J. Biochem. 122, 1024-1033 (1997)

Characterization of the Binding of Nuclear Envelope Precursor Vesicles and Chromatin, and Purification of the Vesicles

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Received for publication, June 30, 1997

The binding of nuclear envelope precursor vesicles and chromatin was characterized by using an *in vitro* system constituted from a *Xenopus* egg extract and demembranated *Xenopus* sperm chromatin. The results of binding studies in the presence of salts, urea, and a chelator showed that the binding involves an ionic interaction. Chemical modification studies suggested that a protein(s) in the vesicles, which is responsible for the binding with chromatin, has essential lysine, histidine, and methionine residues. The vesicle protein could not be extracted from vesicles with 1 M KCl, 2 M urea, or 0.1 M Na_2CO_3, suggesting that it is an intrinsic membrane protein. The protein was denatured with 8 M urea and 0.1 M Na_2CO_3, and could be renatured by incubation at 23°C, suggesting that the native conformation of the protein is important for the binding. Affinity purification of nuclear envelope precursor vesicles was achieved by binding to chromatin and dissociation with 0.24 M NaCl. The vesicle fraction thus obtained exhibited the ability to form nuclear envelope on incubation with chromatin in *Xenopus* egg cytosol without any other membrane fraction. These results suggested that there is a nuclear envelope precursor vesicle population containing both a chromatin targeting protein and vesicle fusion machinery.

Key words: nuclear envelope, nuclear envelope precursor vesicle, nuclear envelope reconstitution, nuclear membrane, *Xenopus* egg extract.

Breakdown of the nuclear envelope at the prophase and reformation at the telophase are normal steps of cell cycle progression. Analysis of nuclear envelope formation has been greatly facilitated by the use of cell-free systems derived from eggs of *Xenopus* (*1-4*) and *Drosophila* (*5-7*) acting upon homologous or heterologous demembranated sperm chromatin. In *Xenopus*, nuclear envelope formation *in vitro* comprises three steps: nuclear membrane vesicle binding, fusion, and growth of the fused membrane. The binding of the vesicles to decondensed chromatin does not require the cytosol (*3, 4*) and is insensitive to *N*-ethylmaleimide, a sulfhydryl modifier (*4*); however, the binding is inhibited by protease treatment of the chromatin (*4*) and vesicles (*2, 4*). Nuclear membrane vesicle binding is independent of ATP and GTP in *Xenopus* (*4*), but not in *Drosophila* (*5*), and is regulated by phosphorylation (*8-10*). Recently, it was shown that the fusion of chromatin-bound vesicles requires inositol 1,4,5-trisphosphate receptors (*11*), ATP, and hydrolysis of GTP (*3*) for complete nuclear envelope assembly.

It was suggested that lamins, which form a filamentous meshwork lining the nucleoplasmic surface of the inner nuclear membrane, are required for nuclear membrane vesicle binding to chromatin because they bind directly to chromatin *in vitro* (*12-14*), and anti-lamin antibodies suppress the vesicle binding to chromatin in *Drosophila* embryos and CHO cell-free extracts (*7, 15*). Experiments performed with extracts from *Xenopus* eggs suggested that the vesicle binding is not mediated by lamin because the binding occurs in lamin L_{III}-depleted extracts (*16*). However, the discovery of lamin isoforms, and the membrane association of some of both lamins L_I and L_{II} in the egg extract has necessitated re-evaluation of the hypothesis that nuclear envelope reassembly occurs independently of lamins (*17*). Integral membrane protein p58, referred to as the lamin B receptor, and lamina-associated polypeptides (LAPs) have been detected on the inner nuclear membrane and may also be involved in vesicle-chromatin interactions, because these proteins can bind to DNA fragments (*18*) and mitotic chromosomes (*19*). Recently, it was suggested that a kinase exhibiting nuclear membrane-releasing activity regulates the nuclear membrane-chromatin interaction

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Abbreviations: chloramine T, *p*-toluenesulfonchloramide; DEPC, diethylpyrocarbonate; DHCC, 3,3′-dihexyloxacarbocyanine iodide; DTT, dithiothreitol; EGTA, *O,O′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid; EtBr, ethidium bromide; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; HOECHST, Hoechst 33342, bisbenzimide; LAP, lamina-associated polypeptide; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SITS, 4-acetamido-4′-isothiocyanostilbene-2-2′-disulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Triton X-100, polyoxyethylene(10)octylphenyl ether.

together with a phosphatase during nuclear envelope assembly and disassembly (*8, 10*). Although potential roles of the inner nuclear membrane proteins in the interaction of the nuclear membrane and chromatin have been reported, as mentioned above, the question of what kinds of interaction occur between vesicles and chromatin remains unanswered. Therefore, further characterization of the mode of binding of the vesicles and chromatin is necessary as a basis for studies on the mechanism of targeting of nuclear envelope precursor vesicles.

Understanding of the nuclear envelope reassembly mechanism will require the purification of precursor vesicles. Vigers and Lohka performed differential and density gradient centrifugation of a *Xenopus* egg extract, and obtained two vesicle populations necessary for nuclear envelope formation (*20*). From the sea urchin egg system, three kinds of vesicle populations required for nuclear envelope formation were separated (*21*). These vesicles were purified by sucrose density gradient centrifugation, which is useful for analyzing vesicle populations. However, affinity-purified nuclear envelope precursor vesicles are necessary for further analysis of the nuclear envelope reassembly mechanism.

We investigated in this study the mode of binding of nuclear membrane vesicles and chromatin in the *Xenopus* egg extract system, and found that the binding involves an ionic interaction and is reversible. The binding was inhibited irreversibly by the chemical modification of lysine, histidine, and methionine residues. Nuclear envelope precursor vesicles exhibiting the chromatin binding activity were purified by a chromatin-affinity method utilizing the reversible interaction. It was shown that affinity-purified vesicles contain not only chromatin-targeting protein but also fusion machinery for nuclear envelope formation.

EXPERIMENTAL PROCEDURES

Preparation of Demembranated Sperm Chromatin—Demembranated sperm chromatin consists of *Xenopus* sperm treated with lysolecithin to remove the plasma and nuclear membranes without the highly condensed chromatin being affected. Chromatin was prepared as described, using buffer T (0.2 M sucrose, 15 mM NaCl, 5 mM EDTA, 7 mM $MgCl_2$, 80 mM KCl, and 15 mM Pipes/KOH, pH 7.4), and stored at −70°C at a concentration of 40,000/μl (*22*).

Preparation of a Xenopus Egg Extract—*Xenopus* eggs were collected, dejellied, and then lysed to prepare an interphase extract essentially as described (*23*). The egg lysis buffer for the preparation of interphase extracts consisted of 0.25 M sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, and 50 mM Hepes/KOH, pH 7.7, supplemented with 2 mM 2-mercaptoethanol, 10 μg/ml aprotinin, and leupeptin immediately before use. Eggs were packed into tubes by brief centrifugation for several seconds at 6,000 × *g*. Excess buffer above the packed eggs was removed and then the eggs were crushed by centrifugation at 15,000 × *g* for 10 min. The crude extract, the supernatant between the lipid cap and pellet, was collected and mixed with 10 μg/ml cytochalasin B, and then further separated into cytosol, membrane-rich and gelatinous pellet fractions by ultracentrifugation at 200,000 × *g* for 4 h in an RP55S rotor (Hitachi). The cytosol generated was then re-centrifuged at 200,000 × *g* for 30 min to remove residual membranes, divided into 30-μl aliquots, frozen in liquid nitrogen, and stored at −70°C until use. The membrane fraction was resuspended in 10 volumes of cold lysis buffer supplemented with 1 mM dithiothreitol (DTT), and 5 μg/ml aprotinin and leupeptin, and then centrifuged at 200,000 × *g* for 1 h at 4°C, the resuspension and centrifugation steps being repeated twice to remove residual cytosolic components. The membranes were resuspended in egg lysis buffer containing 0.5 M sucrose to a final volume corresponding to ~10% of the original volume of the crude extract (10× membranes), divided into 10-μl aliquots, frozen in liquid nitrogen, and then stored at −70°C until use.

Assay of Vesicle Binding to Chromatin—To observe nuclear vesicle binding to the chromatin surface, essentially as described (*4*), 1 μl of demembranated sperm chromatin (10,000/μl) was incubated in 10 μl of heated cytosol containing 1 μl of membranes at 23°C for 1 h. After incubation, a 2-μl aliquot of the mixture was removed and diluted with 2 μl of Hoechst/DHCC buffer (buffer T containing 20 μg/ml bisbenzimide DNA dye (Hoechst 33342; Calbiochem-Novabiochem), a lipid dye, 3,3′-dihexyloxacarbocyanine iodide (DHCC; Aldrich Japan), and 3.7% formaldehyde) on a glass slide. The fixed sample, covered with a glass coverslip and sealed with nail varnish, was examined by phase contrast and fluorescence microscopy. An Axioplan (Carl Zeiss)-equipped fluorescence microscope with exciter-barrier reflector combinations suitable for DHCC and bisbenzimide was used.

Nuclear Assembly Assays—Assays were performed as described by Smythe and Newport (*22*). The precipitated membrane vesicle fraction was suspended in 10 μl of cytosol supplemented with 0.5 μl of an ATP regeneration system (333 mM phosphocreatine, 67 mM ATP, pH 7.0, and 1.7 mg/ml creatine kinase), and then mixed with demembranated sperm chromatin. The standard reaction mixture consisted of 10 μl cytosol and a vesicle fraction derived from 10 μl of crude extract, and 1,000 chromatin/μl of cytosol. After incubation at room temperature (23°C) for 1.5 h, an aliquot of the reaction mixture was removed, and observed by phase contrast and fluorescence microscopy as described above. In some cases, assembled nuclei were subjected to electron microscopy after fixation with glutaraldehyde according to the method described by Macaulay and Forbes (*24*).

Affinity Purification of Nuclear Precursor Vesicles—Demembranated sperm chromatin (corresponding to about 4×10^5 nuclei) was incubated for 2 h in 100 μl of a freshly prepared crude extract of eggs and then chromatin-bearing nuclear precursor vesicles were precipitated through 0.5 M sucrose in v-buffer (0.25 M sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 100 μg/ml cycloheximide 5 μg/ml cytochalasin B, and 1 mM DTT) by centrifugation at 1,000 × *g* for 15 min. The precipitate was suspended in v-buffer and the NaCl concentration was brought to 0.24 M. After dissociation of the vesicles at 4°C for 30 min, the mixture was layered on top of 1 M sucrose in v-buffer and centrifuged at 13,000 × *g* for 5 min to remove chromatin. Affinity-purified vesicles in the supernatant fraction were collected by centrifugation at 100,000 × *g* for 1 h as a pellet.

Pretreatment of Membranes with Various Reagents—A membrane suspension, 1-3 μl, was added to 100-300 μl of egg lysis buffer supplemented with 10 μg/ml aprotinin and

leupeptin, and each chemical modification reagent, of which the concentration is given in the figures and under "RESULTS." After incubation at 23°C for 30 min, each mixture was diluted to 1 ml with cold lysis buffer containing 1 mM DTT, and 5 μg/ml aprotinin and leupeptin, and then centrifuged at 100,000×g for 1 h, the resuspension and centrifugation steps being repeated twice to stop the chemical modification and to remove residual reagents.

Protease treatment of membranes was performed at 4°C in 100-300 μl of egg lysis buffer containing 1 mM DTT and 15 μg/ml trypsin (Worthington Biochemical). The membrane suspension, 1-3 μl, was added and then incubation was performed at 4°C for 30 min. The final membrane protein concentration in the protease digest mixture was 0.4-0.6 mg/ml. To stop the protease digestion, protease inhibitors (aprotinin and leupeptin; final concentrations, 100 μg/ml each) were added to the digestion tube and mixed, followed by incubation for an additional 10 min at 4°C. Then the mixture was centrifuged as described above.

Other membrane treatments were performed in 1 ml of medium at 4°C for 30 min. After incubation with each reagent, of which the concentration is given in the figures or under "RESULTS," each reaction mixture was centrifuged without dilution, resuspended and re-centrifuged as described above.

To determine nuclear vesicle binding to the chromatin surface, each treated membrane sample was incubated with demembranated sperm chromatin and heated cytosol, as described above.

Pretreatment of Decondensed Chromatin with Various Reagents—Demembranated sperm chromatin (10,000/μl, 1 μl) was incubated and decondensed in 10 μl of heated cytosol at 23°C for 30 min. The decondensed chromatin was added, followed by incubation with 100 μl of each reagent, as described above. To separate treated chromatin from the reaction mixture, a sample was layered onto 200 μl of 1 M sucrose, followed by centrifugation for 3 min in an RT15S rotor (Hitachi) at 13,000×g. The pelleted chromatin was resuspended in 10 μl of egg lysis buffer supplemented with 1 mM DTT, and 5 μg/ml aprotinin and leupeptin.

Protease treatment of decondensed chromatin was performed, after pelleting through 1 M sucrose as described above, at 4°C for 30 min in 10-20 μl of egg lysis buffer containing 1 mM DTT and 15-100 μg/ml trypsin or chymotrypsin (Sigma Chemical). To stop the protease digestion, protease inhibitors (aprotinin and leupeptin or chymostatin; final concentrations, 100 μg/ml each) were added to each digestion tube and mixed, followed by incubation for an additional 10 min at 4°C.

To observe nuclear vesicle binding to the chromatin surface, 1-3 μl of membranes was added to each sample of treated chromatin, followed by incubation, as described above.

Treatment of Vesicle-Chromatin Complexes with Chemical Modification Reagents—Demembranated sperm chromatin (10,000/μl, 1 μl) was incubated at 23°C for 1 h in 10 μl of heated cytosol containing 1 μl of membranes and a final 10 U/ml of apyrase. Then, each reagent, at the final concentration given in the tables or under "RESULTS," was added to the mixture containing vesicle-chromatin complexes, followed by incubation at 23°C for 30 min, and an aliquot of the mixture was removed and examined by fluorescence microscopy.

Assay of Vesicle Binding to Chromatin in the Presence of Reagents—Demembranated sperm chromatin was incubated in heated cytosol, decondensed, and then pelleted through 1 M sucrose as described above. The decondensed chromatin was suspended in 20 μl of buffer N (20 mM EGTA, 15 mM $MgCl_2$, and 25 mM Hepes-KOH, pH 7.5) containing various concentrations of NaCl, sodium phosphate (pH 7.5), Na_2SO_4 or 2 M urea, and then incubated with membranes as described above to determine the nuclear vesicle binding to the chromatin surface. In the case of 10 mM EDTA, egg lysis buffer was used instead of the above medium.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Silver Staining—These were carried out according to the methods of Laemmli (*25*) and Morrissey (*26*), respectively.

RESULTS

Characterization of the Nuclear Membrane Vesicle Protein(s) Responsible for Chromatin-Binding—To characterize the vesicle protein(s) required for the binding of nuclear membrane vesicles and chromatin, a vesicle fraction isolated from a *Xenopus* egg crude extract was treated with various reagents, and the chromatin binding activity was examined (Fig. 1). The activity was lost on pretreatment with trypsin (Fig. 1B), showing that a proteineous component(s) is required for the binding of nuclear membrane vesicles to chromatin, as reported previously (*2*). In contrast, the activity was not affected by washing with a buffer containing 1 M KCl, 0.24 M sodium phosphate, 2 M urea, 10 mM EDTA, or 0.1 M Na_2CO_3 (pH 11) at 4°C for 30 min (Fig. 1, C-G). These results suggested that the vesicle protein(s) responsible for the binding to chromatin is an integral membrane protein or a protein tightly associated with the membrane.

The effects of pretreatment of vesicles with protein denaturants on the chromatin-binding activity were examined next. Vesicles were exposed to 10% TCA, 8 M urea, or 0.1 M Na_2CO_3, and then pelleted, the binding activity being determined immediately. Chromatin-binding activity was completely suppressed in all cases (data not shown). However, when vesicles were incubated in an appropriate buffer at 23°C, but not 4°C, for 2 h prior to the binding assay, the activity was recovered (Fig. 2). The suppressed binding activity could also be recovered on incubation with chromatin at 23°C for more than 3 h, but not on preincubation at 4°C for 6 h (data not shown). These results suggested that the binding of vesicles to chromatin depends on renaturation of a membrane protein structure; in other words, the native conformation of a protein tightly associated with the membrane is important for the binding to chromatin.

To determine what kinds of amino acid residues in the membrane protein participate in the binding to chromatin, vesicles were treated with 10 mM chemical modification reagents at 23°C for 30 min, and then the binding activity to chromatin was assayed (Fig. 3). *N*-Ethylmaleimide (NEM) had no effect on the binding activity, as reported previously (Fig. 3B) (*4*). In contrast, dimethylpimelimidate, a chemical modifier of amino groups, diethylpyrocarbonate (DEPC), a chemical modifier of histidine residues, and sodium *p*-toluenesulfonchloramide (chloramine T), which

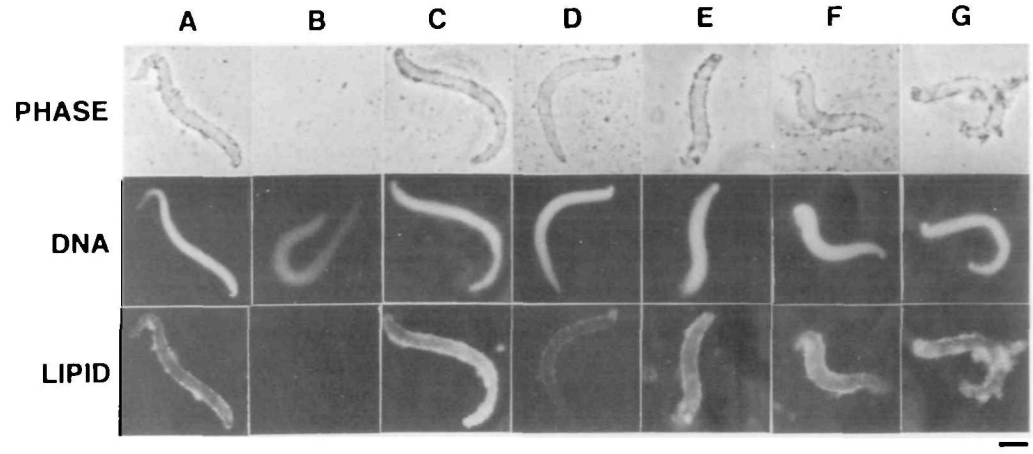

Fig. 1. **Binding of chromatin and nuclear membrane vesicles pretreated with some reagents.** Membranes were treated with 0.1 M Na_2CO_3 (G), or egg lysis buffer (A) containing 15 μg/ml trypsin (B), 1 M KCl (C), 0.24 M sodium phosphate (pH 7.5) (D), 2 M urea (E), or 10 mM EDTA (F), as described under "EXPERIMENTAL PROCEDURES," and then incubated with demembranated sperm chromatin in the presence of heated cytosol. After incubation at 23°C for 1 h (3 h in G), an aliquot of each mixture was removed and examined by fluorescence microscopy. Top, nuclear membrane vesicles bound around decondensed chromatin were examined by phase-contrast microscopy. Middle, DNA fluorescence of the same structures visualized with HOECHST. Bottom, membranes visualized through fluorescence of the lipophilic dye, DHCC. Bar, 10 μm.

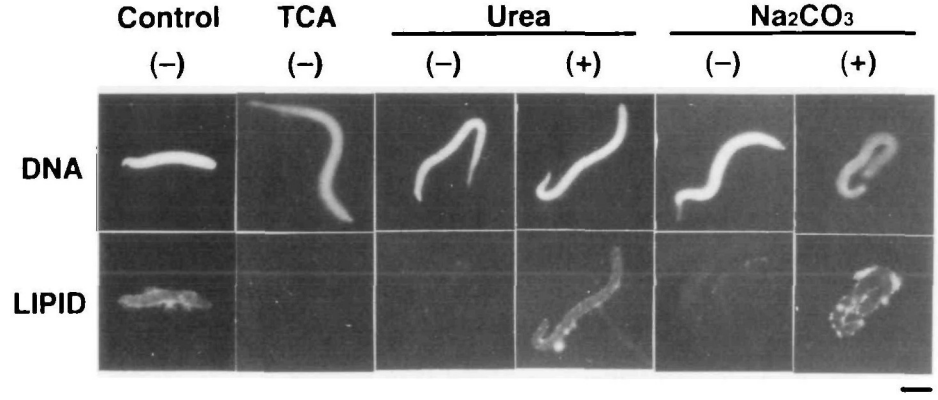

Fig. 2. **Effects of protein denaturants on the binding of nuclear membrane vesicles to chromatin.** Membranes treated with egg lysis buffer (Control), 10% TCA (TCA), 8 M urea (Urea), or 0.1 M Na_2CO_3 (Na_2CO_3), as described under "EXPERIMENTAL PROCEDURES," were incubated at 4°C (−) or 23°C (+) for 2 h in egg lysis buffer and then collected by centrifugation. The treated membranes were incubated with demembranated sperm chromatin in the presence of heated cytosol at 23°C for 1 h, and then an aliquot of each mixture was examined by fluorescence microscopy. Top, DNA fluorescence of the decondensed chromatin structures visualized with Hoechst dye. Bottom, nuclear membrane vesicles bound around the same chromatin structures were stained with DHCC. Bar, 10 μm.

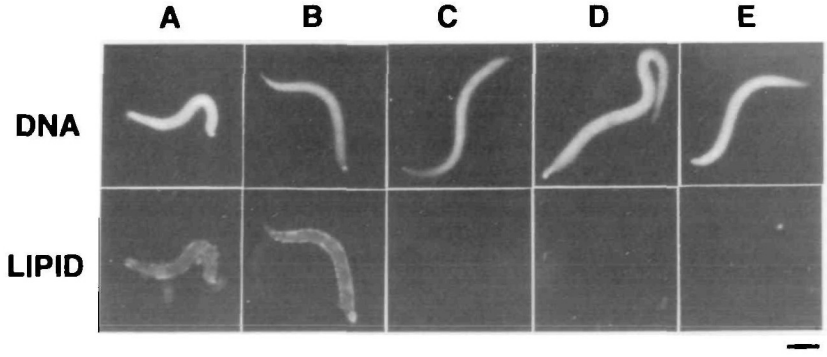

Fig. 3. **Effects of chemical modification of nuclear membrane vesicles on the binding to chromatin.** Membranes were treated with egg lysis buffer (A) containing 10 mM NEM (B), 10 mM dimethylpimelimidate (C), 10 mM DEPC (D), or 10 mM chloramine T (E), as described under "EXPERIMENTAL PROCEDURES," and then incubated with demembranated sperm chromatin in the presence of heated cytosol. After incubation at 23°C for 1 h, an aliquot of each mixture was examined by fluorescence microscopy. Top, DNA fluorescence of the decondensed chromatin structures visualized with Hoechst dye. Bottom, nuclear membrane vesicles bound around the same chromatin structures were stained with DHCC. Bar, 10 μm.

oxidizes methionine residues, inhibited the binding (Fig. 3, C-E). The binding was also inhibited by methylacetimidate, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), and 2,4,6-trinitrobenzenesulfonic acid (TNBS), chemical modifiers of amino groups (data not shown). Fifty percent inhibition of the binding to chromatin was achieved with 0.1-0.5 mM dimethylpimelimidate, SITS, TNBS, DEPC, and chloramine T, respectively (data

not shown). These results suggest that lysine, histidine, and methionine residues of the protein on nuclear membrane vesicles are important for the binding to chromatin.

Characterization of the Chromatin Protein(s) Responsible for Nuclear Membrane Vesicle-Binding—To determine what kinds of components of chromatin are responsible for the binding to nuclear membrane vesicles, chromatin decondensed with heated cytosol was treated with protease and subsequently assayed for vesicle-binding activity (Table I). The activity was lost on treatment with trypsin, showing that a proteineous component of chromatin is required for the binding to nuclear membrane vesicles, as reported previously (*4*). However, the protein(s) was not susceptible to chymotrypsin.

To determine whether any nucleic acids of chromatin are directly involved in the binding of chromatin with nuclear membrane vesicles, decondensed chromatin was treated with 15 μg/ml DNase I or RNase A and then assayed for vesicle-binding activity. The activity was not affected by RNase A treatment of the decondensed chromatin (Table I). However, we could not judge whether or not the vesicle-binding activity of chromatin was affected by DNase I, because chromatin treated with DNase became fragmented and thus could not be subjected to the binding assay (data not shown). Then we examined the effects of DNA-binding reagents on the interaction of vesicles and chromatin. Decondensed chromatin was treated with 0.25 mM ethidium bromide (EtBr) and 9-aminoacridine, which are intercalators strongly accommodated between nucleic acid base-pairs, and Hoechst 33342 (bisbenzimide), which is a compound that strongly binds to the minor grooves of DNA-double helices, and then assayed for vesicle-binding activity (Table I). Although the chromatin structure was more decondensed by these reagents, the binding to vesicles was not affected by any of them.

Decondensed chromatin was also treated with some reagents that disrupt weak protein-protein interactions or weak protein-nucleic acid interactions, and then assayed for the binding activity: 0.24 M sodium phosphate, 2 M urea, 10 mM EDTA, and 1% Triton X-100 (Table I). However, none of these treatments inhibited the binding. These results show that the chromatin protein participating in vesicle-binding is tightly associated with the chromatin structure.

To determine what kinds of amino acid residues in the chromatin protein contribute to the binding, decondensed chromatin was pretreated with NEM, dimethylpimelimidate, DEPC, or chloramine T, and then assayed for binding activity (Table I). However, these treatments had no effect on the binding of nuclear membrane vesicles and chromatin. These results show that cysteine, lysine, histidine, and methionine residues in the chromatin protein are not important for the binding.

Characterization of the Mode of Binding of Nuclear Membrane Vesicles and Chromatin—Pretreatment of vesicles with trypsin, dimethylpimelimidate, DEPC, and chloramine T inhibited the binding to chromatin (Fig. 3). Then we examined whether or not these reagents can dissociate vesicles from chromatin. As shown in Table II, vesicles bound to chromatin could be released by treatment with trypsin. However, other treatments did not release vesicles from the chromatin surface. Methylacetimidate, a chemical modifier of amino groups, also did not release them (data not shown). The amino acid residues of the vesicle protein responsible for the chromatin binding, which are modified in the absence of chromatin and are important for the binding to chromatin (Fig. 3), seemed to be covered by chromatin and thus could not be modified.

To determine what kind of interaction participates in the binding of vesicles and chromatin, decondensed chromatin

TABLE I. **Binding of nuclear membrane vesicles to chromatin pretreated with various reagents.** Decondensed chromatin was pretreated with the various reagents indicated, as described under "EXPERIMENTAL PROCEDURES," washed with a medium, and then incubated with membrane vesicles at 23°C for 1 h. The binding of nuclear membrane vesicles was assayed as in Fig. 1.

Reagent	Vesicle-binding
Buffer (control)	+
15 μg/ml trypsin	−
15 μg/ml chymotrypsin	+
20 μg/ml RNase A	+
0.25 mM EtBr	+
0.25 mM 9-aminoacridine	+
0.25 mM Hoechst 33342	+
0.24 M sodium phosphate	+
2 M urea	+
10 mM EDTA	+
1% Triton X-100	+
10 mM NEM	+
10 mM dimethylpimelimidate	+
10 mM DEPC	+
10 mM chloramine T	+

TABLE II. **Dissociation of nuclear membrane vesicles from chromatin on chemical modification.** Membrane vesicles were bound to chromatin by preincubation of demembranated sperm chromatin in the presence of heated cytosol and 10 U/ml apyrase at 23°C for 1 h, and then one of the chemical modification reagents indicated was added to the mixture. After incubation at 23°C for 30 min, nuclear membrane vesicle dissociation from chromatin was determined by microscopy, as in Fig. 1.

Reagent	Dissociation
Buffer (control)	No
15 μg/ml trypsin	Yes
10 mM NEM	No
10 mM dimethylpimelimidate	No
10 mM DEPC	No
10 mM chloramine T	No

TABLE III. **Effects of salts, urea, and EDTA on the binding of nuclear membrane vesicles and chromatin.** Decondensed chromatin was incubated with membranes in 20 mM EGTA, 15 mM $MgCl_2$, and 25 mM Hepes-KOH, pH 7.5 (ion strength = 0.10), supplemented with sodium salts and urea at the indicated concentrations at 23°C for 1 h, and then nuclear membrane vesicles bound to chromatin were examined as in Fig. 1. In the case of EDTA, egg lysis buffer was used instead of the above medium.

Reagent	Concentration (M)	Ionic strength	Vesicle-binding
− (Control)	0	0.10	+
NaCl	0.10	0.20	+
	0.24	0.34	−
Sodium phosphate	0.043	0.20	+
	0.10	0.34	−
Na_2SO_4	0.033	0.20	+
	0.080	0.34	−
Urea	2.0	0.10	+
EDTA	0.010	0.12	+

was incubated with vesicles in the presence of sodium salts, urea or EDTA, and then examined by microscopy to determine whether or not vesicles are bound to the chromatin surface (Table III). The binding of vesicles and chromatin was inhibited in the presence of high concentrations of salts, but not in the presence of 2 M urea or 10 mM EDTA, showing that an ionic interaction, not hydrophobic, hydrogen-bonding, or chelating interaction, is important for the binding of nuclear membrane vesicles and chromatin. Moreover, the inhibition occurred at the same ionic strength, but not the same molar concentration, of three kinds of sodium salts, showing that the interaction is not dependent on the ionic species. Added sodium salts should directly affect the interaction between vesicles and chromatin, since prewashing of the vesicles and chromatin with 0.24 M sodium phosphate (ionic strength = 0.56) had no effect on the binding. Vesicles bound to the chromatin surface in the presence of heated cytosol were dissociated from the chromatin by dialysis against 0.24 M sodium phosphate (ionic strength = 0.56), and could be re-associated with the chromatin by dialysis against a low ionic strength buffer (Fig. 4). These results suggest that nuclear membrane vesicles active as to chromatin binding can be

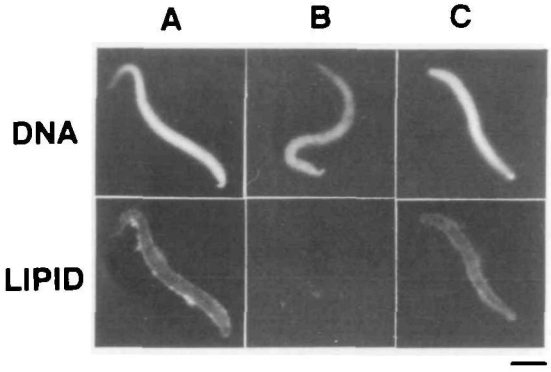

Fig. 4. **Reversible binding of nuclear membrane vesicles to chromatin.** Membrane vesicles were bound to chromatin by incubation in the presence of heated cytosol and 10 U/ml apyrase at 23°C for 1 h (A), and then the reaction mixture was dialyzed against 0.24 M sodium phosphate buffer (pH 7.5) supplemented with 1% 2-mercaptoethanol and 0.1 mM PMSF at 23°C for 6 h (B). The dialyzed mixture was again dialyzed against egg lysis buffer at 4°C for 12 h (C). After each dialysis procedure, an aliquot of each mixture was removed, and examined by phase contrast and fluorescence microscopy. Top, DNA of the decondensed chromatin visualized with Hoechst dye. Bottom, nuclear membrane vesicles bound to the chromatin were stained with DHCC. Bar, 10 μm.

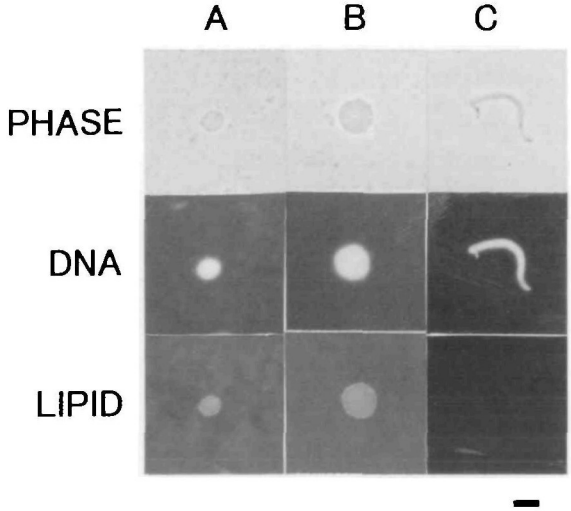

Fig. 5. **Nuclear formation with an affinity-purified vesicle fraction.** A: Nuclear envelope precursor vesicles were purified by the chromatin-affinity method. The vesicle fraction was incubated at 23°C for 1.5 h with *Xenopus* sperm chromatin and a cytosol fraction prepared from a *Xenopus* egg extract. The nuclei thus formed were examined by fluorescence microscopy, as in Fig. 1. B: Sperm chromatin was incubated as in A, with the crude membrane and cytosol fractions of a *Xenopus* egg extract, and the nuclei formed were observed. C: Sperm chromatin was incubated as in A in the absence of the vesicle fraction. Bar, 10 μm.

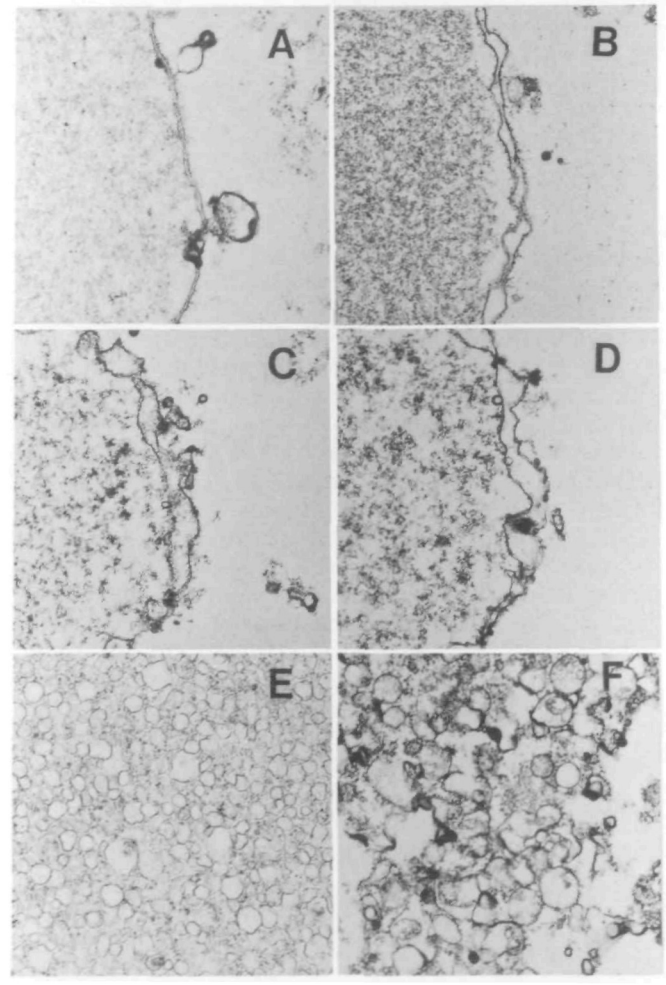

Fig. 6. **Nuclear envelope precursor vesicles and their fusion on the surface of chromatin.** A and B, parts of nuclei assembled with a crude extract of *Xenopus* eggs and sperm chromatin; C, a part of a nucleus assembled with the crude membrane and cytosol fractions of eggs and sperm chromatin; D, a part of a nucleus assembled with an affinity-purified nuclear envelope precursor vesicle fraction, and cytosol of eggs and sperm chromatin; E, a crude membrane fraction of eggs; F, an affinity-purified nuclear envelope precursor vesicle fraction. Samples were fixed with glutaraldehyde, postfixed with OsO_4, stained with uranyl acetate and lead citrate, and then observed under a transmission electron microscope. Bar, 1 μm.

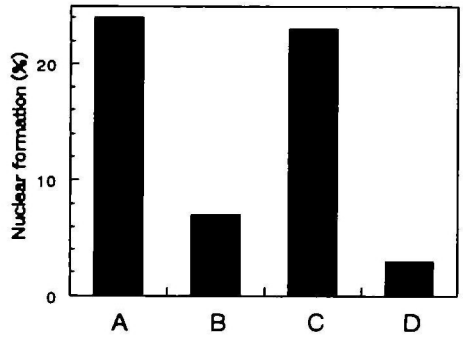

Fig. 7. **Nuclear formation activity of an affinity-purified vesicle fraction.** A: A standard amount of crude membrane fraction separated from a *Xenopus* egg extract (27 μg protein/tube) was incubated in the cytosol fraction with *Xenopus* sperm chromatin under the standard conditions except that incubation was carried out for 3 h. Then the sample was observed by fluorescence microscopy after staining as in Fig. 5 to determine the nuclei formed. "Nuclear formation" in the figure shows (formed nuclei/observed chromatin) × 100 (%). B: The same as in A except that a 1/5 amount of membrane protein/tube was used. C: The same as in B except that an affinity-purified vesicle fraction was used instead of the crude membrane fraction. D: The same as in C except that a vesicle fraction obtained by affinity purification procedures in the absence of chromatin was used.

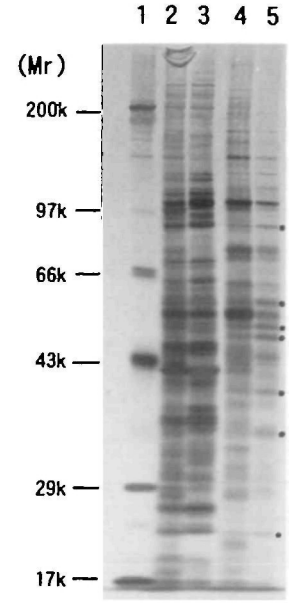

Fig. 8. **SDS-PAGE of an affinity-purified vesicle fraction.** Samples prepared from a *Xenopus* egg extract were electrophoresed in a 8-12% linear gradient gel and stained with silver. Lanes: 1, marker proteins; 2, crude extract fraction; 3, cytosol fraction; 4, membrane fraction; and 5, affinity-purified nuclear envelope precursor vesicle fraction. The bands concentrated in lane 5 are indicated by dots.

isolated from other vesicles by precipitation with chromatin following dissociation with 0.24 M sodium phosphate or other salts.

Affinity Isolation of Active Nuclear Envelope Precursor Vesicles—We tried to isolate active nuclear envelope precursor vesicles from the membrane fraction of *Xenopus* egg extract by means of the above chromatin-affinity method. However, the method was unsuccessful because membrane vesicles very easily lose their activity and aggregate during prolonged treatment. This means that the simplest possible procedures should be used to get active precursor vesicles. Therefore, we tried to use a fresh crude extract of eggs directly without isolation of the membrane fraction. Demembranated chromatin was incubated with a crude extract and the chromatin-vesicle complexes thus formed were precipitated through a 0.5 M sucrose layer. The complexes were dissociated with 0.24 M NaCl, and chromatin was removed by centrifugation. The nuclear envelope formation activity of the vesicles thus obtained was determined. As shown in Fig. 5, the vesicle fraction exhibited the ability to form nuclei on incubation with chromatin and a *Xenopus* egg cytosol fraction without any other membrane fraction. As a control experiment, when a heated cytosol fraction of the eggs was used instead of the native one, the affinity-purified vesicles exhibited only chromatin binding (data not shown). In the other control experiment, sperm chromatin was incubated with a cytosol fraction in the absence of the affinity-purified vesicles. Nucleus or chromatin bearing membrane vesicles were not found under these conditions (Fig. 5C). These results suggested that these vesicles had the ability of not only chromatin binding but also of fusing with each other and of forming nuclear membrane in the presence of the cytosol fraction. The fusion of the vesicles was confirmed by electron microscopy (Fig. 6). These purified nuclear envelope precursor vesicles exhibited about 4-times higher nuclear formation activity than the crude membrane fraction (Fig. 7). The diameters of nuclei formed with the affinity-purified vesicles were 12.5 ± 2.5 μm, in comparison with 25 ± 2.5 μm in the case of those formed with the crude membrane fraction. The sizes of the nuclei formed with affinity-purified vesicles were not increased by prolonged incubation. These results suggest that some component necessary for growth of the nuclear membrane is lacking in this vesicle fraction (discussed in more detail below).

To determine whether or not some protein components are concentrated in the affinity-purified precursor vesicle fraction, the fraction was analyzed by SDS-PAGE (Fig. 8). Lane 5 in Fig. 8 contains precursor vesicle proteins bound to chromatin. When lanes 4 and 5 were compared with each other, several bands, marked by dots, were found to be concentrated in lane 5. These proteins are enriched in the nuclear envelope precursor vesicle fraction. A protein(s) responsible for the binding to chromatin should also be enriched in the fraction.

DISCUSSION

In many eukaryotic cells the nuclear envelope dissociates from chromatin at the time of mitosis, and then is targeted back to the surface of chromatin at the completion of mitosis as the first step towards reassembly of an intact envelope. In this study we examined the mode of binding of nuclear membrane vesicles and chromatin using an *in vitro* assay system derived from a *Xenopus* egg extract. It was shown that an ionic interaction, not a hydrophobic, hydrogen-bonding, or chelating interaction, is important for the binding (Table III and Fig. 4). The results obtained with protein denaturants suggested that the native conformation of the membrane protein(s) responsible for the interaction is important (Fig. 2). Inhibition of the interaction by

treatment with dimethylpimelimidate or methylacetimidate, with which the gross charges of modified amino groups are not altered, suggested that a specific lysine residue(s) of the membrane protein(s), but not the gross positive charge, is important for the binding to chromatin. Histidine and methionine residues of the membrane protein(s) are also important for the binding to chromatin (Fig. 3). Moreover, it was suggested that these amino acid residues are present in the chromatin-binding site of the vesicle protein(s) responsible for the interaction (Fig. 3 and Table II). On the other hand, it was suggested that these kinds of amino acid residues in the chromatin protein are not essential for the binding (Table I). The chromatin protein(s) responsible for the binding to nuclear membrane vesicles seemed to be tightly associated with the chromatin structure, because the binding activity was not affected by treatment with structural perturbants (Table I). Whether or not nuclear membrane vesicles interact directly with DNA besides chromatin proteins in nuclear envelope assembly is not clear. However, base-pair moieties and minor grooves of DNA double helices may not be important in the interaction of chromatin and nuclear membrane vesicles, because intercalators and a reagent bound to the minor grooves had no effect on the vesicle binding (Table I). We found that membrane vesicles associate with and dissociate from chromatin reversibly with a change in the ionic strength of the medium (Fig. 4). This means that nuclear envelope precursor vesicles active as to chromatin binding can be separated from other vesicles by means of very simple procedures.

Experiments performed with extracts of *Drosophila* embryos and mammalian tissue culture cells at mitosis suggested that membrane-bound and soluble lamins are involved in the targeting of nuclear membrane vesicles (*7, 27*). In contrast, the results of experiments on *Xenopus* egg extracts demonstrated that soluble components, including soluble lamins and membrane-bound lamin B_3, which has only been found in *Xenopus* eggs, are not required for the vesicle binding (*16*). However, Lourim and Krohne reported recently that *Xenopus* eggs contain another B-type lamin, that is similar or identical to the *Xenopus* somatic cell B-type lamin B_2 (*28*). Therefore, whether or not some membrane-bound lamins are required for the vesicle-binding in *Xenopus* egg nuclear assembly remains unclear. It is known that most lamins bound to the nuclear membrane of interphase nuclei of mammalian somatic cells can be extracted by treatment with 0.1 M NaOH or 4 M guanidine-HCl (*29*). Therefore, we examined the binding activity of vesicles pretreated with 0.1 M NaOH or 4 M guanidine-HCl, as described (*29*), and observed complete inhibition of the binding activity (data not shown). However, we could not judge from the results whether the inhibition reflects extraction of peripheral membrane proteins and/or irreversible denaturation of integral membrane proteins by such treatment, because the chromatin-binding activity was not recovered at all in either case on incubation of extracted vesicles with the extract at 23°C prior to the binding assay (data not shown). Then, we applied slightly milder conditions, 0.1 M Na_2CO_3 washing, to remove most peripheral membrane proteins from the surface of nuclear membrane vesicles (*30*). The chromatin-binding activity of nuclear membrane vesicles was not destroyed by the treatment (Fig. 2). These results support the idea that soluble proteins and peripheral membrane proteins including a B-type lamin(s) are not necessary for the binding of nuclear membrane vesicles to chromatin in the *Xenopus* egg system, in contrast to the *Drosophila* embryo system (*7*).

It is known that several integral membrane proteins of the inner nuclear membrane bind to chromatin or DNA *in vitro*. Four LAPs, 75, 68, 55, and 53 kDa, of the inner nuclear membrane have been characterized in rat cells (*29, 31*). All these proteins are intrinsic membrane ones specific to the inner nuclear membrane (*29, 31*). Among these proteins, the 53 kDa LAP2 protein associates with chromosomes prior to the assembly of most lamins, which suggests that the protein may play some role in initial events of nuclear envelope reassembly (*31*). Another intrinsic inner nuclear membrane protein, lamin B receptor, contains three putative DNA-binding motifs in its N-terminal domain (*32, 33*), and binds to DNA fragments (*18*). Pyrpasopoulou *et al.* reported recently that the lamin B receptor provides essential chromatin docking sites on the nuclear envelope in mammalian and bird cell systems (*34*). Collas *et al.* found recently, using an antibody against the human lamin B receptor, that a lamin B receptor-like integral membrane protein mediates the targeting of vesicles to chromatin in a sea urchin egg system (*35*). We showed recently that the amino terminal region of the lamin B receptor binds directly to *Xenopus* sperm chromatin *in vitro* (*33*). The binding was suppressed by treatment of the chromatin with diluted trypsin, at the same concentration as used in Table I, prior to the binding assay (unpublished observation). Therefore, in the *Xenopus* egg system, the lamin B receptor is the most probable candidate for the vesicle protein which targets vesicles to chromatin.

It was reported by Vigers and Lohka (*20*) that two kinds of nuclear envelope precursor vesicle fractions, NEP-A and NEP-B, are necessary to assemble the nuclear envelope around chromatin and to grow it to a normal size in a *Xenopus* egg extract. Vesicles in NEP-B, which were isolated from a 200,000 × *g* supernatant fraction, can bind to chromatin but not fuse with each other in the absence of NEP-A (*20*). Conversely, vesicles in NEP-A cannot bind to chromatin, but are necessary for the formation of fused nuclear envelopes, together with NEP-B (*20*). These results suggested that a protein necessary for vesicle targeting to chromatin is included in distinct vesicles from those containing membrane fusion machinery. However, our nuclear envelope precursor vesicles isolated directly from a *Xenopus* egg crude extract should contain both targeting proteins and fusion machinery, because these vesicles bind directly to chromatin and can fuse with each other on the chromatin surface in the cytosol fraction without any other membrane fraction (Figs. 5 and 6). These results suggested that our nuclear precursor vesicle fraction contains a new type of vesicles.

Buendia and Courvalin showed that distinct membrane domains of the nuclear envelope, the inner membrane and the pore membrane, were vesiculated separately in mitosis in human T lymphoblastoma cells (*36*). These two types of vesicles of differ in size distribution: pore membrane-derived vesicles, which contain gp210, are smaller than inner membrane-derived vesicles, which contain the lamin B receptor and LAP-2. In the *Xenopus* egg system, NEP-B

was isolated from a 200,000×*g* supernatant fraction of a *Xenopus* egg extract from which large vesicles had been removed (*20*). Moreover, it is known that the density of nuclear pore complexes in the reassembled nuclear envelope is dependent on the amount of added NEP-B (*20*). Therefore, the majority of vesicles in NEP-B seem to be precursor vesicles for the pore membrane domain. On the other hand, vesicles of another type, which were found in our vesicle fraction and purified from a crude extract, seem to be precursor vesicles for the inner and outer membranes of the nuclear envelope because (i) these vesicles contained chromatin-targeting protein and fusion machinery, and have larger diameters, (ii) ribosome-like dots were observed on the surface of the vesicles (Fig. 6F).

We characterized in this study the binding mode of membrane vesicles and chromatin, and showed that the binding comprises an ionic interaction and is reversible. We dissociated membrane vesicles from chromatin by means of a high salt buffer and purified them. The vesicle fraction could form small nuclei without any other membrane fraction. It was shown that the vesicles are nuclear envelope precursor vesicles. Studies to determine whether the addition of NEP-A or NEP-B to our vesicle fraction can cause tight association of two nuclear membranes with each other and the growth of nuclei to the normal size are now under way.

We wish to thank Drs. H. Takisawa and Y. Kubota, Osaka University, for advice on the nuclear assembly assay involving *Xenopus* egg extracts. We also wish to thank Kazuko Hasegawa for her help in preparing the pictures.

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