

In vitro reassembly of nuclear envelopes and organelles in *Xenopus* egg extracts

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We reconstituted bilayer nuclear membranes, multilayer membranes, and organelles from mixtures of *Xenopus laevis* egg extracts and demembrated *Xenopus* sperm nuclei. Varying proportions of the cytosolic and vesicular fractions from the eggs were used in the reconstitution mixtures. A cytosol:vesicle ratio of 10:1 promoted reassembly of the normal bilayer nuclear membrane with inserted nuclear pore complexes around the decondensed *Xenopus* sperm chromatin. A cytosol:vesicle ratio of 5:1 caused decondensed and dispersed sperm chromatin to be either surrounded by or divided by unusual multilayer membrane structures with inlaid pore complexes. A cytosol:vesicle ratio of 2.5:1 promoted reconstitution of mitochondria, endoplasmic reticulum networks, and Golgi apparatus. During reassembly of the endoplasmic reticulum and Golgi apparatus, vesicular fragments of the corresponding organelles fused together and changed their shape to form flattened cisternae, which were then stacked one on top of another.

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Introduction

During mitosis, a cell needs to partition its duplicated genetic material into two daughter cells under very strict control in a program that comprises several checkpoints. Equally accurate partitioning of the intracellular organelles that sustain vital cellular function is also required [1]. At the onset of mitosis (or meiosis), the nuclear envelope breaks down, as do the endoplasmic reticulum, the Golgi apparatus, and the mitochondria. At telephase, all of the organelles are rapidly reassembled from their fragments, which are present in each daughter cell. Membranes of most cellular organelles, including the nuclear envelope, the endoplasmic reticulum and Golgi cisternae, and the mitochondrial cristae, have complicated shapes in which two membranes are closely apposed to one another. How

these structures are generated and maintained is largely unknown [2].

Although organelle disassembly and reassembly has been the focus of considerable research, an integrated picture of the way in which components interact to form a functioning cell is still lacking. The two principal obstacles to research in this field are the complexity of the mechanisms of cell division and proliferation, and the absence of techniques for tracing and controlling the process of cell division *in vivo*.

Cell-free systems have proven to be powerful tools for understanding the molecular basis of cellular function. Because cell-free preparations are amenable to a wider range of manipulation than are intact cells, they are particularly useful in biochemical investigations of the cell cycle. It has been shown that *in vitro* nuclear assembly spontaneously in *Xenopus* egg interphase extracts a double nuclear membrane with nuclear pore forms around added chromatin, whether natural animal or plant sperm chromatin or exogenously added procaryotic DNA is used [3-5]. Some information concerning reassembly of nuclear membranes, organelles, and individual nucleosomes has

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arisen from the use of cell-free extracts derived from eggs of *Xenopus* or sea urchin, and somatic cells and embryos of *Drosophila* or *Nicotiana* [2, 6-14].

The Golgi apparatus is one of the most complex intracellular organelles. Its basic units are disc-shaped cisternae, each of which features a flattened central portion that is closely apposed to adjacent cisternae in a stack. Each face of the stack is apposed to an extensive tubular reticulum termed the *cis*- and *trans*-Golgi network [15]. In mammalian cells, four to eight cisternae are stacked in a polarized manner, and numerous such stacks are connected [16]. The Golgi apparatus is disassembled at the onset of mitosis and reassembled at the end of mitosis. The disassembly-reassembly process is generally believed to be essential for equal partitioning of the Golgi apparatus into daughter cells [17]. During mitosis, the Golgi apparatus fragments into tens of thousands of vesicles and short tubules that disperse throughout the cytoplasm. The number and random dispersion of these fragments ensures accurate partitioning. At telephase, a Golgi apparatus is rapidly reassembled from its fragments within each daughter cell.

The endoplasmic reticulum is a structure within eucaryotic cells that is highly variable in shape. Sometimes it exists as a continuum, and sometimes it may be observed as a number of isolated components that can fuse to form a continuum. Structural modifications of the ER, including dilation-vesiculation of rough cisternae, as well as smooth tubule proliferation can also coincide with changes in cytoskeleton organization and changes in cell cycle [7]. At the end of mitosis, membranes are recruited to the chromatin, probably in the form of ER sheets, and fuse side-to-side, encompassing the DNA with an intact double nuclear membrane [18].

Each *Xenopus* egg contains a stockpile of all components of the nucleus and the cytoplasm, including histones, lamins, pore complex components, and membrane vesicles, sufficient to assemble more than 4 000 somatic nuclei. These nuclei are derived from the rapid division of zygote 12 times, which take place in the early embryo [19]. The endoplasmic reticulum, Golgi apparatus, and mitochondria of fertilized ova are present in small membrane tubules and vesicles that, at some point during early development, must fuse to reform the endoplasmic reticulum network, Golgi apparatus, and mitochondria. The machinery for the formation of these structures must also be stored in the egg because there is no synthesis of new material during the first cell divisions [2]. From a biochemical point of view, fragmentation of the cell structures allows their homogenization, with the added advantage that it occurs in such a way as to permit reassembly of the original organelle. If it was possible to mimic reassembly of these

cellular structures using a cellular homogenate *in vitro*, identification of the components underlying organelle structure would be greatly facilitated [20]. *Xenopus* egg extracts provide an extraordinary opportunity for us to achieve this aim.

In the present report, we describe the formation of bilayer or multilayer nuclear envelopes *in vitro* using *Xenopus* egg extracts. This cell-free system was also used to successfully reconstitute many kinds of organelles, including mitochondria, endoplasmic reticulum networks, and Golgi apparatus, by varying the cytosol:vesicle ratio in the reconstitution mixture. Of particular interest is the fact that this report is the first published account of the successful reassembly of mitochondria *in vitro*.

Materials and methods

Preparation of egg extracts

The following protocol was modified from Newport and Spamm [21] and Sider *et al.* [22]. Mature *Xenopus* females were induced to lay eggs by injections of HCG (human chorionic gonadotrophin) the night before use and were placed in 3 L of 110 mM NaCl. The eggs were degelled with three egg volumes of 2% cysteine (pH 7.8) dissolved in 110 mM NaCl for 5-10 min until the eggs were closely packed. The degelled eggs were rinsed three times with MMR (0.1 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 0.1 mM ethylenediaminetetraacetic acid, 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), pH 7.8) to remove cysteine. They were then rinsed twice in S-lysis buffer [250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 100 µg/mL cycloheximide, 5 µg/mL cytochalasin B, 1 mM dithiothreitol (DDT), 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5]. All centrifugation steps were conducted at 4 °C. The eggs were packed by centrifugation at 100 ×g for 30 s, and excess S-lysis buffer was removed thoroughly. The eggs were then crushed by centrifugation at 15 000 ×g for 30 min. As a result, the egg components were distributed in the centrifuge tube with the yolk at the bottom, the crude extract in the middle, and a lipid layer at the top. The crude extract was removed, placed into a new tube, and centrifuged again for 15 min to remove the remaining contaminating yolk and large particulate material. The crude extract was then centrifuged at 200 000 ×g for 2 h using a TLS-55 swinging-bucket rotor at 55 000 rpm in a Beckman Optima TL and TLX. After ultracentrifugation, the crude extracts were laminated into seven layers. These layers (from the top to the bottom of the tube) were lipid, cytosol (egg extracts), membrane vesicles, mitochondria, ribosomes, glycogen, yolk, and pigment granules. The cytosol layer was carefully transferred into a new tube and centrifuged again at 200 000 ×g for 20 min to remove residual membrane vesicles. The membrane vesicle layer was resuspended in S-lysis buffer containing 0.5 M sucrose and centrifuged at 26 000 ×g for 15 min to pellet cytosol-depleted membrane vesicles. The cytosolic and membrane vesicle fractions were quick-frozen in liquid nitrogen as 50 µL and 5 µL aliquots, respectively, and stored at -70 °C.

Preparation of sperm nuclei

Testes were dissected from sexually mature *Xenopus laevis* males and rinsed free of blood. Sperm were released by gently squeezing the

testes in 1 mL of nuclear isolation buffer (NIB) consisting of 15 mM NaCl, 60 mM KCl, 15 mM Tris, 1 mM DDT, 0.5 mM spermin, 0.25 M sucrose, pH 7.5. Somatic tissue was removed by centrifugation at 100 $\times g$ for 1 min at 4 °C. The supernatant fraction containing sperm was then centrifuged at 1 500 $\times g$ for 10 min at 4 °C. We slowly resuspended the white part of the sediment in NIB, carefully avoiding the red cell pellet. Sperm were collected by centrifugation and incubated at 22 °C in 1 mL of NIB containing 0.05% lysolecithin for 8-10 min, at the time when three volumes of cold NIB containing 3% bovine serum albumin were added to the sperm suspension to stop the lysolecithin reaction. Nuclei were then washed three times in NIB. The lysolecithin treatment removed the sperm plasma membrane and nuclear envelope, while leaving the chromatin in its highly condensed state. The density of the demembrated sperm nuclei was adjusted to 4×10^7 /mL, and the suspension was quick-frozen in liquid nitrogen in 5- μ L aliquots and stored at -70 °C.

Nuclear reassembly

Demembrated *Xenopus* sperm (5 μ L) was mixed with 45 μ L of a mixture containing *Xenopus* egg extract and membrane vesicles in an ATP-regenerating system containing 2 mM ATP, 20 mM phosphocreatine, and 50 μ g/mL creatine phosphokinase. The mixture was incubated at 22 °C. Three different ratios of *Xenopus* egg cytosol to membrane vesicle fractions were used: 10:1, 5:1, and 2.5:1.

Fluorescence microscopy

At 15-30-min intervals, a 2- μ L aliquot of the nuclear reconstitution mixture was placed on a slide containing 2 μ L of 2.5% glutaraldehyde and 1 μ L of 5 μ g/mL of the fluorescent DNA dye 4, 6-diamidine-2 phenylindole. The process of sperm pronuclear assembly was observed using a Leica fluorescence microscope.

Transmission electron microscopy

At 30-min intervals, a 10- μ L aliquot of the nuclear reassembly reaction was placed in a tube containing 200 μ L of 2.5% glutaraldehyde, and incubated for 2 h at 4 °C. Samples were washed three times with phosphate buffer (pH 7.4), and post-fixed for 2 h in 1% osmium tetroxide at 4 °C. The fixed mixtures were dehydrated using a graded concentration series of acetone, and embedded in Epon 812. Sections were made at 50-60 nm and stained with uranyl acetate followed by lead citrate. The samples were observed and photographed at 80 kV under a JEM 1010 transmission electron microscope.

Results

Nuclear reassembly at a 10:1 ratio of cytosol to vesicle fractions

When observed using fluorescence microscope, demembrated *Xenopus* sperm (Figure 1A) were found to undergo a series of morphological changes during incubation with *Xenopus* egg extract mixture. The sperm began to expand after 15-30 min of incubation with the egg extract mixture, and they became plumper and shorter than their original long, tadpole-like shape (Figure 1B and 1C). After an additional 15-30 min of incubation, the sperm DNA started to disperse (Figure 1D and 1E). After 1.5 h of

incubation, the sperm DNA was completely decondensed and dispersed, and the sperm became spherical (Figure 1F).

When observed using transmission electron microscope, demembrated *Xenopus* sperm were found to be long and to have densely packed chromatin (Figure 2A). After 30 min of incubation, the demembrated sperm swelled and shortened in length (Figure 2B). The membrane vesicles started to recruit to the rim of the swollen demembrated *Xenopus* sperm. After 60 min of incubation, the sperm became elliptical or spherical, and the bilayer nuclear envelope was partially assembled (arrowheads in Figure 2C). A pair of vesicles about to fuse was observed (arrow in Figure 2C). After incubation for 1.5 h, the reassembled nucleus was observed with double nuclear membrane inserted nuclear pore complexes (Figure 2D). One of the nuclear pore complexes reconstituted was enlarged and is shown (arrow in Figure 2D-1) (Figure 2D-1 was the magnifying figure of right-side nuclear pore complex in Figure 2D). A cytosol:vesicle ratio of 10:1 was optimal for the formation of a normal bilayer membrane containing nuclear pore complex.

Nuclear reassembly at a 5:1 ratio of cytosol to vesicle fractions

Observation by transmission electron microscope showed that the nuclear reassembly process of the

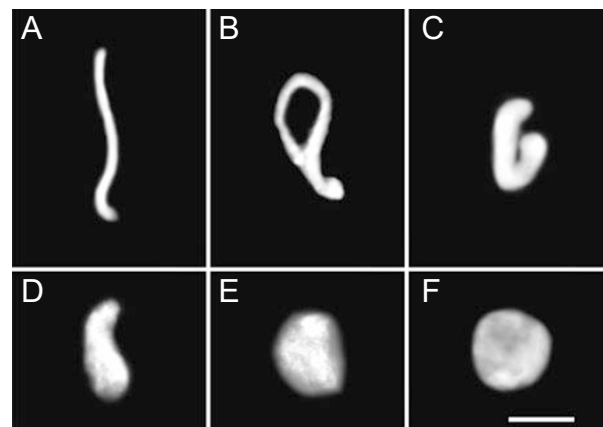


Figure 1 Nuclear reconstitution from demembrated *X. laevis* sperm in *Xenopus* egg extracts visualized using fluorescence microscope. The egg cytosol:vesicle ratio in the reconstitution mixture was 10:1. (A) Demembrated sperm. (B) Demembrated sperm after a 15-min incubation with egg extract. (C) Swollen sperm (30-min incubation). (D) Expanded and dispersed sperm (45-min incubation). (E) Decondensed sperm chromatin (60-min incubation). (F) Completely decondensed chromatin in spherical sperm (90-min incubation). Bar=10 μ m.

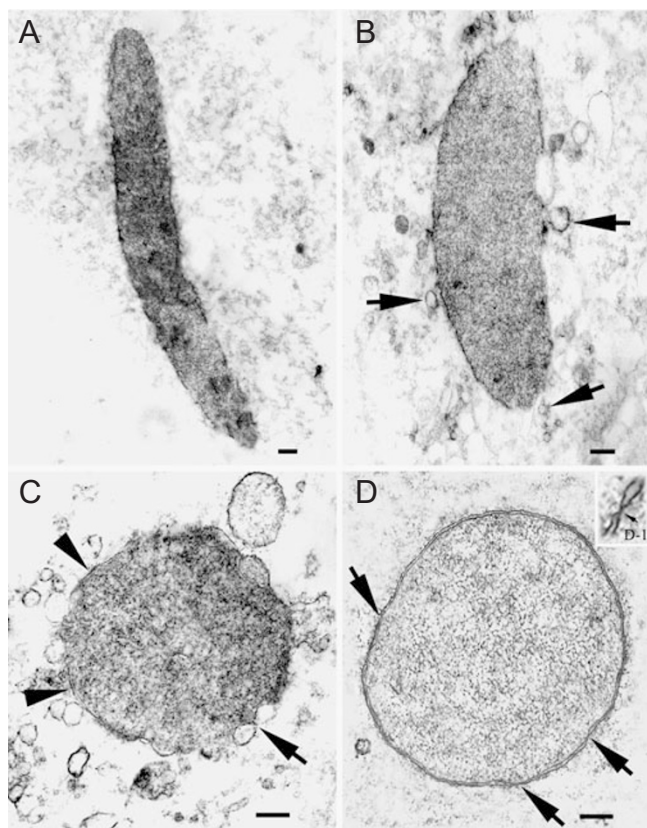


Figure 2 Nuclear reconstitution from demembrated sperm in egg extracts visualized using transmission electron microscope. The egg cytosol:vesicle ratio in the reconstitution mixture was 10:1. **(A)** Demembrated sperm. **(B)** After a 30-min incubation with egg extract, the sperm began to swell, and membrane vesicles (arrowhead) were recruited to the sperm edge. **(C)** After a 60-min incubation, the sperm continued to decondense, and became spherical. A bilayer nuclear membrane (arrowhead) was partially assembled at the sperm periphery. The arrow indicates a pair of fusing vesicles. **(D)** The reassembled nucleus has a double envelope with inserted nuclear pore complexes (arrows). **(D-1)** A nuclear pore complex enlarged from right-side nuclear pore complex **(D, arrow)**. Bar=1 μ m.

demembrated *Xenopus* sperm in a mixture composed of a 5:1 ratio of egg cytosolic to vesicular fractions was quite different from that described above for a 10:1 ratio. After incubation for 30 min, a nuclear reassembly intermediate was observed. This intermediate had dispersed chromatin, and was surrounded by many vesicles (Figure 3A). After incubation for 1 h, another, more highly organized intermediate appeared. This intermediate was surrounded by a membrane (arrow) and contained two small, reassembled “nuclei” (arrowheads in Figure 3B). After 1.5 h of incubation, many unusual structures appeared (Figure 3C and 3D). These structures usually consisted

of multilayer membranes with inserted nuclear pore complexes (arrows in Figure 3C and 3D, and Figure 3C-1 and 3D-1 were, respectively, the section-magnifying figures of Figure 3C and 3D). Sometimes, a bilayer or multilayer membrane separated the chromatin into different areas within the unusual nucleus-like structures (Figure 3C and 3D).

Reassembly of endoplasmic reticulum, mitochondria, and Golgi apparatus at a 2.5:1 ratio of cytosol to vesicle fractions

For the demembrated sperm mixture incubated with an egg cytosol:vesicle ratio of 2.5:1, we observed reconstitution of organelles other than nuclei (endoplasmic reticulum network, Golgi apparatus, and mitochondria). Most of the demembrated sperm chromatin remained condensed after 2 h of incubation (Figure 4A). We did not observe pronucleus-like structures with reassembled nuclear membranes under transmission electron microscope in the 2.5:1 mixture.

Reassembling endoplasmic reticulum structures were observed after 30-60 min of incubation (Figure 4B and 4C). The endoplasmic reticulum reassembly process appeared to be the reverse of the disassembly process. First, the fragmented endoplasmic reticulum vesicles grouped together, and two or more vesicles fused together (1, 2 in Figure 4B). This event was followed by a morphological change to form short cisterna structures. A larger vesicle arising from the fusion of smaller vesicles was observed to be simultaneously changing the shape to form a cisterna and fusing with another vesicle (3 in Figure 4B). These short cisterna structures fused to produce longer and more flattened cisternae, and overlapped each other to form layered stacks of cisternae. An endoplasmic reticulum that is reassembling after 30 min of incubation is shown in Figure 4B. Two sets of stacked cisternae in the process of maturing are shown in Figure 4B-4 and 4B-5. Their appearance is that of ribbon-like structures stacked together. In the assembling endoplasmic reticulum, many ribosome particles are visible in the newly formed cisternae (arrows in Figure 4B). Most of the vesicles that fused to form the endoplasmic reticulum were similar and carried many ribosomes (arrowheads in Figure 4B). The section-magnifying picture of Figure 4B shows ribosomes clearly (arrows in Figure 4B-1). In addition, we discovered that when the proportion of egg cytosol in the reassembly mixture was low, the vesicles fused to form larger vesicular structures rather than the more highly organized cisternal structures (6 in Figure 4B).

A mature, artificially reconstituted endoplasmic reticulum that formed after 1 h of incubation is shown in Figure 4C. It has a complex network structure, as well as

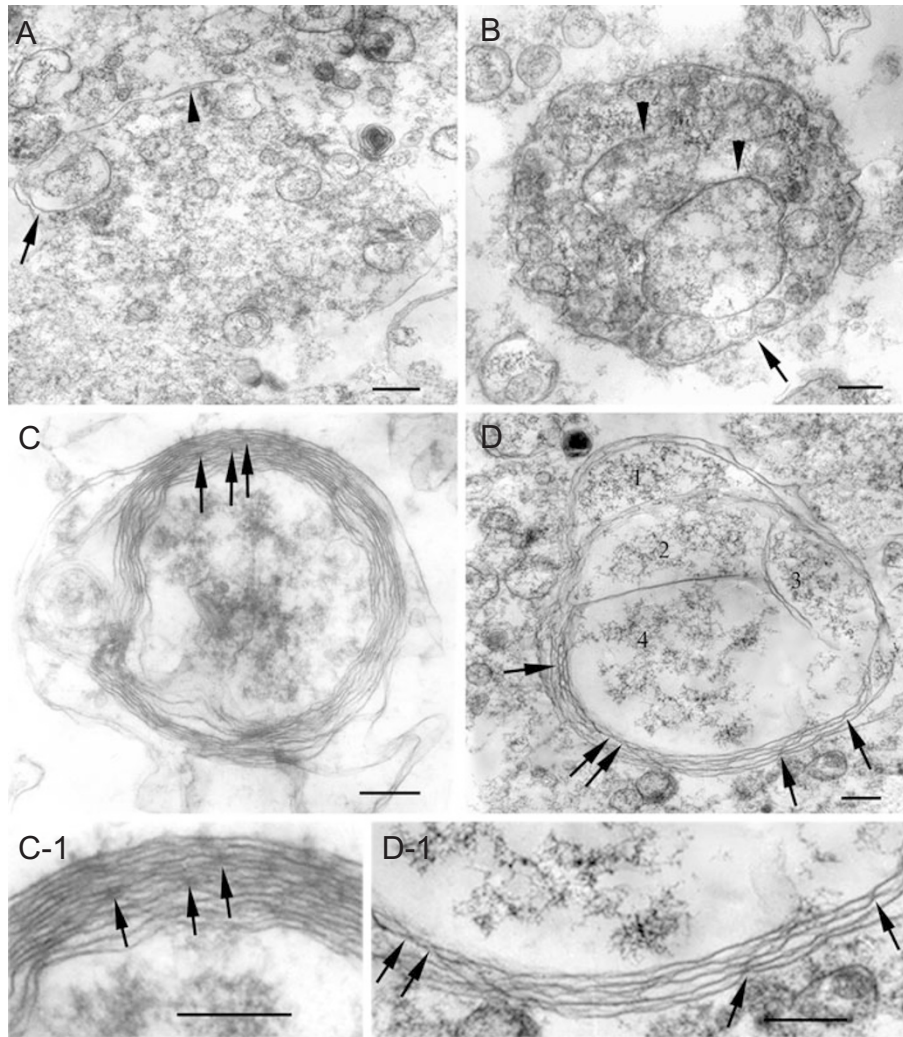


Figure 3 Nuclear reconstitution from demembrated sperm in egg extracts visualized using transmission electron microscope. The egg cytosol:vesicle ratio in the reconstitution mixture was 5:1. **(A)** Demembrated *Xenopus* sperm after a 30-min incubation with egg extract. Many vesicles were recruited to the decondensed *Xenopus* sperm, and some vesicles fused to form larger vesicles (arrow). The membrane structure was partially reassembled (arrows). **(B)** A reconstitution intermediate composed of two reassembled “nuclei” (arrows) surrounded by many vesicles, and embedded within a larger, reconstituting nucleus (arrow; 60-min incubation). **(C)** The reassembled nucleus has a multilayer concentric membrane with inserted nuclear pore complexes (arrowheads; 90-min incubation). **(C-1)** The magnifying figure of section with nuclear pore complexes in **(C)**, arrow. **(D)** A reconstituted nucleus divided into four parts (1-4) by a reassembled membrane decorated with nuclear pore complexes (arrows; 90-min incubation). **(D-1)** The magnifying figure of section with nuclear pore complexes in **(D)**, arrow. Bar=1 μ m.

cisternal junctions. Because sufficient time was elapsed for the reassembly reaction to be complete, junctions (arrow in Figure 4C) between the flattened cisternae had formed.

We observed the reassembly of mitochondrial structures in our cell-free system when the egg cytosol: vesicle ratio was 2.5:1. In Figure 4D, a reassembled mitochondrion (arrowhead) is shown near another that is ready for reassembly (arrow). We presumed that the latter

mitochondrion would complete the assembly process if more vesicles and incubation time were provided. The cristae of the reassembled mitochondrion were well defined and transparent.

We also observed the reassembly of a Golgi apparatus in the 2.5:1 mixture (Figure 4E). Six cisternae were formed and stacked in a polarized manner. Abutting each side of the stacks was a tubular/vesicular network, the *cis*- and

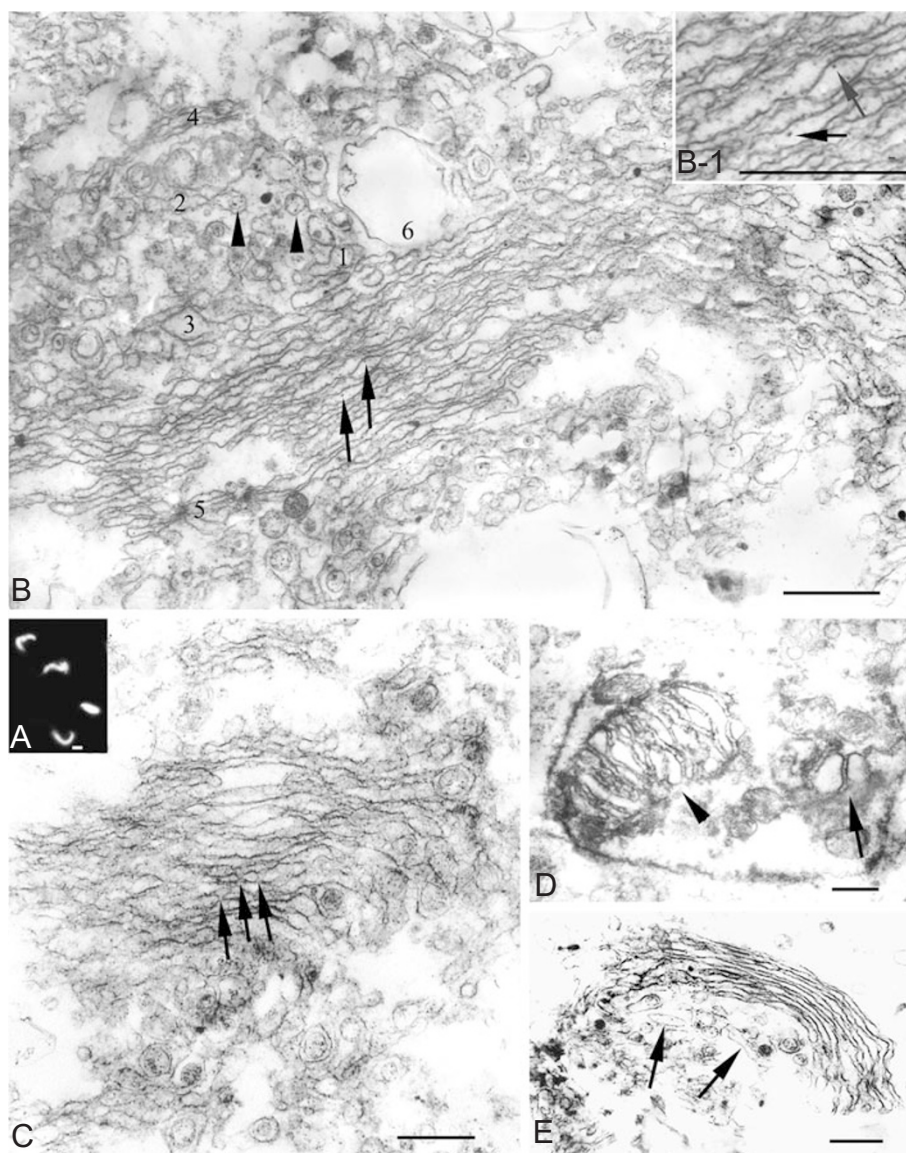


Figure 4 Reconstitution ER, Golgi apparatus, and mitochondria in egg extracts visualized using transmission electron microscope. The egg cytosol:vesicle ratio in the reconstitution mixture was 2.5:1. **(A)** Most of the demembrated sperm chromatin remained condensed after a 2-h incubation with egg extract. **(B)** The endoplasmic reticulum reassembly process. Many ribosomes are present in the reassembled cisternae (arrowheads). Most of the vesicles were of the same type, and carried ribosome particles (arrow). 1-6 in **(C)** show as follows: 1, a pair of vesicles fusing; 2, three vesicles fusing; 3, a larger fused vesicle in the process of simultaneously changing to a flattened shape with a longer cisterna and fusing with another vesicle; 4 and 5, two sets of stacked cisternae being processed; 6, a larger vesicles arising from the fusion of small vesicles. **(B-1)** Section-magnifying picture of **(B)** shows ribosomes clearly (arrows). **(C)** A reassembled endoplasmic reticulum. The arrows indicate the junctions formed between endoplasmic reticulum cisternae (1-h incubation). **(D)** A reassembled mitochondrion (arrowhead) with well-defined, transparent cristae. A second, partially reassembled mitochondrion (arrow) presumably could finish assembly if sufficient time, space, and vesicles were provided. **(E)** A reconstituted Golgi apparatus with six cisternae stacked in a polarized manner. Bar=1 μm .

trans-Golgi networks. The Golgi cisternae were derived from vesicular fusion followed by a change in shape (arrows in Figure 4E). This process was similar to that of ER reassembly (Figure 4B).

Discussion

The technological method to extract cytosol and membrane vesicles from *Xenopus* eggs has

assured the elimination of all organelles because of the ultracentrifugation at 200 000 $\times g$ for 2 h and the breakdown of membrane system triggered by free Ca^{2+} release as a result of egg rupture [23]. Meanwhile, the cytosol and membrane vesicles were checked carefully with transmission electron microscope. No residual organelles were observed in the two parts. The organelles, that are observed in the paper, were derived from the *in vitro* reconstitution in cell-free system.

The endoplasmic reticulum, Golgi apparatus, and mitochondrion reconstituted *in vitro* have similar features of structures with the same kinds of organelles from intact *Xenopus* eggs and other cells according to morphological characteristics [24-26]. The identifications of organelle structures reconstituted in the paper are tentative and based only on apparent morphological similarity.

The observations of organelle reassembly in *Xenopus* egg extracts described in this report suggest that the reassembly of the endoplasmic reticulum and Golgi apparatus is essentially the reverse of the disassembly process that has been elucidated by research *in vivo*. The endoplasmic reticulum and Golgi apparatus appear to have similar reconstitution mechanisms. During the Golgi apparatus and endoplasmic reticulum reassembly processes, only few vesicles from the fragmented organelles first fuse with each other and change their shape to form cisternae (Figure 4B and 4E). These cisternae then stack on top of one another to reconstitute the complex structure of their parent organelles.

In general, repeated fusion of membrane vesicles may be expected to form progressively larger vesicles. Instead, the end products of fusion and assembly are flattened cisternae that are morphologically distinct from the starting material. How does the structure of the flattened cisternae arise? The membrane vesicles may first fuse to form large vesicular structures. These large vesicles then undergo a morphological change, becoming more tubular, and then condense or flatten during the stacking process. Some products of vesicular fusion change their shape to the cisterna shape after the fusion event (3 in Figure 4B), and some of them change their shape at the time of fusion, without time intermission (1 and 2 in Figure 4B).

At the onset of mitosis, the Golgi ribbon is broken down into discrete stacks that undergo fragmentation to yield clusters of vesicles (50-70 nm diameter), large vesicles and tubules (150-250 nm diameter), and cisternal remnants [1, 27]. Disassembly of the Golgi apparatus occurs in two steps, the first of which generates discrete stacks [28]. In the second step, the stacks fragment to yield small vesicles and short tubules, and eventually free vesicles are distributed at random throughout the cytoplasm of the mitotic cell. Up to 10 000 Golgi vesicles are produced, which would,

theoretically, and in a stochastic manner, provide almost every daughter cell with $50\% \pm 1.5\%$ of the original Golgi membrane [29, 30]. The proteins that determine the size and shape of the organelles are unknown, in part because biochemical dissection of the process is always difficult.

Similarly, we conclude that vesicles carrying ribosomes (arrows in Figure 4B and 4C) fuse to reconstitute the rough endoplasmic reticulum. However, the force driving the grouping together of huge populations of endoplasmic reticulum vesicles (Figure 4B) and the mechanisms employed for endoplasmic reticulum reassembling remain unknown.

How are the endoplasmic reticulum and Golgi membrane structures generated? A seemingly important clue comes from the fact that in the absence of cytosol, the membranes can fuse, but they form large, spherical vesicles rather than networks (6 in Figure 4B). Furthermore, cytosol must be present *during* the fusion reaction to mediate tubular network formation. Specific components of the cytosol may thus be required for the vesicle shape change. Thermodynamically, spheres are probably the most stable result of fusion; therefore, one or more cytosolic factors may modify this default reaction to convert it into a fusion reaction that results in a network [2]. In contrast, a low cytosol:vesicle ratio is unfavorable to the formation of the normal bilayer nuclear envelope, instead of the multilayer membrane or multi-double membrane (Figure 3C and 3D).

In our experiments, cytosol was necessary for the reassembly of complex organelle structures, probably because it contains proteins that are involved in vesicle fusion and shape change. High cytosol:vesicle ratios (10:1) were unfavorable to reassembly of organelles other than nuclei, however. One possible explanation for this is that the vesicles may not be at a high enough concentration. Since endoplasmic reticulum networks, Golgi complexes, and mitochondria are composed principally of vesicle components, a certain minimal concentration of vesicles may be essential for the assembly of organelles. Another possible explanation is that a specific balance of cytosolic and vesicular components may be required.

Pharmacological studies indicate that calmodulin is required for late-stage vacuole fusion *in vitro* and *in vivo* [31]. A large proportion of the bound calmodulin is released from the vacuole at Ca^{2+} concentrations below 500 nM, and the Ca^{2+} concentration in the cytosol of living yeast cells is 100-150 nM [32], which is within this range. Released calmodulin can be recovered from the supernatant. Freshly isolated vacuoles (in the presence of 3 μM Ca^{2+}) carry about 0.15 ng calmodulin per microgram vacuolar protein, corresponding to 20-100 molecules per vacuole [31]. Sullivan *et al.* [33] consider that calcium mobilization

is required for nuclear vesicle fusion. An elevated free calcium wave follows fertilization in eggs [34].

Research with various Ca^{2+} chelators suggests that Ca^{2+} fluctuations of $\sim 1 \mu\text{M}$ may be important for endoplasmic reticulum network formation. A chelator with a binding constant in this range had a stronger inhibitory effect than that of chelators with lower or higher binding constants [2]. In other systems, similar observations are explained by the fact that cytosolic Ca^{2+} gradients can be most effectively dispersed by chelator with a binding constant in the range of the average Ca^{2+} concentration in the gradient [33]. It is thus tempting to speculate that a Ca^{2+} -dependent step regulates the fusion event that leads to network formation. A regulatory role for Ca^{2+} has been established in many other fusion reactions. For example, it controls the fusion of secretory vesicles and of vacuoles [31]. We do not observe the obvious differences of free Ca^{2+} levels in all the three different ratios of cytosol to vesicles (data not shown).

In a mature *Xenopus* egg, all of the materials that control membrane breakdown and reassembly are stockpiled in the cell, as shown by the fact that a *Xenopus* zygote can form over 4 000 somatic nuclei [19] during early cell divisions *in vitro*. The extremely plentiful *Xenopus* egg can independently and meticulously regulate the disassembly and reassembly of cell structure during early embryonic development *in vivo*. The components necessary for reassembly of cellular structure are present and stored in *Xenopus* egg extracts, and thus we can imitate this assembly process by using a cell-free system to better understand the mechanism of cell reconstitution.

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