

Figure 1. Decondensation of sperm chromatin in a heat-treated extract. The soluble fraction of a nuclear assembly extract was incubated for 5 min in 100°C water bath. After this, precipitated proteins were removed by centrifugation. The remaining soluble material is referred to as a heat-treated extract and contains primarily Nucleoplasmin. (*A*) Demembranated sperm chromatin before incubation in heat-treated extract. (*B*) After incubation in heat-treated extract. Both *A* and *B* were stained for DNA with HOESCHT and observed under a fluorescent microscope. Note that after incubation in heat extract the sperm chromatin is substantially decondensed. Bar, 10 μm.

have found that when condensed sperm chromatin is added to a heat treated extract highly enriched in nucleoplasmin the extract causes the sperm chromatin to decondense ~25–30-fold based on visual measurements of the chromatin volume before and after addition to the heat-treated extract (Fig. 1, *A* and *B*). The dimensions of the cylindrical sperm chromatin before addition of extract are 1 μm in diameter and 10 μm long, whereas after extract treatment they enlarge to ~3 μm in diameter and 30 μm in length. The decondensation process is extremely rapid occurring in <1 min at 23°C. It occurs in heat-treated extracts which have been either extensively dialyzed or treated with hexokinase (50 U/μl) and glucose (20 mM) to remove ATP and, therefore, does not require ATP hydrolysis. We find that the extent of decondensation decreases when the sperm concentration exceeds 3,000 sperm/μl of heat extract, and little visible decondensation of chromatin is observed at sperm concentrations above 20,000 sperm/μl of extract. Therefore, the decondensation process itself may result from the stoichiometric displacement of protamines by the acidic tail of Nucleoplasmin (Dingwall et al., 1987; Pfaller et al., 1991).

Using the heat extract to induce partial sperm chromatin decondensation we investigated whether such partial decondensation was sufficient to allow the purified membranes to bind to the surface of chromatin in the absence of the proteins present in the soluble fraction. For this purpose, demembranated sperm nuclei were incubated in heat extract for 20 min at room temperature and then an aliquot of the partially purified membrane fraction was added to this reaction. The binding of membrane to chromatin was monitored after staining the membrane with the hydrophilic fluorescent dye DECC. As shown in Fig. 2 (*D–F*), under these conditions membranes did indeed bind to and cover the entire surface of the partially decondensed sperm chromatin. The interaction of membrane with chromatin was apparent both by the increased membrane fluorescent staining present around sperm in reactions containing membranes versus reactions lacking membranes (Fig. 2, *C* and *F*), and by the appearance of a phase-dense boundary around chromatin in reactions containing membranes that was noticeably absent in reactions lacking membrane vesicles (Fig. 2, *B* and *E*). To determine if membrane binding required ATP heat extracts were depleted of ATP by either extensive dialysis against buffer (see Materials and Methods) or by pretreating the extract for 30 min with hexokinase (50 U/μl) and glucose (20 mM). We found that the density of membranes bound to chromatin in extracts lacking ATP was identical to the density of membranes bound to chromatin in extracts containing ATP. Based on these observations we conclude that chromatin decondensation exposes sites on chromatin that can interact with membrane vesicles and that this interaction is a spontaneous event not requiring ATP.

To investigate further the nature of the interaction of the membrane bound to chromatin, reactions containing decondensed chromatin with membrane bound were fixed and observed by EM. As shown in Fig. 3, sections of the chromatin–membrane substrate revealed that the chromatin template was surrounded by an ~0.5-μM-thick layer composed of numerous membrane vesicles. The majority of the bound vesicles appeared to stain darkly. Measurements of several hundred of these vesicles revealed that they were quite homogenous in size with an average diameter of 74 nM. As shown in Fig. 3 *B* many of the bound vesicles were in direct contact with chromatin. A second class of vesicles which was larger, heterogenous in size, and did not stain darkly, although present in the membrane preparation did not appear to interact with chromatin. These observations indicate that our partially purified membrane fraction contains a class of 74-nM-diam vesicles which can interact specifically with the decondensed chromatin in the absence of proteins present in the soluble portion of the nuclear assembly extract.

To determine how many vesicles were bound per sperm we counted the number of vesicles bound directly to the surface of the sperm chromatin in EM sections. With this value, the

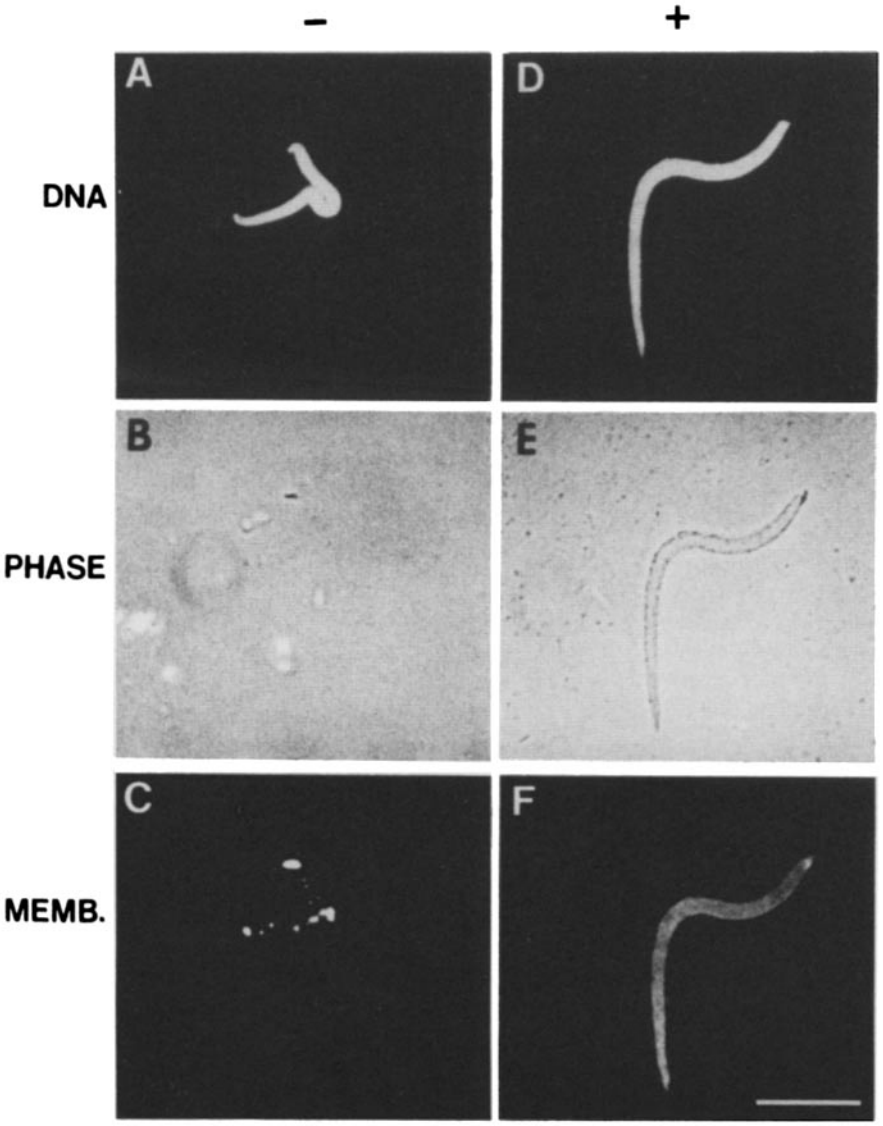

Figure 2. Membrane vesicles bind to sperm chromatin decondensed in heat extract. Sperm chromatin (1,000 sperm/μl of extract) was added to heat extract and allowed to decondense for 20 min. After this the extract was divided into 2 equal 25-μl aliquots. A 2.5-μl aliquot of partially purified membranes was added to one aliquot. After a further 20-min incubation the sperm were stained with HOESCHT and DECC to stain for DNA and membrane, respectively, and then observed in a fluorescent microscope under the appropriate excitation wavelengths. *A*, *B*, and *C* a sperm nucleus incubated in heat extract lacking membranes. *D*, *E*, and *F* a sperm nucleus incubated in heat extract containing membranes. Note the absence of membrane staining in *C* as compared with *F*. Also note the lack of a phase-dense boundary around the sperm in *B* as compared with *E*. Bar, 10 μm.

dimensions of a decondensed sperm, and the thickness of the EM sections (70 nM) we could calculate how many vesicles were bound per sperm. These calculations indicated that there were 2.8 ($\pm$2.0) $\times$ 10^4 vesicles bound per sperm. *Xenopus* sperm contains $\sim$2.8 $\times$ 10^9 bp of DNA; therefore, there is approximately one vesicle binding site on sperm chromatin every 100,000 bp of DNA. Because we do not know if all chromatin sites are occupied, this number represents a minimum estimate of the number of chromatin binding sites available to interact with the membrane vesicles. However, these results indicate that during envelope formation at the end of mitosis DNA can be in direct contact with the envelope as often as every 100,000 bp.

Is the Chromatin–Membrane Interaction Specific?

The results presented above demonstrate that when sperm chromatin is induced to decondense, by addition to a heat-treated egg extract, it can interact with a subset of membrane vesicles isolated from eggs. Such interactions could be due to either specific or nonspecific interactions between the chromatin and the vesicles. For example, the observed chromatin–membrane interaction could be due to charge–charge interactions between chromatin and a subset of vesicles. Such nonspecific interactions may be irrelevant to nuclear envelope formation. Alternatively, the chromatin–membrane interactions observed could represent an intermediate step in the nuclear envelope assembly pathway. If the latter is true, then we would expect that the membranes associated with chromatin would, under the appropriate conditions, be able to fuse and form an intact double nuclear envelope. To determine if the vesicles bound to chromatin are capable of forming an envelope, we did the following. Sperm chromatin was decondensed by incubation in a heat-treated extract for 20 min. After this, partially purified membranes were added to the decondensed sperm and allowed to bind for 30 min. The chromatin with membranes bound was then separated from unbound membranes by gently centrifuging (500 *g* for 5 min) the chromatin through a 1-M sucrose cushion. This isolated chromatin–membrane substrate was then added to the soluble fraction of a nuclear assembly extract. Under these conditions we observed that the initially elongated

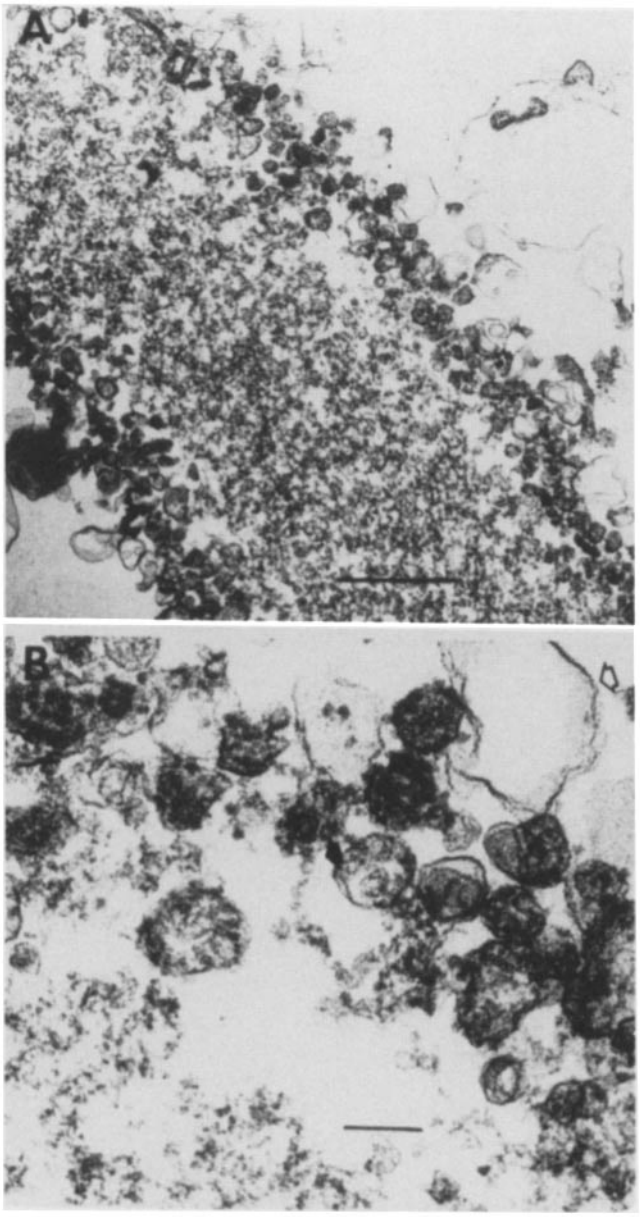

Figure 3. Electron micrographs of membrane vesicle binding to sperm chromatin. (*A*) Sample containing heat extract, sperm chromatin, and membranes was prepared as described in Fig. 2. The material was then fixed, sectioned, and stained as described in Materials and Methods. In *A* numerous small vesicles can be observed bound to the outer surface of the sperm chromatin. (*B*) High magnification photograph of vesicles bound to chromatin. Note that the small, uniformly sized, darkly staining vesicles appear to be in direct contact with chromatin (black filled arrow). Larger vesicles although present in the membrane preparation seldom make direct contact with the chromatin (open arrow). Bars: (*A*) 1 μM; (*B*) 0.1 μM.

chromatin–membrane substrate became spherical in shape and formed nuclei which were 3–5 μM in diameter (Fig. 4, *A–C*). To determine whether the vesicles initially bound to the chromatin had fused together to form a completely intact envelope under these conditions we added fluorescently labeled dextran (100 μg/ml) to the extract and determined whether the dextran was excluded from entering the nuclear interior (Newmeyer et al., 1986). We found by fluorescent microscopy that the dextran surrounded the nuclei but was unable to enter (Fig. 4 *E*), demonstrating that the vesicles initially bound to the chromatin had fused to form an intact nuclear envelope which excluded dextran. Previously we have demonstrated that an intact nuclear envelope is a prerequisite for the efficient replication of sperm chromatin added to *Xenopus* extracts (Newport, 1987; Sheehan et al., 1988; Newport et al., 1990). To determine if the nuclei formed from prebound vesicles were functional with respect to replication, we assayed for DNA replication by measuring incorporation of radiolabeled dCTP into DNA (see Materials and Methods). As shown in Fig. 4 *D*, sperm chromatin that was decondensed in heat extract in the absence of membranes did not act as a substrate for DNA replication when incubated in the soluble fraction of an assembly extract. By contrast, when isolated membrane–chromatin substrates were added to the soluble fraction of an assembly extract they did replicate efficiently. Taken together, the change in shape, the exclusion of dextran, and the activation of DNA replication demonstrate that the vesicles initially bound to the sperm chromatin in the heat extract are able to fuse together and form an intact functional nuclear envelope.

During mitosis the nuclear envelope in eukaryotes dissociates from the chromatin. Therefore, if the association between sperm chromatin and membrane vesicles is specific then we would expect that the addition of this complex to an extract that was in mitosis would cause membranes to be released from the chromatin. A nuclear assembly extract can be converted to mitosis by addition of MPF kinase (Miake-Lye et al., 1983; Miake-Lye and Kirschner, 1985; Dunphy and Newport, 1988). To test whether the membranes bound to chromatin in a heat extract would be released under mitotic conditions, we added isolated chromatin–membrane complexes assembled in a heat extract to the soluble fraction of an assembly extract that had been converted to mitosis by addition of MPF. As shown in Fig. 5 membrane vesicles that were initially bound to the chromatin in the heat-treated extract were released from the chromatin within 30 min after addition to a mitotic extract. Thus, the membranes that bind to sperm chromatin in a heat-treated extract appear to do so specifically by several criteria: (*a*) they are homogeneous in size and appear to represent a subset of the membranes vesicles present in the partially purified membrane fraction; (*b*) they can fuse to form an intact functional nuclear envelope; and (*c*) their association with chromatin appears regulated and is cell cycle dependent.

To determine if the binding of membrane vesicles to decondensed chromatin was mediated by the interaction between chromatin-bound and membrane-bound proteins, we examined how tryptic digestion of each of these substrates affected the binding process. To do this, the membrane fraction was first digested with trypsin (Wilson and Newport, 1988) followed by inactivation of the trypsin by addition of trypsin inhibitor. When these protease-treated membranes were added to a heat extract containing decondensed sperm chromatin we found that the digested membranes were now unable to bind to the chromatin. Similarly, we found that decondensed sperm chromatin digested with trypsin was unable to bind membrane vesicles (results not shown). These results

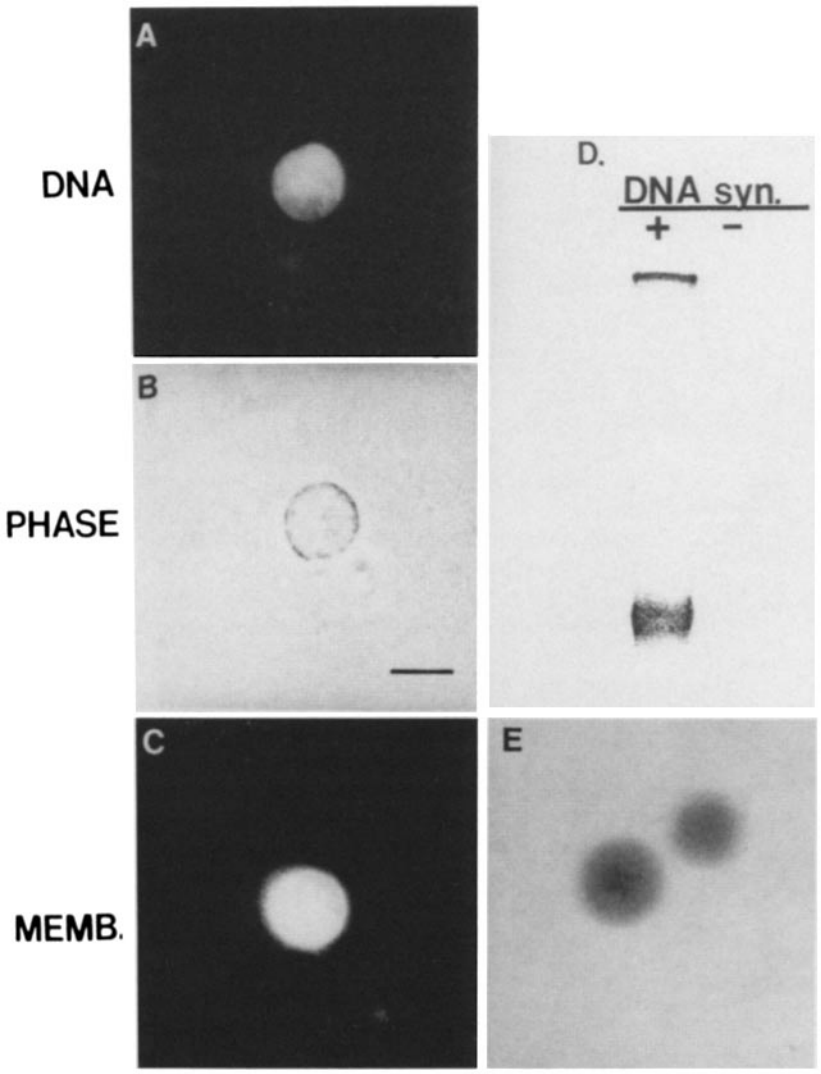

Figure 4. Prebound membrane vesicles fuse to form intact nuclear envelopes. Membranes were bound to decondensed sperm chromatin as described in Fig. 2. After this the vesicle-coated sperm chromatin was separated from unbound vesicles by gentle centrifugation through a 1-M sucrose cushion. The resuspended membrane–chromatin substrate was then added to the soluble components of a nuclear assembly extract and observed under the microscope. After 60 min the initially elongated sperm chromatin rearranged to form a sphere containing a smooth phase-dense boundary (*B*) and membrane (*C*). (*A*) DNA staining with HOESCHT; (*B*) same nucleus under phase optics; and (*C*) same nucleus stained for membrane with DECC. (*D*) Autoradiograph of radioactive dCTP incorporated into nuclei precoated with membranes (+) or lacking membranes (−). Note that extensive DNA replication occurs when chromatin is precoated with vesicles whereas little replication occurs on chromatin-lacking membranes. (*E*) Exclusion of fluorescently labeled dextran from nuclei demonstrating that the prebound vesicles fuse to form an intact envelope when incubated in the soluble fraction. Bar, 3 μM.

strongly suggest that the interaction between decondensed chromatin and membrane vesicles in a heat extract requires both chromatin-bound and membrane-bound proteins.

Fusion of Chromatin-bound Vesicles Is GTP Dependent

The results presented above show that a subclass of nuclear envelope membranes will bind to the decondensed chromatin present in a heat extract. However, we found that these bound vesicles would not fuse together in the absence of the soluble fraction of an assembly extract. This indicates that by boiling the extract we have either denatured and precipitated a protein(s) required for vesicle fusion, or destroyed some molecule needed for the fusion process. Recent evidence from numerous laboratories have demonstrated that the fusion events involved in both the endo- and exo-cytosis pathways involve GTP hydrolysis (Melançon et al., 1987; Goud et al., 1988; Orci et al., 1989; for review see Balch, 1990). Because GTP breaks down rapidly when heated, we investigated whether the inability of chromatin-bound vesicles to fuse in a heat extract was due to GTP degradation during preparation of the heat extract (5–10 min boiling). For this purpose, we supplemented the heat extract with exogenous GTP and then examined the membrane around the decondensed sperm chromatin. In the absence of GTP the chromatin-bound membrane vesicles appear to be loosely distributed in a thick layer surrounding the chromatin (Fig. 6 *A*). A similar staining pattern is observed when metaphase chromosomes, isolated from CHO cells, are added to a heat extract and membranes are allowed to bind (Fig. 6 *C*). However, within 5 min after the addition of GTP (50 μM) to such an extract, the attached vesicles had changed conformation and appeared to form continuous membrane regions along the surface of either the sperm chromatin or metaphase chromosome (Fig. 6 *B* and *D*). That the GTP-dependent change in membrane fluorescent staining pattern was indeed due to fusion of the chromatin-bound vesicles to each other was confirmed by fixing samples 20 min after the addition of GTP and then observing the sectioned samples by EM. As shown in Fig. 7 *A*, after addition of GTP to the extract the previously bound but unfused membrane vesicles (Figs. 3 *A* or 7 *B*) have fused together into large regions of double membrane attached to chromatin. These regions of double membrane ranged in size from 0.3 to 6 μm in length indicating that they are the result of from 10 to as many as 80 vesicles fusing together to form a double membrane region. To determine if GTP hydrolysis is required for vesicle fusion we

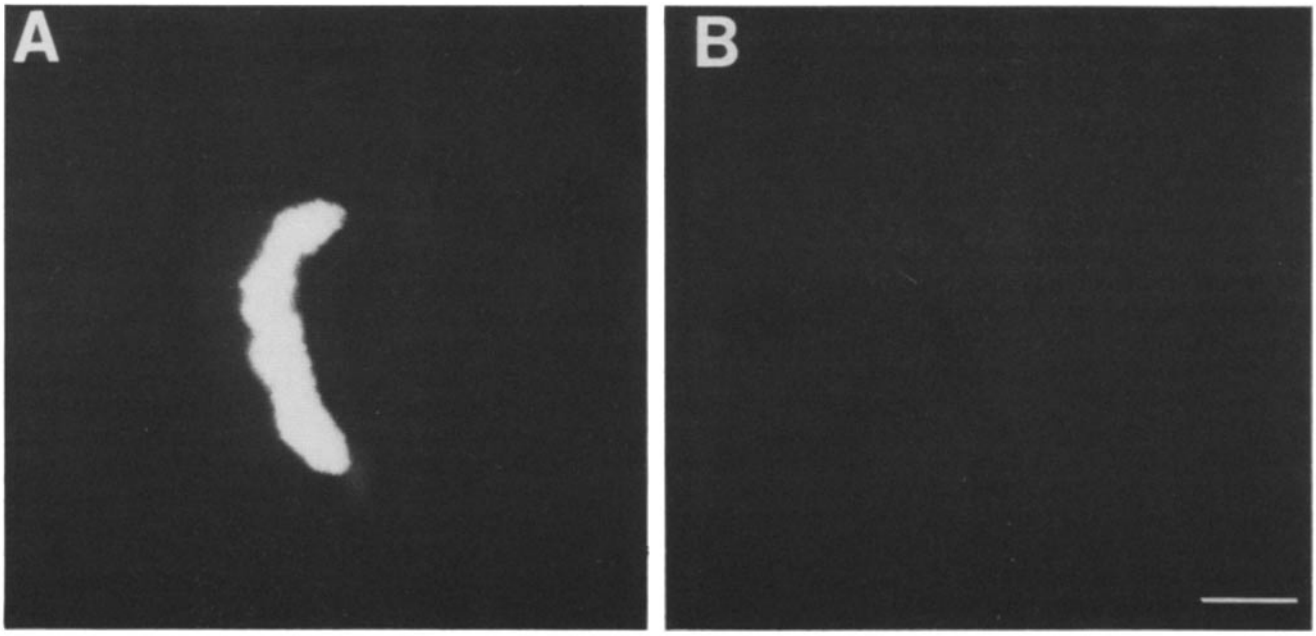

Figure 5. Vesicles dissociate from chromatin when incubated in mitotic extracts. Membrane vesicles were bound to decondensed sperm chromatin incubated in heat extract as described in Fig. 2. An aliquot of this material was then added to the soluble fraction of an egg extract which had been treated with MPF to induce it to enter mitosis (see Materials and Methods). After 30 min a sample of this mixture was removed and stained for DNA with HOESCHT (*A*) and for membrane with DECC (*B*), and examined under fluorescent illumination. The chromatin substrate was initially covered with membrane vesicles (Fig. 2 *F*). After a 30-min incubation in mitotic extract membrane vesicles had been quantitatively released from the chromatin (*B*). Bar, 10 μM.

investigated whether the nonhydrolyzable GTP analogue, GTPγS, blocked the fusion process. As shown in Fig. 7 *B*, when 50 μM GTP and 10 μM GTPγS are both present in a heat-treated extract, vesicles bind to the surface of chromatin but fusion is completely blocked. This result demonstrates that GTP hydrolysis is required for fusion of chromatin-bound vesicles.

To ask whether the fusion process also requires ATP, a

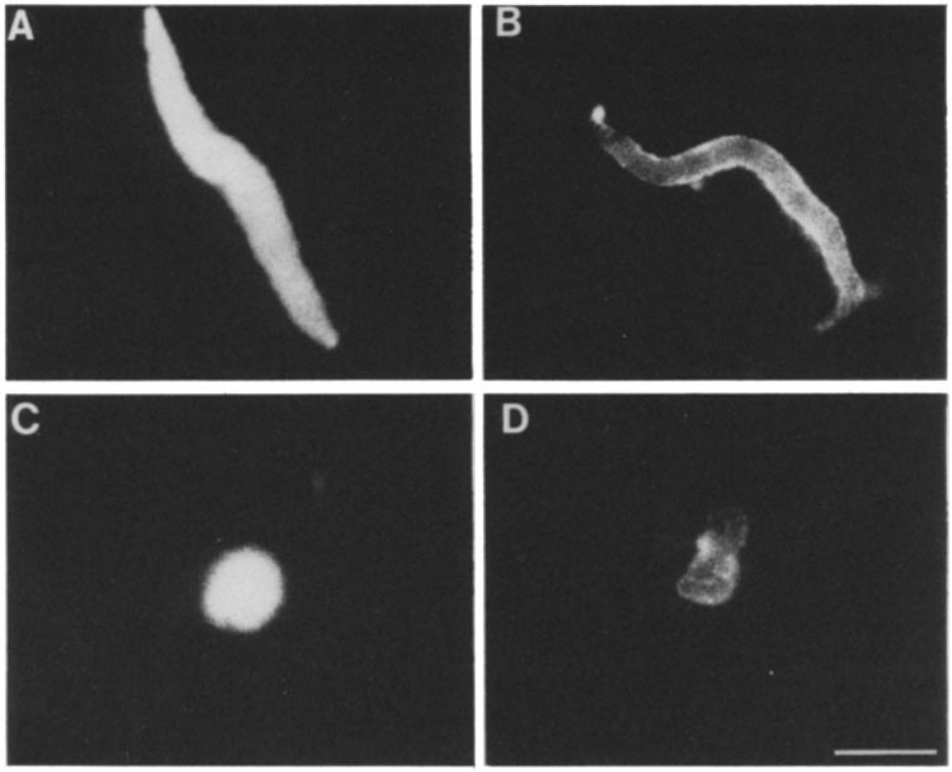

Figure 6. Chromatin-bound vesicles fuse in the presence of GTP. Sperm (*A* and *B*) or metaphase chromosomes (*C* and *D*) were added to a heat extract containing membranes. The chromatin was allowed to decondense and bind membrane for 40 min. When stained for membrane binding at this time the sperm chromatin (*A*) and metaphase chromosomes (*C*) appeared to be surrounded by a diffuse cloud of membrane. Within 20 min after addition of 50 μM GTP to such a sample the membrane staining rearranged such that the membrane was now located in close association with the periphery of the chromatin; sperm (*B*), mitotic chromosome (*D*). In both phase and fluorescent microscopy the membrane now appeared to be incorporated into a smooth continuous envelope. Bar, 10 μM.

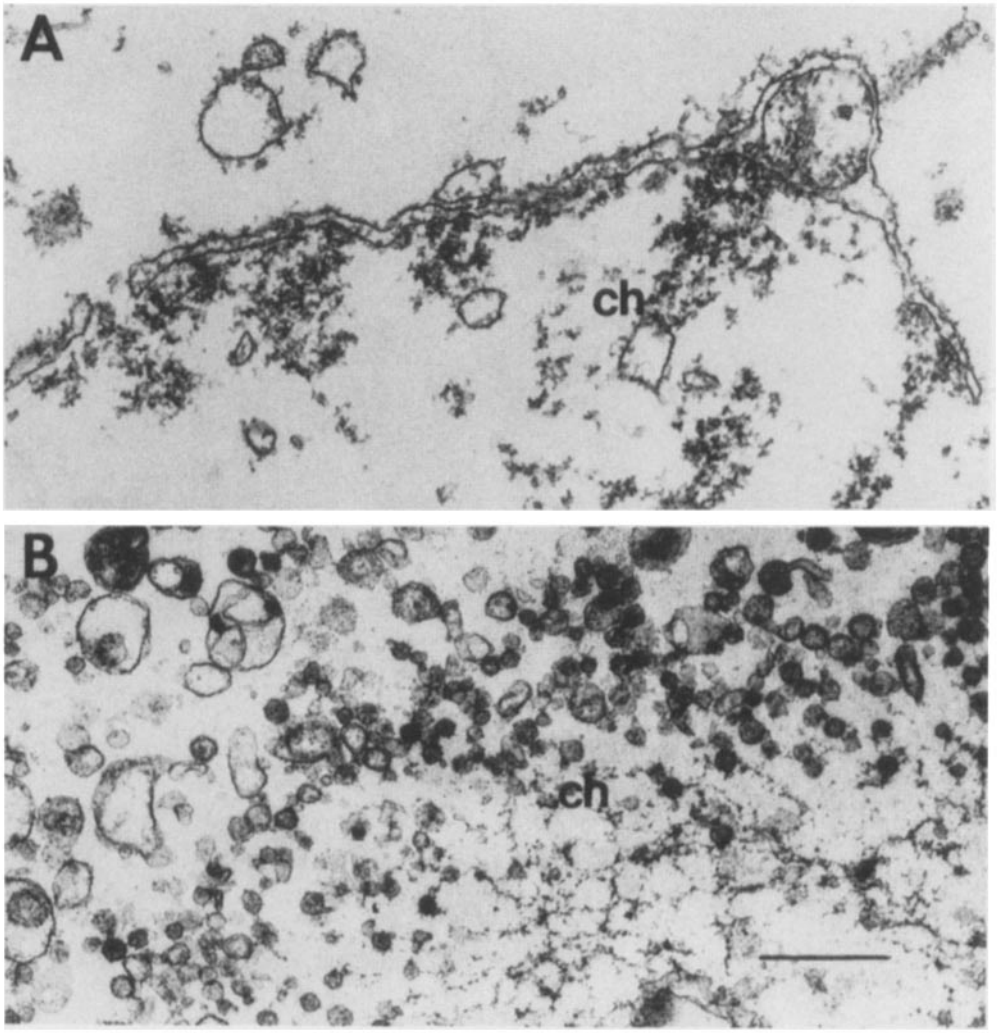

Figure 7. Regulation of vesicle fusion by GTP. Sperm chromatin was incubated with membranes in heat extract as described in Fig. 2. After this GTP (50 μM) was added and the extract incubated another 20 min before being fixed and prepared for electron microscopy. After addition of GTP the chromatin-bound vesicles fuse together (*A*) to form regions of continuous double membrane which is attached to chromatin. If γ-GTP (10 μM) is added at the same time as TGP (50 μM) vesicles remain bound to the chromatin but do not fuse to each other (*B*). Bar, 1 μM.

heat-treated extract was either dialyzed extensively or pretreated with a hexokinase and glucose ATP depletion system to remove ATP. As described above, both chromatin decondensation and vesicle binding continues in extracts depleted of ATP. We further found that when GTP alone was added back to an ATP-depleted heat extract containing chromatin and membrane vesicles, fusion did not occur. However, when both ATP and GTP were added, fusion occurred rapidly. Thus, while membrane binding requires neither ATP nor GTP, membrane fusion appears to require both these nucleoside triphosphates.

To further characterize the molecular components involved in the membrane binding and fusion reactions, the membrane fraction was treated with 5 mM *N*-ethylmalamide (NEM), a sulphydryl modifier, for 20 min and then the remaining NEM was quenched by addition of 10 mM DTT (Block et al., 1988; Malhotra et al., 1988). These NEM-treated membranes were then added to decondensed sperm chromatin in a heat-treated extract and examined for their ability to interact with chromatin via fluorescent microscopy. Results from this experiment demonstrated that NEM-treated membranes could bind to sperm chromatin as well as untreated membranes. Furthermore, when chromatin with NEM-treated membranes bound was isolated via centrifugation and added to a mitotic extract, the NEM-treated membranes were released from chromatin with the same kinetics as untreated membranes. Thus, it appears that NEM treatment of the membranes does not affect the proteins involved in binding of membranes to chromatin. By contrast, when GTP was added to a heat extract containing NEM-treated vesicles bound to chromatin, little if any change in membrane vesicle organization was apparent. Therefore, while the membrane proteins involved in chromatin binding are insensitive to NEM, at least one protein involved in vesicle–vesicle fusion is inactivated by NEM treatment.

Fusion of Nonchromatin-binding Membranes Vesicles

Previously we have shown that the partially purified membrane fraction described here contains both nuclear envelope membrane and endoplasmic reticulum (Wilson and Newport, 1988). We have also shown that the bulk of the endoplasmic reticulum membrane present in this fraction does not bind to chromatin. Above we have shown that the vesicles bound to chromatin require (*a*) ATP and GTP to fuse, (*b*) that this fusion occurs in the absence of soluble proteins, and (*c*) that fusion of these vesicles is NEM sensitive. To determine if the fusion properties of the bulk of the vesicles in our membrane fraction are similar to the chromatin-bound vesicles the following experiments were done. To determine the nucleotide requirements for fusion of these vesicles the

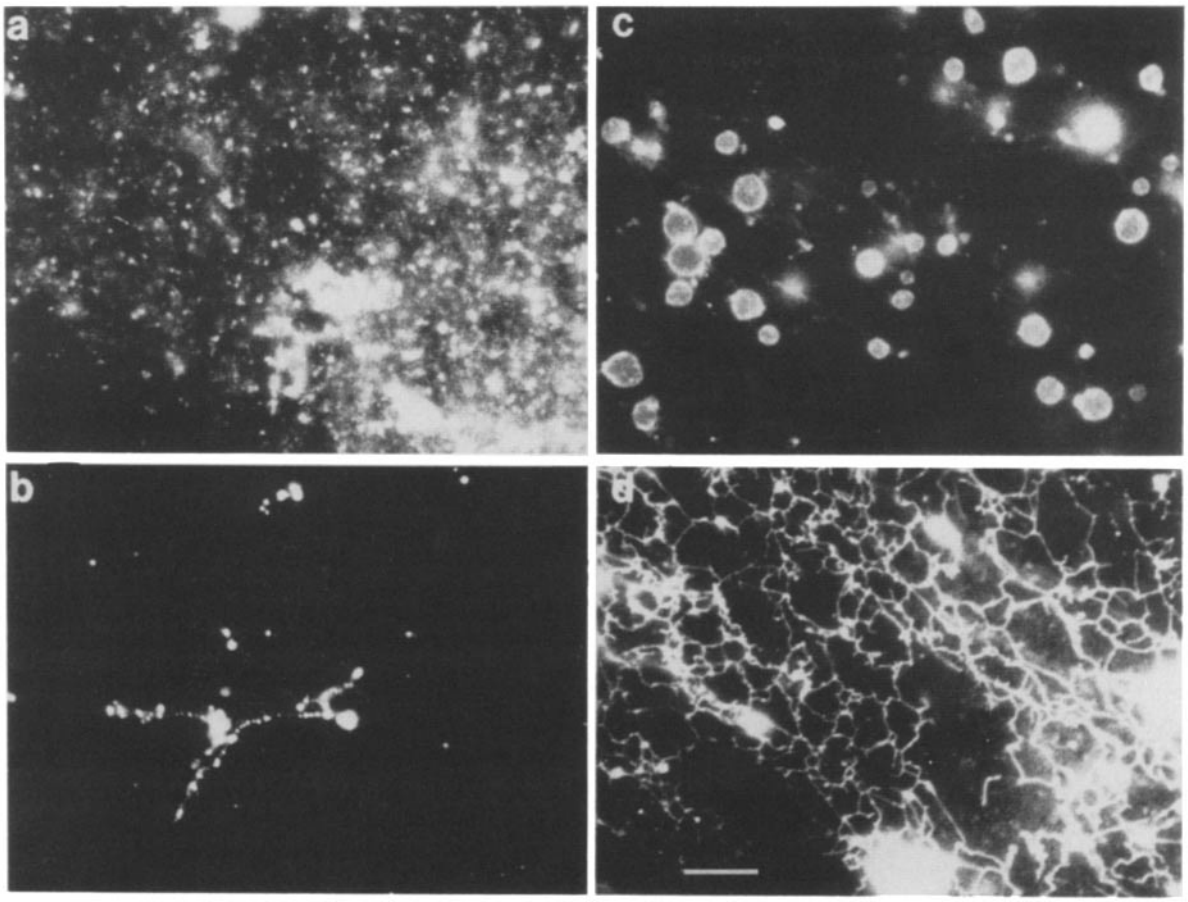

Figure 8. Fusion of nonchromatin-bound vesicles. Isolated membranes (1 μl) were added to 20 μl of a buffer containing 10 mM Hepes, pH 7.0, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT. Samples were removed, stained with DECC, and observed under a microscope at different times. Without added ATP (1 mM) and GTP (100 μM) membranes did not fuse (*a*). 5–10 min after addition of ATP and GTP vesicles aggregated to form linear assemblies made up from numerous associated vesicles (*b*). 30–60 min later many of these linear arrays had collapsed to form large vesicles (*c*). However, on many occasions these linear arrays appeared to be stable and formed an extensive network of interconnected tubules (*d*). Bar, 5 μm.

purified membranes were diluted 10-fold in a buffer containing 10 mM Hepes, pH 7.0, 50 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT. When these vesicles were stained with DECC and observed under the microscope little evidence for vesicle fusion was observed (Fig. 8 *a*). However, when both ATP (1 mM) and GTP (100 μM) were added to this reaction the vesicles rapidly (5–10 min) associated with each to form linear assemblies which appeared to be made up of numerous vesicles (Fig. 8 *b*). If these linear assemblies were incubated for a further 30–60 min they often formed large spheres (Fig. 8 *c*) indicating that fusion of vesicles had occurred. Alternatively, if a small drop of the linear assemblies was put on a microscope slide and then stretched by placing a coverslip on top they produced a linear matrix of interconnected tubules which looked very similar to the structural organization of endoplasmic reticulum in cells (Fig. 8 *d*). Like fusion of nuclear vesicles we found that fusion of these nonnuclear vesicles required both ATP and GTP, was inhibited by GTPγS, and was sensitive to NEM (not shown). Thus, it appears that the fusion properties of much of the membrane vesicles present in our membrane fraction are similar to the fusion properties of the nuclear binding vesicles. Interestingly, when the fused structures shown in Fig. 8 *d* were added to a soluble fraction that had been treated with MPF and was, therefore, in mitosis, no changes in structure were observed (not shown). This indicates that the bulk of the endoplasmic reticulum in *Xenopus* eggs may not dissociate into small vesicles at mitosis.

Discussion

In many eukaryotic cells the nuclear envelope dissociates from chromatin at mitosis and then is targeted back to the surface of chromatin at the completion of mitosis in a first step towards reassembling an intact envelope. In this report we have shown that the association of membrane vesicles with chromatin can be studied using chromatin and a partially purified membrane fraction from *Xenopus* eggs. We have shown that once sperm chromatin is partially decondensed it can associate with purified membrane vesicles. We have also shown by protease digestion of either chromatin or membrane that this binding reaction requires proteins on both the chromatin and membrane. Our results indicate that the binding event is independent of ATP and is insensitive to NEM. The association of membrane vesicles with chromatin in this simple system appears to represent a significant and specific interaction based on several criteria. First, the bound vesicles appear to be uniform in size (74 nM) and rep-

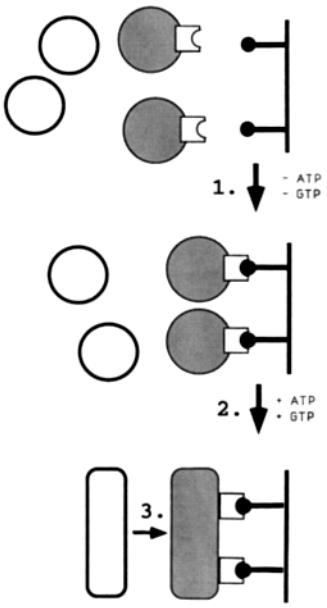

Figure 9. Model for nuclear envelope assembly. In step 1 vesicles containing a receptor bind to chromatin by interacting with a chromatin-bound ligand. This step is independent of ATP and GTP. In step 2 these chromatin-bound vesicles fuse to each other in a process which requires both ATP and GTP. In step 3 further membrane is incorporated into nuclei via fusion of nonreceptor-containing membrane to the outer membrane of the nuclear envelope.

resent a distinct subclass of vesicles within the membrane fraction. Second, when this membrane–chromatin complex is isolated away from unbound vesicles and then incubated with a soluble fraction the vesicles fuse to form an intact membrane which can exclude dextran and allow DNA replication to occur. Third, when this same chromatin–membrane complex is incubated in a soluble fraction that is in mitosis the initially bound vesicles dissociate from chromatin. Thus, the bound vesicles are both capable of forming an intact functional nuclear envelope and respond appropriately to normal cell cycle controls. Unlike membrane binding we have presented evidence that fusion of membrane vesicles to each other is dependent on ATP and GTP, and is sensitive to NEM. Interestingly, our results indicate that these washed membranes can fuse to each other in the absence of soluble factors. This finding implies that the factor(s) required for membrane fusion are associated with the membrane vesicles. Overall, our results indicate that the assembly of the nuclear envelope at the end of mitosis is mediated by a specific class of 74-nM-diam membrane vesicles that specifically interact with chromatin via protein sites that occur at least every 100 kb along the length of the DNA.

The results of our studies are summarized in the model presented in Fig. 9 (see also Wilson and Newport, 1988; Vigers and Lohka, 1991). According to this model the first step in nuclear membrane formation after mitosis consists of an ATP-independent association of membrane vesicles with chromatin (step 1). The interaction between these two components is mediated by the interaction between a vesicle-bound receptor with a chromatin-bound protein. After binding the second step (step 2) of nuclear envelope formation consists of fusion between adjacent vesicles. This fusion step requires both ATP and GTP. Further envelope growth beyond this point would involve fusion of vesicles directly to the outer nuclear envelope (step 3).

Based on our model the first stage in envelope assembly involves fusion of vesicles bound directly to a chromatin-associated protein. We estimate that these chromatin sites occur on average every 100 kb of DNA or $\sim$2–3 $\times$ 10^4 sites per sperm. Using these numbers we calculate that the fusion of all of the 74-nM vesicles bound directly to chromatin in the first step of membrane assembly would produce a double envelope that is 2 μM in diameter. The experiment described in Fig. 4 is a test of this calculation and produces nuclei that are 3–5 μM in diameter. The relatively close agreement between calculated and observed sizes further supports our conclusion concerning the number of vesicle chromatin–binding sites. Nuclei made from vesicles bound directly to chromatin are able to exclude dextran and carry out DNA replication indicating that the envelope is intact and sufficient for nuclear function. These results indicate that membrane incorporated into nuclei via direct binding to chromatin may be sufficient for most nuclear functions to be activated. Despite this observation it is clear that when sperm chromatin is incubated in an assembly extract under conditions in which membrane is in excess the nuclear envelope grows to $\sim$5–10 μM in diameter (Wilson and Newport, 1988). This additional growth must occur via fusion of membrane directly to the outer nuclear envelope. Comparison of the diameter of nuclei formed when this "outer membrane fusion process" is occurring and when it is absent leads us to conclude that in a typical nucleus assembled in vitro 10–30% of the nuclear envelope originates from fusion of chromatin-bound vesicles and 70–90% of the envelope is due to fusion of vesicles to the outer nuclear envelope. Whether this 70–90% of the envelope is required for nuclear function is currently not known.

The membrane receptor and the chromatin-bound ligand that it binds to have yet to be identified. However, based on their membrane association properties and their location at the periphery of assembled nuclei several known proteins may be good candidates for the receptor proteins. These include lamin B (Gerace and Blobel, 1980); the proposed lamin B receptor (Worman et al., 1988); Otefin, a conserved nuclear envelope protein originally found in *Drosophila* (Padan et al., 1990), and several membrane proteins associated with the inner nuclear envelope (Fields and Shaper, 1988; Senior and Gerace, 1988). In many cells a proportion of the lamin B is associated with membranes. The recent demonstration that the soluble mammalian lamins A and C can bind directly to metaphase chromosomes indicates that the membrane-bound lamin B may do so as well. If so, it would be an excellent candidate for the receptor in mammalian cells. However, in *Xenopus* embryos the single known lamin (L_{III}) is soluble (Krohne et al., 1981; Benavente et al., 1985; Krohne and Benavente, 1986) and its depletion from an extract does not block envelope formation (Newport et al., 1990). Therefore, the precise role of the lamin protein during nuclear envelope formation in *Xenopus* is unclear. Currently no good candidate exists for the chromatin bound protein that interacts with the receptor although several proteins that stain the periphery of metaphase chromosomes have been described (McKeon et al., 1984; Chaley et al., 1984).

At mitosis the interaction between the receptor and chromatin must be broken in order for the nuclear envelope to breakdown. Recently we have presented evidence that the dissociation of these two components from each other occurs as a result of the phosphorylation of the membrane-bound receptor (Pfaller et al., 1991). Our evidence indicates that at the onset of mitosis the critical mitotic regulator MPF either activates the kinase or attenuates the phosphatase which

regulates receptor phosphorylation. As a result of phosphorylation the receptor dissociates from the chromatin. At the completion of mitosis dephosphorylation of the receptor causes the receptor to associate with chromatin again in the first step towards assembly of the nuclear envelope.

Although it is clear that at the onset of mitosis chromatin–membrane interactions are broken and at the completion of mitosis there may be as many as one such association every 100 kb of DNA it is not clear how many of these mitotically initiated associations remain during an extended interphase period. It is possible that during interphase these associations are regulated and contribute to chromatin organization and function. For example, in interphase cells most heterochromatin appears to be associated with the nuclear envelope while euchromatin is located within the nucleus. Similarly, recent observations have demonstrated that DNA replication occurs at sites located within the nucleus rather than at the envelope (Leno and Laskey, 1990). These observations may indicate that dissociation of membrane–chromatin association is part of a regulatory pathway leading to activation of chromatin for transcription and DNA replication. In the nuclei of oocytes in many organisms it is clear that all chromatin–membrane interactions have been broken and that the lampbrush chromosomes of these nuclei lie within the nuclear interior well away from the nuclear envelope (Callan, 1963). This observation again argues that chromatin–membrane associations during interphase may be dynamic. The system described here may be useful for identifying the proteins involved in forming and controlling these associations both at mitosis as well as during interphase.

Regulation of Vesicle Fusion

The membrane vesicles that bind to chromatin in our assay are remarkably homogeneous in size (74 nM). Their conserved size indicates that these vesicles like coated pits (Pearse and Bretscher, 1981) and Golgi vesicles (Orci et al., 1986) are formed by a process involving the binding of a coating protein. The absence of a coat on chromatin-bound vesicles examined at high magnification indicates that the coat proteins may have been removed either during or before their isolation.

It is well documented that membrane fusion throughout the secretory pathway is regulated by several ATP-dependent steps and involves a large number of GTP-binding proteins. We have shown that the fusion of both the unbound and chromatin-bound vesicles requires the presence of ATP and GTP and is strongly inhibited by NEM and the nonhydrolyzable GTP analogue GTPγS. This indicates that fusion of membrane vesicles to form the nuclear envelope may be regulated by mechanisms similar to those described for membrane fusion in the secretory pathway (Goud et al., 1988; Orci et al., 1989; for review see Balch, 1990). It should be noted that our in vitro fusion assay is distinct from all other fusion assays described, thus far, in that in our hands vesicle fusion occurs in the absence of proteins present in the soluble fraction of an egg cytosol. This suggests that the vesicles we isolate contain all of the proteins required for fusion and, as such, represents a potentially useful system for investigating membrane–membrane fusion mechanisms under relatively simple and controlled conditions.

In summary, we have developed a simple system composed of isolated membrane vesicles and chromatin for investigating early events in nuclear envelope assembly. Our results indicate that there is a population of vesicles that contains a receptor which can recognize and bind to a protein ligand bound to chromatin. On sperm chromatin this chromatin-bound ligand occurs on average at least every 100 kb of DNA. We propose that the first step in the formation of the nuclear envelope at the end of mitosis is mediated by the fusion of vesicles bound to these chromatin sites. Our results show that fusion of these vesicles requires ATP and GTP, indicating that it is regulated by processes similar to those described for membrane fusion in the secretory pathway. In the future, this simple system should be useful for identifying the proteins involved in both the binding and fusion events.

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