Roles of Cytosol and Cytoplasmic Particles in Nuclear Envelope Assembly and Sperm Pronuclear Formation in Cell-free Preparations from Amphibian Eggs

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ABSTRACT A cell-free cytoplasmic preparation from activated *Rana pipiens* eggs could induce in demembranated *Xenopus laevis* sperm nuclei morphological changes similar to those seen during pronuclear formation in intact eggs. The condensed sperm chromatin underwent an initial rapid, but limited, dispersion. A nuclear envelope formed around the dispersed chromatin and the nuclei enlarged. The subcellular distribution of the components required for these changes was examined by separating the preparations into soluble (cytosol) and particulate fractions by centrifugation at 150,000 *g* for 2 h. Sperm chromatin was incubated with the cytosol or with the particulate material after it had been resuspended in either the cytosol, heat-treated (60 or 100°C) cytosol or buffer. We found that the limited dispersion of chromatin occurred in each of these ooplasmic fractions, but not in the buffer alone. Nuclear envelope assembly required the presence of both untreated cytosol and particulate material. Ultrastructural examination of the sperm chromatin during incubation in the preparations showed that membrane vesicles of ~200 nm in diameter, found in the particulate fraction, flattened and fused together to contribute the membranous components of the nuclear envelope. The enlargement of the sperm nuclei occurred only after the nuclear envelope formed. The pronuclei formed in the cell-free preparations were able to incorporate [^{3}H]dTTP into DNA. This incorporation was inhibited by aphidicolin, suggesting that the DNA synthesis by the pronuclei was dependent on DNA polymerase-α. When sperm chromatin was incubated >3 h, the chromatin of the pronuclei often recondensed to form structures resembling mitotic chromosomes within the nuclear envelope. Therefore, it appeared that these ooplasmic preparations could induce, in vitro, nuclear changes resembling those seen during the first cell cycle in the zygote.

In many species, the cell cycles initiated in the early embryo by fertilization consist of rapidly alternating periods of DNA replication and mitosis (1–3). During the first cell cycle, which is usually longer than succeeding ones, the nucleus of the fertilizing sperm undergoes a transformation into an interphase nucleus (for review, see references 4 and 5). Shortly after the sperm fuses with the plasma membrane of the egg, the nuclear envelope surrounding the sperm chromatin breaks down, the highly condensed sperm chromatin becomes dispersed and a new nuclear envelope is assembled at the periphery of the chromatin to form the male pronucleus. The male pronucleus increases in size, synthesizes DNA, associates with the female pronucleus, and enters mitosis.

The changes in the sperm nucleus following fertilization and during the first cell cycle of zygotes are similar to the changes in the nuclei of other proliferating cells. In both cases, nuclear envelope assembly, chromatin decondensation, DNA synthesis, and chromosome condensation occur in a similar manner. Cell fusion experiments have shown that in proliferating cells these events are controlled by cytoplasmic factors that are active at specific phases in the cell cycle (6). Similarly, the induction of chromatin decondensation, DNA synthesis, and mitosis in nuclei transplanted from embryonic or nondividing somatic cells into activated eggs, suggests that cytoplasmic factors control nuclear behavior in the zygote (4–9). Therefore, nuclear behavior may be controlled by similar

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cytoplasmic substances in both zygotes and proliferating cells.

The investigation of the molecular basis of the cytoplasmic control of nuclear behavior would be facilitated if the nuclear behavior observed during the cell cycle of intact cells could be reproduced in a cell-free system. Eggs may offer an ideal source of material for such a cell-free system since during oogenesis they accumulate a large store of cellular components required for the rapid cell proliferation that follows fertilization (10). In previous studies, ooplasmic preparations have been shown to induce, in vitro, the decondensation of hen erythrocyte (11) and sea urchin sperm chromatin (12), as well as the initiation of DNA synthesis in isolated somatic cell nuclei (13). We have reported that a cytoplasmic preparation from activated *Rana pipiens* eggs can induce demembranated *Xenopus laevis* sperm nuclei to transform into pronuclei and then mitotic chromosomes (14). In the present experiments, we show that the transformation of the sperm nuclei into pronuclei requires both the soluble and particulate cytoplasmic components found in a heavy ooplasmic fraction and that the particulate material contributes membrane vesicles that form a nuclear envelope in the presence of soluble ooplasmic factors.

MATERIALS AND METHODS

Preparation of Sperm Nuclei: Sperm nuclei were prepared as previously described (14). Testes were dissected from sexually mature *X. laevis* that had been injected with of 100 I.U. human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO) and kept for 1 h at 22°C. They were washed free of blood and incubated overnight at 18°C in 200% Steinberg's solution containing antibiotics (15) and human chorionic gonadotropin (10 I.U./ml). Sperm were released by gently squeezing the testes, collected by centrifugation at 1,500 *g* for 10 min, and treated for 5 min at 22°C with nuclear isolation medium (16), which contained 0.5% lysolecithin and 1 mg/ml soybean trypsin inhibitor (both from Sigma Chemical Co.). Lysolecithin-treated sperm were washed once with ice-cold nuclear isolation medium + 3% bovine serum albumin (BSA; fraction V, Sigma Chemical Co.), three times with nuclear isolation medium + 0.4% BSA and once with buffer (see below) before use. The suspension contained ~95% sperm nuclei and 5% nuclei from other cells, mostly erythrocytes. For some experiments the isolated sperm nuclei were stored at −80°C in 30% glycerol in nuclear isolation medium. Nuclei stored in this manner were washed extensively with buffer (see below) before use.

Preparation of Cytoplasmic Fractions: Female *R. pipiens* were primed by an injection of 1/6 of a pituitary, kept at 18°C for 24 h and then induced to ovulate by injection of one pituitary and 1 mg progesterone dissolved in corn oil. 40–48 h later, eggs were removed from the ovisac and enzymatically dejellied, in 1% $Na_2HPO_4 7H_2O$, 0.5% crude papain (crude powder, type II; Sigma Chemical Co., St. Louis, MO) and 0.4% cysteine-HCl (Sigma Chemical Co.). The eggs were washed well in 0.1 M NaCl and 200% Steinberg's solution and any damaged or activated eggs were removed. While in 200% Steinberg's solution, the dejellied eggs were activated by an electric shock (80 V, 200 ms), and then incubated in 20% Steinberg's at 19 ± 1°C for 1 h.

Cytoplasmic fractions were prepared as previously described (14). The activated dejellied eggs were washed in ice-cold buffer which consisted of 250 mM sucrose, 200 mM KCl, 1.5 mM $MgCl_2$, 2.0 mM β-mercaptoethanol and 10 mM Tris-HCl at pH 7.5 and transferred to 5 ml centrifuge tubes containing ice-cold buffer. After the excess medium was withdrawn, the eggs were crushed, without homogenization, by centrifugation at 9,000 *g* for 15 min at 2°C. In most experiments, the layer of heavy supernatant above the packed pigment and yolk (Fig. 1*A*) was transferred to an 0.8-ml tube and centrifuged at 9,000 *g* for 30 min to remove most of the pigment. In cytoplasmic preparations made in a similar manner the buffer contributed approximately one-third of the soluble part of the preparation (17). Lysolecithin-treated sperm was incubated at 18°C in 200 μl of the cytoplasmic fraction to give a concentration of 5×10^4 to 1×10^5 sperm/ml.

The soluble and particulate cytoplasmic components were separated by centrifuging the cytoplasmic preparation in a small tube (0.8 ml, Beckman Instruments, Inc., Palo Alto, CA) at 150,000 *g* for 2 h (Fig. 1*B*). The fluffy part of the pellet, which contains cytoplasmic vesicles, was resuspended, before mixing with the sperm nuclei, in either the supernatant or the supernatant that had been heated at 60°C or 100°C for 10 min and centrifuged at 1,500 *g* for 10 min to remove the precipitated material (heat-treated supernatant), or 1/3 buffer. The final volume of each mixture was adjusted to equal its initial volume.

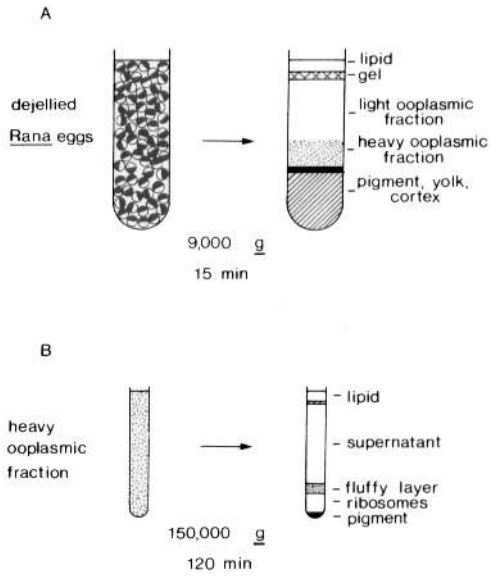

FIGURE 1 Diagram of the ooplasmic fractions obtained following the centrifugation of dejellied *R. pipiens* eggs. The centrifuge used was a Beckmann L3-4 with an SW-50.1 rotor.

Assay for Nuclear DNA Synthesis: To determine whether the sperm nuclei were induced to synthesize DNA, we mixed 100 μl of the ooplasmic preparation with an equal volume of 1/3 buffer containing [methyl-^{3}H]thymidine-5′-triphosphate ([^{3}H]dTTP[1]; 44 Ci/mmol, Amersham) at a concentration of 40 or 80 μCi/ml. Aphidicolin (a gift from Dr. J. Rossant) was dissolved in dimethysulfoxide at a concentration of 5 mg/ml and added to the 1/3 buffer at twice the desired concentration. As a control, dimethylsulfoxide alone was added to the 1/3 buffer. The ooplasmic preparation was then diluted with an equal volume of 1/3 buffer containing both the label and the drug. Freshly prepared sperm nuclei were suspended in the ooplasmic preparations and incubated at 18°C. Samples taken from the incubation mixture at various times were fixed and processed for autoradiography.

Cytological and Histological Procedures: Aliquots of the incubation mixture were fixed in a cold mixture of ethanol and acetic acid (3:1). The fixed material was stained by the Feulgen procedure and portions were transferred to a droplet of 50% acetic acid on a microscope slide and squashed. The squashed preparations were frozen in a mixture of dry ice and ethanol, the coverslips were removed and the slides were air-dried. The specimens were restained with 2% Giemsa in 0.01 M phosphate buffer at pH 6.8 and after drying covered with Pro-Texx (Scientific Products, McGaw Park, IL). The area of the sperm nuclei in the squashed preparations was determined using the formula for the area of an ellipse, after the lengths of the long and short axes had been measured.

In some cases, portions of the incubation mixture were fixed with Smith's solution, stained and sectioned for histological examination as previously described (18).

Autoradiographic Procedures: The squashed specimens were washed twice in cold 5% trichloroacetic acid, rinsed in running water for 1 h, air-dried, and coated with Kodak NTB2 emulsion. Slides were exposed for 14 to 17 d at 4°C and developed in Kodak D-19.

The relative amount of [^{3}H]dTTP incorporated by a sperm nucleus was expressed as the corrected grain count per nucleus. The corrected grain count per nucleus was obtained by counting the number of grains in a 100- or 400-μm^2 area in which the nucleus was located and subtracting from this value the number of grains found in an equivalent area surrounding the nucleus.

Preparation for Electron Microscopy: Freshly prepared sperm nuclei were incubated at 18°C in an undiluted ooplasmic preparation or in one of its cytoplasmic fractions. Aliquots of the incubation mixture were fixed overnight in ice-cold 2% glutaraldehyde buffered with 0.05 M phosphate buffer at pH 7.4. Nuclei were recovered by centrifugation at 1,500 *g* for 5 min, washed three times with phosphate buffer, postfixed for 2 h at room temperature in 1% osmium tetroxide, dehydrated through a graded concentration series of ethanol to propylene oxide, and embedded in Epon 812. Sections were made at 50 to 60 nm, stained with uranyl acetate, followed by lead citrate and examined at 60 kV on a Philips 201 electron microscope.

[1] *Abbreviations used in this paper*: [^{3}H]dTTP, [^{3}H]thymidine triphosphate.

RESULTS

Behavior of Sperm Chromatin in Ooplasmic Fractions

Crushing activated *R. pipiens* eggs by centrifugation at 9,000 *g* for 15 min resulted in a crude separation of ooplasmic components (Fig. 1*A*). Yolk, cortex and some pigment were found in the pellet. The supernatant obtained by this centrifugation consisted of two layers: an upper layer, which we call the "light ooplasmic fraction," and a more viscous and heavily pigmented lower layer, which we call the "heavy ooplasmic fraction." When 5 ml of eggs are crushed by centrifugation, ~1.4 to 1.6 ml of the light ooplasmic fraction and 0.6 to 0.8 ml of the heavy ooplasmic fraction is obtained. The centrifugation buffer is thought to contribute about one-third of the volume of the supernatants (17).

The lysolecithin-treated sperm nuclei were incubated either in the light or in the heavy ooplasmic fraction or in the buffer used in the preparation of these fractions (Table I). The sperm nuclei incubated in the heavy ooplasmic fractions underwent a series of changes in their morphology. In all cases, at the start of the incubation, the sperm chromatin, found in a long, thin form that is characteristic of the sperm nucleus, could be stained deeply with Giemsa (Fig. 2*A*). We refer to nuclei with this morphology as type A nuclei. Within 1 h, the nuclei changed to a round or oval shape, but their chromatin could still be stained deeply (type B nuclei; Fig. 2*B*). These nuclei then began to enlarge, as their peripheral chromatin became more decondensed, to form nuclei with an inner core deeply staining chromatin and a peripheral portion of lightly staining chromatin (type C nuclei; Fig. 2*C*). The process of nuclear enlargement gradually continued until the chromatin was completely decondensed, forming pronuclei whose chromatin was uniformly stained lightly by Giemsa (type D nuclei, Fig. 2*D*). The heavy ooplasmic fraction was able to transform both freshly prepared sperm nuclei and those that had been stored at −80°C in 30% glycerol into pronuclei (type D nuclei) during a 3-h incubation. This effect of the heavy fraction was observed not only with *Xenopus* sperm nuclei, but also with the nuclei isolated from *R. pipiens* sperm (Table I). In contrast, the morphology of the sperm nuclei did not change when they were incubated for 3 h in the buffer used for preparing the ooplasmic fractions or in the buffer that was diluted to one-third its original concentration. Similarly, the chromatin of sperm nuclei that were incubated in the light ooplasmic fraction remained condensed during a 3-h incubation, although in this case the nuclei often changed to a round or oval shape (Table I). These results clearly indicate that the heavy ooplasmic fraction contains the components that could induce the formation of the sperm pronucleus and that these components are lacking or greatly reduced in the light ooplasmic fraction.

We examined the effect of diluting the heavy ooplasmic fraction on its ability to decondense sperm nuclei (Table I).

TABLE I

Behavior of Sperm Nuclei during 3-h Incubation in Cell-free Preparations of Activated R. pipiens Eggs

Incubation conditions	No. of nuclei	Percentage of nuclei			
		Type A and B*	Type C*	Type D*	Other‡
Xenopus sperm					
Light fraction	905	68	4	1	27
Heavy fraction	464	1	16	82	1
Diluted heavy fraction (1/2)	703	17	32	47	4
Diluted heavy fraction (1/4)	655	91	8	0	1
Buffer	636	95	0	0	5
1/3 buffer	1,230	94	0	0	6
Rana sperm					
Heavy fraction	557	0	10	89	1
1/3 buffer	558	95	0	0	5

* Refer to text.
‡ Damaged or nonsperm nuclei.

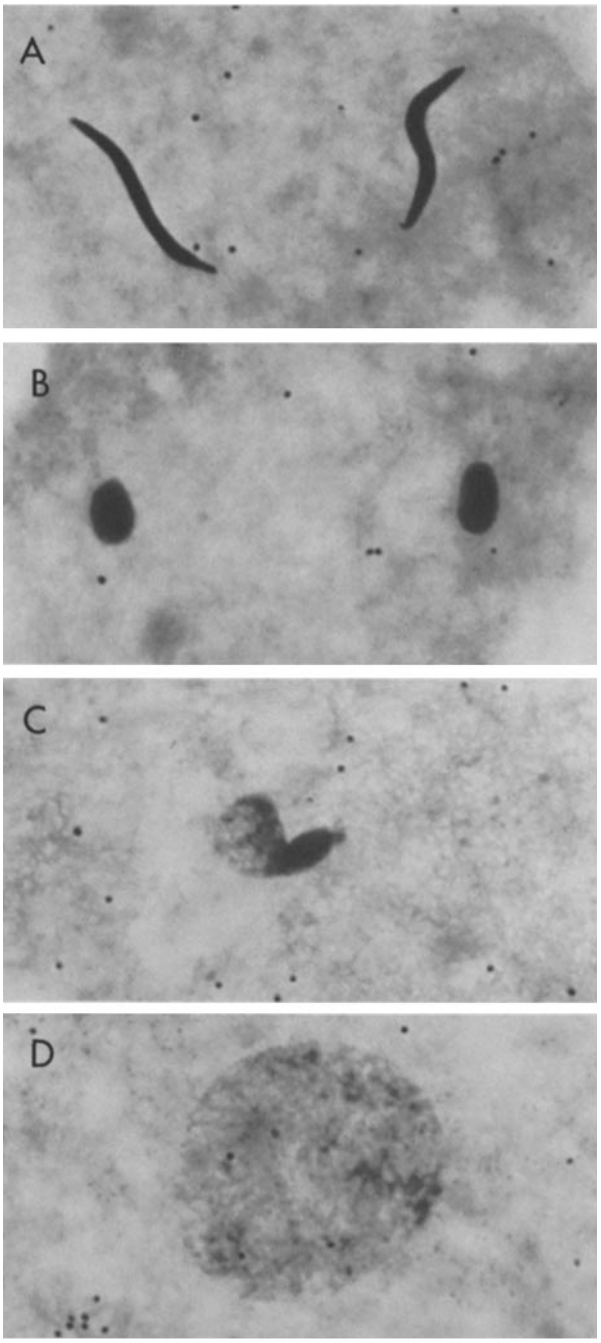

FIGURE 2 The morphology of *X. laevis* sperm nuclei at various times during incubation in ooplasmic preparations. (*A*) Condensed sperm nuclei (type A nuclei), 0 min. (*B*) Round sperm nuclei (type B nuclei), 60 min. (*C*) Partially decondensed sperm nucleus (type C nucleus), 90 min. (*D*) Completely decondensed sperm nucleus (type D nucleus), 180 min. × 1,000.

Sperm nuclei were incubated for 3 h in equal volumes of the undiluted heavy fraction or in the fraction after it was diluted to one-half or one-quarter of its original concentration with 1/3 buffer. The results show that the ability to induce pronuclear formation was dependent upon the concentration of the heavy fraction. In the undiluted heavy fraction, 82% of the sperm nuclei formed pronuclei within 3 h. On the other hand, only 47% of the sperm nuclei formed pronuclei in this fraction after it was diluted by one-half, and none of the sperm nuclei formed pronuclei in the fraction diluted to one-quarter (Table I).

As the sperm chromatin decondensed during incubation in the heavy ooplasmic fraction, the nuclei enlarged. This change could be quantitatively expressed as an increase in the area of the sperm nuclei in the squash preparations. The areas of pronuclei (type D nuclei) that formed during a 3-h incubation in undiluted or half-diluted heavy fractions was determined. The results showed that while the average area of the decondensed nuclei varied among experiments, in each experiment the mean area of the nuclei incubated in the undiluted heavy ooplasmic fraction was consistently about twice that of the nuclei incubated in the diluted fraction. For example, the average areas of 50 pronuclei formed during a 3-h incubation in undiluted preparations were 119 ± 50, 188 ± 97 and 352 ± 124 μm^2 in three different experiments, whereas the areas of those formed in half-diluted preparations in the same experiments were 61 ± 17, 86 ± 36, and 154 ± 53 μm^2, respectively. These results indicate that the ooplasmic components in the heavy fraction induce pronuclear formation and that their effect is concentration-dependent.

Assembly of the Nuclear Envelope

To further investigate the processes of pronuclear formation in vitro, we observed sperm nuclei incubated in the heavy ooplasmic fraction at an ultrastructural level. Previously, we found that treatment of *X. laevis* sperm with lysolecithin removed most of their plasma membrane and nuclear envelope, leaving the highly condensed chromatin intact (14). The morphology of these sperm nuclei remained unchanged for at least 3 h if they were incubated either in the buffer or in the buffer diluted to one-third of its initial concentration. In contrast, the sperm chromatin incubated in the heavy ooplasmic fraction was uniformly dispersed to become a less electron-dense, fibrous structure within 5 min. However, it should be noted that while the sperm chromatin was found to be dispersed within 5 min when it was examined ultrastructurally, no change in the chromatin could be discerned when the nuclei were examined under the light microscope at this time. Instead, the chromatin retained its elongate form and could still be stained deeply by Giemsa.

During incubation, a nuclear envelope began to assemble at the periphery of the sperm chromatin from vesicles that had previously been dispersed in the heavy ooplasmic fraction. From the morphology of vesicles observed near the periphery of the chromatin in samples incubated for 30 min, the sequence of changes that lead to the formation of the nuclear envelope was constructed (Fig. 3, *A–H*). Initially, the periphery of the dispersed chromatin was devoid of nuclear envelope and therefore directly exposed to the cytoplasmic components (Fig. 3*A*). Cytoplasmic vesicles having a diameter of 180 to 200 nm and containing electron-opaque material, were soon found at many sites along the periphery of the sperm chromatin (Fig. 3*B*). These vesicles fused to each other and flattened (Fig. 3*C*) to form the double membrane of the nuclear envelope. Electron-dense material (Fig. 3*E*) accumulated on the outer membrane of the flattened vesicles. Nuclear pore structures were formed in the flattened vesicles, possibly from the material that adhered to the outer membranes, at sites where the inner and outer membranes had coalesced (Fig. 3, *E–F*). Vesicles added to the periphery of the chromatin continued to fuse and flatten at the edges of the nascent nuclear envelope (Fig. 3*F*) until a continuous nuclear envelope, containing pores, enclosed the chromatin (Fig. 3*G*). The pores were also seen in sections tangential to the membrane (Fig. 3*H*). Although our observations clearly indicate that membrane vesicles that had a single membrane and contained electron-opaque material contributed membrane material to the formation of the nuclear envelope around the sperm chromatin, we cannot rule out the possibility that other membrane vesicles are also involved in nuclear envelope assembly.

The proportion of the perimeter of the sperm chromatin that was covered with nascent nuclear envelope increased with time during incubation in the cytoplasmic preparations. In nuclei examined at 30 min after incubation, only short fragments of nuclear envelope that covered less than one half of the chromatin contour, were found, although cytoplasmic vesicles had aligned at many sites on the chromatin periphery. By 60 min, however, 85% of the nuclei had at least half of the periphery of the chromatin covered with a nascent nuclear envelope, and by 90 min, practically all of the nuclei observed were enclosed by a continuous nuclear envelope.

Although nuclear envelope assembly around the sperm chromatin was usually complete by 90 min, no flattened membranes of any kind, resembling either the nuclear envelope or annulate lamellae, even in a fragmented form, were found in ooplasmic preparations that were examined after incubation for 180 min without sperm chromatin. Therefore, it is highly likely that the presence of sperm chromatin is required for the formation of the nuclear envelope from cytoplasmic membrane vesicles.

Ooplasmic Components Required for Nuclear Decondensation and Nuclear Envelope Assembly

The heavy ooplasmic fraction contains both soluble components and particulate material. Centrifugation of this fraction at 9,000 *g* for 30 min removed most of the pigment without a loss of the ability to induce pronuclear formation. However, as seen in Table II, the supernatant obtained from the heavy ooplasmic fraction after a centrifugation at 150,000 *g* for 120 min was unable to transform sperm nuclei into pronuclei during a 3-h incubation, although the nuclei often changed to a round or oval shape that could still be stained deeply by Giemsa. In contrast, pronuclei were formed when sperm nuclei were incubated for 3 h in the supernatant to which the particulate components, which had been sedimented in the fluffy layer during centrifugation (Fig. 1*B*), were returned and resuspended. These results, taken together with the ultrastructural observations described above, demonstrate that the presence of particulate components including cytoplasmic vesicles are prerequisite for the transformation of sperm nuclei into pronuclei.

To examine the role of soluble components in pronuclear formation, we incubated sperm nuclei with the fluffy part of the pellet after it had been resuspended in one of the following media: (*a*) the supernatant obtained by centrifugation of the

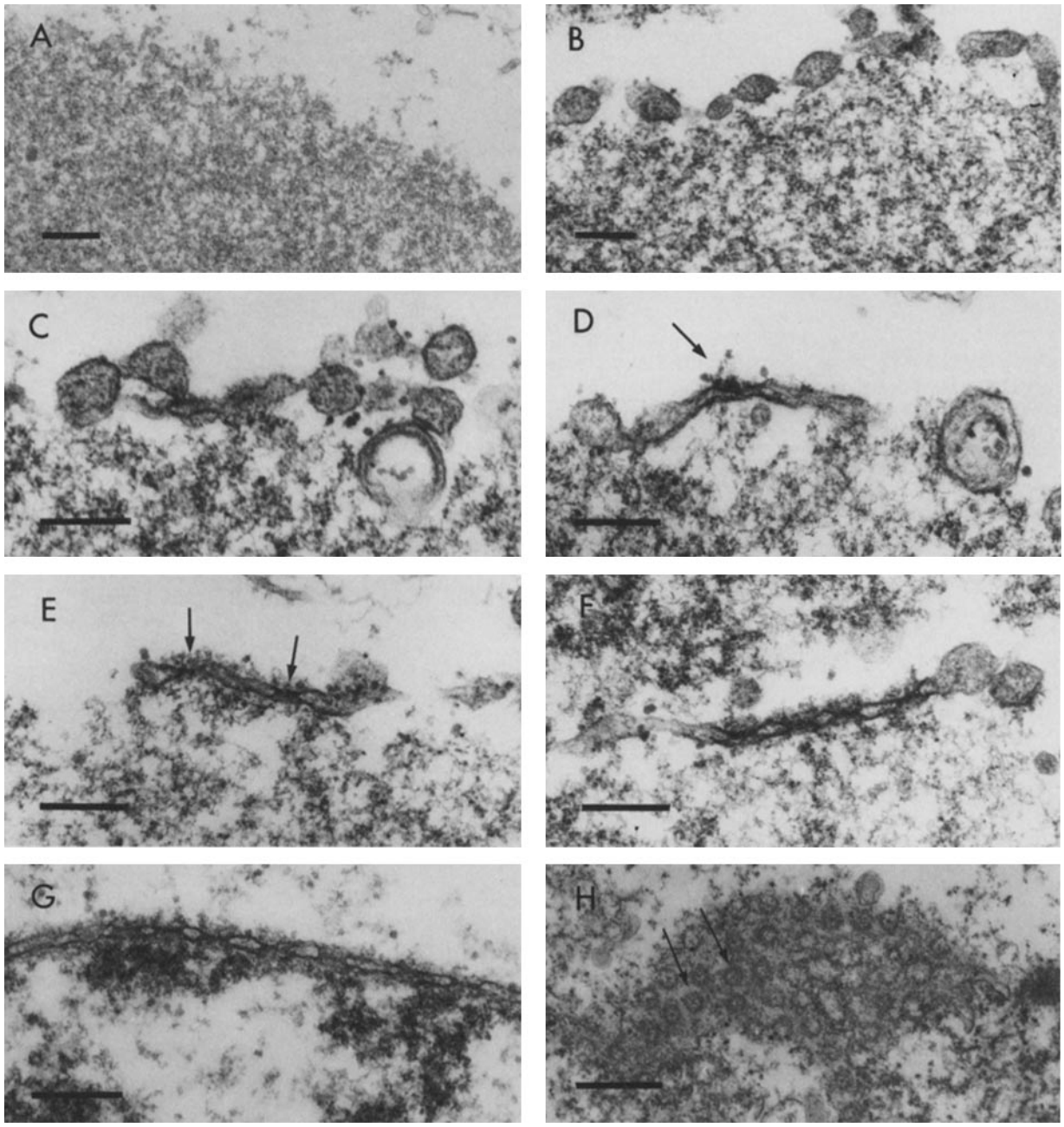

FIGURE 3 The sequence of changes resulting in the formation of a nuclear envelope. (*A*) The periphery of the sperm chromatin is devoid of nuclear envelope. × 35,500. (*B*) Cytoplasmic vesicles of ~200 nm in diameter accumulate at many sites along the periphery of the chromatin. × 35,500. (*C*) Vesicles in the process of fusing to each other and flattening to form the double membrane of the nuclear envelope. × 54,000. (*D*) Electron-dense material (arrow) accumulates on the outer membrane of the flattened vesicle. × 54,000. (*E*) Pores (arrows) form in the flattened vesicles, thereby forming short fragments of nuclear envelope. × 54,000. (*F*) Vesicles continue to fuse to the edges of the fragments of nascent nuclear envelope. × 54,000. (*G*) The nuclear envelope that entirely encloses the sperm chromatin. × 54,000. (*H*) Nuclear pores (arrows) found in a section tangential to a nuclear envelope. × 52,800. (*A*) 5-min incubation; (*B–F*) 30-min incubation; (*G–H*) 120-min incubation. Bars, 0.25 μm.

light ooplasmic fraction at 150,000 *g* for 2 h; (*b*) the heat-treated supernatant from the heavy ooplasmic fraction, obtained by centrifugation at 150,000 *g* for 2 h and then exposed to 60°C for 10 min; or (*c*) 1/3 buffer. In each of these cases, only a small percentage of the sperm nuclei were induced to form pronuclei, usually type C nuclei, except for the 5% that formed type D nuclei in the heat-treated supernatant (Table II). These results indicate that the particulate components are not sufficient to induce pronuclear formation. Rather, both soluble factors and particulate components contained in the heavy ooplasmic fraction are required. The soluble factors responsible for these effects appear to be large, heat-labile molecules, since neither the components in the heat-treated supernatant nor in the supernatant from the light ooplasmic fraction are able to complement the effect of the vesicular components that induces sperm chromatin decondensation.

We also determined the proportion of the sperm chromatin periphery that was covered by nascent nuclear envelope during a 2-h incubation in cytoplasmic fractions similar to those described above. As shown in Table III, continuous nuclear

TABLE II

Behavior of Sperm Nuclei during 3-h Incubation in Fractions of Cytoplasmic Preparations Obtained by Centrifugation at 150,000 g for 2 h

Cytoplasmic fraction	No. of nuclei	Percentage of nuclei			
		Type A and B*	Type C*	Type D*	Other‡
Heavy fraction (uncentrifuged)	2,705	1	5	81	12
Supernatant (heavy fraction)	2,023	83	3	0	14
Supernatant (heavy fraction) + pellet	2,337	0	6	87	7
Supernatant (light fraction) + pellet	1,185	85	5	0	10
Heat-treated supernatant‖ (heavy fraction) + pellet	1,209	81	6	5	8
1/3 buffer + pellet	2,344	95	0	0	5

* Refer to text.
‡ Damaged or nonsperm nuclei.
‖ 60°C for 10 min.

TABLE III

Nuclear Envelope Assembly on Sperm Chromatin Incubated for 2 h in Fractions of the Cytoplasmic Preparation Obtained by Centrifugation at 150,000 g for 2 h

Cytoplasmic fraction	No. of nuclei	Percentage of nuclei			
		Percentage of the chromatin perimeter lined by nuclear envelope			
		0	<50	>50	100
Supernatant	50	100	-	-	-
Heat-treated* supernatant + pellet	50	98	2	-	-
1/3 buffer + pellet	60	92	8	-	-
Supernatant + pellet	39	-	5	20	75

* 100°C for 10 min.

envelopes were assembled around sperm chromatin only when it was incubated with both the untreated supernatant from the heavy fraction and the particulate cytoplasmic material. Sperm nuclei incubated in the supernatant, from which all particulate material had been removed by centrifugation, failed to form nuclear envelopes (Fig. 4*A*). Although these nuclei failed to form interphase nuclei, their chromatin became dispersed and less electron-dense, to the same extent as seen during the initial phase of sperm chromatin dispersion that was induced in the heavy cytoplasmic fraction containing vesicles. In contrast, when the sperm chromatin was incubated in the supernatants to which the vesicle-containing fraction was returned and resuspended, complete nuclear envelopes were found at the periphery of most nuclei (Fig. 4*B*). However, when the chromatin was incubated with the vesicle-containing fraction that had been resuspended either in 1/3 buffer or in the heat-treated supernatant exposed to 100°C for 10 min, the vesicles found at the chromatin periphery had neither fused to each other nor flattened to form a nuclear envelope (Fig. 4, *C–D*), except for a few cases in which only very small segments of nuclear envelope were found (Table III). Under these conditions, the sperm nuclei lacking a nuclear envelope failed to develop to a pronucleus, although the chromatin dispersed to about the same extent as the chromatin incubated in the soluble cytoplasmic components alone. Taken together, these results strongly suggest that interactions among the heat-labile, soluble cytoplasmic components, cytoplasmic vesicles, and chromatin are prerequisite for nuclear envelope assembly.

Other Activities of the Pronuclei

It was previously shown that the pronuclei that formed during a 3-h incubation in the heavy fraction containing [^{3}H]dTTP could synthesize DNA. The accumulation of radioactive label by the decondensed nuclei was examined by autoradiography. Both the completely decondensed sperm nuclei (type D nuclei) and nuclei whose chromatin had not yet decondensed completely, (type C nuclei), could incorporate the radioactive label. In contrast, sperm nuclei whose morphology remained unchanged during the incubation did not incorporate the label, nor did sperm nuclei that were incubated in 1/3 buffer containing [^{3}H]dTTP without the ooplasmic fraction.

Aphidicolin, a specific inhibitor of DNA polymerase-α, has been shown to inhibit the DNA synthesis required for chromosome replication (19). To examine whether or not the DNA synthesis observed here is related to chromosome replication, we tested the effect of aphidicolin on the incorporation of [^{3}H]dTTP by decondensed sperm nuclei. Sperm nuclei were incubated for 3 h in the heavy ooplasmic fraction after it was diluted by one-half with 1/3 buffer containing [^{3}H]-dTTP and either aphidicolin or DMSO, the vehicle solvent for aphidicolin. The ability of the heavy ooplasmic preparation to induce pronuclear formation was not affected by aphidicolin. However, aphidicolin inhibited the incorporation of [^{3}H]dTTP by the decondensed sperm nuclei. When aphidicolin was present at concentrations of 5 μg/ml or 25 μg/ml, <2% of the pronuclei, (type D nuclei), had grains counts that exceeded the background level. In contrast, when aphidicolin was not present, >98% of these pronuclei had grain counts that exceeded the background level. Therefore, we conclude that the DNA synthesis by the pronuclei results from the activity of DNA polymerase-α, the enzyme thought to be involved in chromosomal DNA replication in eucaryotes.

We have shown previously that the chromatin of pronuclei formed during a 3-h incubation in the undiluted heavy ooplasmic fraction often condensed again to form structures that resemble mitotic chromosomes when incubated for >3 h (14). Although these chromatin structures resemble chromosomes formed at metaphase, histological examination showed that the nuclear envelope was found to have remained intact, enclosing the chromosomes. The condensed chromatin was usually attached to the inside of the nuclear envelope. It should also be noted that the recondensation of the sperm chromatin to form mitotic chromosomes occurred only in the undiluted heavy ooplasmic fraction. Sperm nuclei incu-

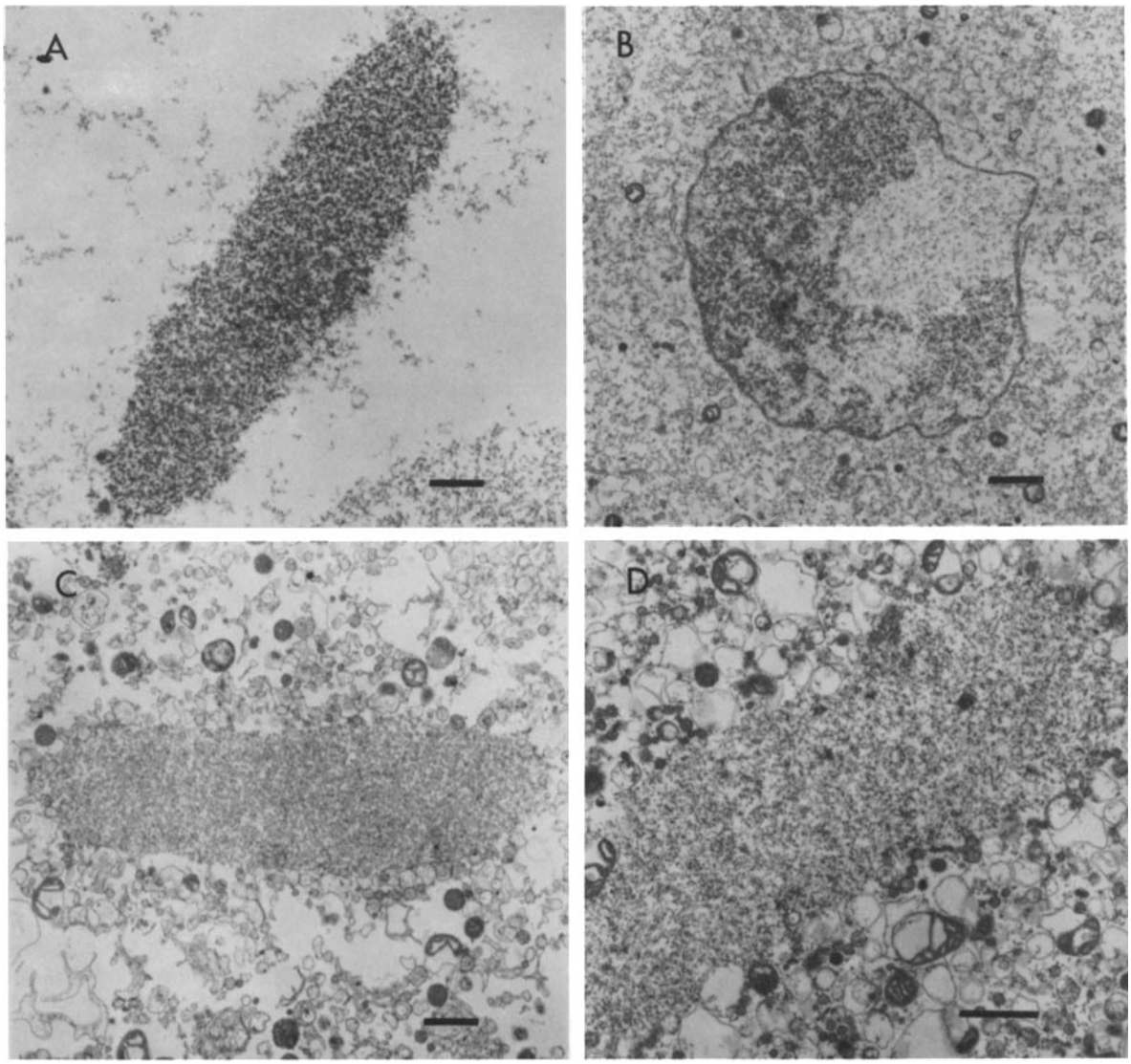

FIGURE 4 The extent of nuclear envelope assembly on lysolecithin-treated sperm nuclei during a 2-h incubation in the supernatant or vesicle-containing fraction obtained by centrifugation of the cytoplasmic preparation. (*A*) Supernatant alone, × 7,500. (*B*) Supernatant and pellet, × 7,500. (*C*) 1/3 buffer and pellet, × 7,500. (*D*) Heat-treated supernatant and pellet, × 10,700. Bars, 1.0 μm.

bated for 6 h in the heavy fraction diluted to one-half failed to condense chromosomes.

DISCUSSION

Numerous investigations have demonstrated that sperm or somatic cell nuclei transplanted into the cytoplasm of activated amphibian eggs are induced to enlarge and synthesize DNA (7–10). It would not be difficult to imagine that the cytoplasmic components acting upon the transplanted nuclei to induce their swelling are the same as those involved in the formation of the male pronucleus. In the present study, we have shown that a cytoplasmic preparation of *R. pipiens* eggs can induce, in vitro, a series of changes in sperm nuclear morphology that are similar to those occurring during pronuclear formation in the intact egg. These changes include (*a*) the initial rapid, but limited, dispersion of the highly condensed sperm chromatin, (*b*) the assembly of a nuclear envelope around the periphery of the sperm chromatin, (*c*) the enlargement of the sperm nuclei accompanied by an extensive decondensation of the sperm chromatin, (*d*) the initiation of DNA synthesis, and (*e*) the recondensation of the chromatin that results in the formation of structures resembling mitotic chromosomes within the nuclear envelope. These observations indicate that the egg cytoplasmic factors necessary for the formation of the sperm pronucleus and its following nuclear cycle can be kept active in this cell-free system.

Upon exposure to the heavy ooplasmic fraction, the sperm chromatin rapidly becomes dispersed and less electron-dense. This change in the sperm chromatin is clearly seen at the ultrastructural level, but it is difficult to observe in the squash preparations under a light microscope, where the sperm chromatin appeared to have been unchanged following a 5-min exposure to the ooplasmic preparations. Similar chromatin dispersion was also observed when sperm nuclei were incubated in the fresh soluble components, as well as in the soluble components that had been exposed to 100°C for 10 min. Furthermore, this type of chromatin dispersion also occurred in 1/3 buffer in which the vesicular components had been resuspended. Since no chromatin dispersion was observed when sperm nuclei were incubated in 1/3 buffer alone, it is likely that the factor responsible for the initial, rapid dispersion of sperm chromatin is a diffusible cytoplasmic substance

contained in the vesicular components as well as in the soluble fraction. However, since the chromatin dispersion occurred even after the majority of proteins were denatured by the heat treatment, it is unlikely that specific, heat-labile cytoplasmic components are responsible for this rapid chromatin dispersion. Rather, a more likely explanation for the phenomenon may be that the initial dispersion of sperm chromatin results from the action of small, heat-stable molecules, including ions, in the cytoplasmic preparations.

A nuclear envelope was assembled in vitro around the periphery of the newly dispersed sperm chromatin. Our results indicate that the formation of the nuclear envelope requires the interaction of cytoplasmic vesicles, heat-labile soluble components and chromatin. The vesicles that we have shown to contribute membrane components to nuclear envelope assembly in this cell-free system, are morphologically similar to some of those that participate in nuclear envelope formation in living cells. Not only were similar vesicles found to be involved in the formation of the sperm pronuclear envelope in eggs of various species (4, 22, 23), but also in the reconstitution of the nuclear envelope at telophase in mitotically dividing cells (24–26). Therefore, the vesicles that participate in nuclear envelope assembly in the ooplasmic preparation may be involved in both the formation of the pronuclear envelope in the zygote, as well as, in the rapid reconstitution of the nuclear envelope in cleaving blastomeres. It may be hypothesized that these vesicles are stored in the frog egg cytoplasm for use as precursors of the nuclear envelope, just as the components required for chromosomal replication are stored in the egg cytoplasm for use during cleavage (10).

The origin of these vesicles is not entirely clear, although it has been supposed that in dividing cells they are derived from the endoplasmic reticulum as well as from remnants of the previous nuclear envelope broken down during mitosis (27). In the fertilized sea urchin egg the endoplasmic reticulum also appears to play a major role in providing components of the sperm pronuclear envelope (28). At present, the origin of the vesicles that contribute to nuclear envelope assembly in the frog egg is unknown. They may originate from the envelope of the germinal vesicle, which is fragmented when the germinal vesicle breaks down during meiotic maturation and later dispersed in the cytoplasm to become indistinguishable from the cisternae of the preexisting endoplasmic reticulum (29, 30). It has been shown that sperm nuclei fail to form pronuclei when they are exposed to the cytoplasm of mature oocytes from which the germinal vesicle has been removed, suggesting that the presence of germinal vesicle material is required for pronuclear formation (21). However, this does not necessarily mean that cytoplasmic membranes derived from sources other than the germinal vesicle are incompetent to assemble nuclear envelopes. In fact, oocytes whose germinal vesicles have been removed become capable of inducing pronuclear formation if they are reinjected with soluble components that were prepared from activated eggs by centrifuging the heavy ooplasmic fraction at 150,000 *g* for 2 h (31). Since the material that was injected in this experiment was completely devoid of particulate and membrane components, the nuclear envelopes of these pronuclei must have been formed from cytoplasmic membranes remaining within the enucleated oocyte.

The major proteinaceous, nonmembranous components of the nuclear envelope have been found to be localized in the nuclear pore complex, situated at the sites where the inner and outer nuclear membranes have joined, as well as in the nuclear lamina lying between the inner membrane of the nuclear envelope and the peripheral chromatin (for review, see references 27, 32). These structures are disassembled during mitosis and reassembled into the nuclear envelope when an interphase nucleus is reformed (33–36). During nuclear envelope assembly in our cell-free system, nuclear pore structures are formed after the membrane vesicles containing electron-opaque material have flattened, leaving the contents between the two membranes. Therefore, it is tempting to propose that at least some of the proteins constituting the nuclear pore complex and lamina originate from the contents of the vesicles, although the incorporation of soluble proteins into these structures is also likely.

Perhaps the most striking change in the sperm nuclei during incubation in the ooplasmic preparation is the dramatic increase in their size. Our results indicate that both soluble and vesicular cytoplasmic components are required to induce the sperm nuclei to form pronuclei and to enlarge. However, since both soluble and vesicular components are also required for nuclear envelope assembly, these results may indicate that nuclear envelope assembly is prerequisite for nuclear enlargement and further dispersion of the chromatin during pronuclear formation. The concept that the sperm nucleus can be induced to enlarge only after a nuclear envelope has formed is supported by the observations that during incubation in the ooplasmic preparation the assembly of the nuclear envelope around the sperm chromatin precedes the enlargement of the nuclei. In interphase nuclei, the pore complex of the nuclear envelope, the peripheral lamina, and the internal matrix form a nuclear skeleton (37). It may be hypothesized that during sperm pronuclear formation the nascent nuclear envelope serves as a support for the assembly of the nuclear skeleton and that assembly of the nuclear skeleton continues while the nuclei enlarge. A similar role could be proposed for the nuclear envelope during telophase when the nuclear envelope is reconstituted around the mitotic chromosomes before they decondense to form interphase nuclei.

When sperm nuclei are induced to enlarge after fertilization, sperm-specific nuclear proteins are replaced by histones and other proteins that were originally found in the egg cytoplasm (38–43). In amphibian eggs, cytoplasmic proteins also move into transplanted nuclei and are thought to induce them to enlarge (44–46). In our experiments, dilution of the ooplasmic preparation reduces both the percentage of nuclei that decondense and the extent to which the nuclei enlarge. This observation supports the idea that cytoplasmic proteins or other components in the ooplasmic preparations move into the sperm nuclei in a concentration-dependent manner to induce them to enlarge. Although at present, the identity and the mode of action of the proteins that move into the sperm nuclei is not known, we may assume that they include the germinal vesicle material found in the soluble ooplasmic fraction (31).

The initiation of DNA synthesis in dividing cells is regulated by cytoplasmic factors that are active in S-phase of the cell cycle. When the nuclei of nondividing cells are exposed to the cytoplasm of cells in S-phase, by cell fusion or nuclear transplantation, they are induced to synthesize DNA (6). Our results indicate that the sperm nuclei incubated in a cell-free preparation of egg cytoplasm are induced to synthesize DNA as well. The inhibition of DNA synthesis by aphidicolin indicates that it is dependent on DNA polymerase-α, the enzyme that is necessary for chromosomal DNA replication in eucaryotes (19). In intact eggs, the movement of cytoplasmic proteins into the transplanted nuclei may be necessary for DNA synthesis to be initiated (44–46). Similarly, DNA

synthesis induced in the cell-free cytoplasmic preparations may depend on the migration into the sperm nuclei of the factors that initiate DNA synthesis in the zygote. However, at present, the possiblity cannot be ruled out that DNA polymerase-α and other enzymes that may play a role in the initiation of DNA synthesis are associated with the *Xenopus* sperm nuclei before their exposure to the ooplasmic preparations.

The cell fusion and nuclear transplantation experiments have also provided evidence that the condensation of chromosomes during mitosis also is controlled by cytoplasmic factors (6, 16, 46). These experiments clearly showed that the cytoplasm of cells in mitosis can induce the formation of mitotic chromosomes in interphase nuclei. When sperm nuclei are incubated in the ooplasmic preparation for 3 to 6 h, the chromatin of those nuclei that had once been induced to decondense to interphase could condense again to form structures that resemble mitotic chromosomes. This recondensation of chromatin does not appear to result from a degenerative change in the nuclei, since in some experiments the condensed chromosomes, if incubated further, could decondense again to return to interphase (14). Also, sperm nuclei were decondensed completely when incubated in preparations made from eggs immediately after activation, but, unlike those incubated in preparations made from eggs 1 h after activation, they did not recondense to form mitotic chromsomes during a 6-h incubation (unpublished results). Therefore, it may be a transient appearance of specific factors in the ooplasmic preparation made from eggs 1 h after activation that is responsible for the condensation of the decondensed chromatin to structures resembling metaphase chromosomes. In all probability, the putative ooplasmic factors responsible for the recondensation of chromatin in vitro are similar to those that regulate chromosome condensation during mitosis in intact cells. These factors may enter the decondensed sperm nuclei to induce chromatin condensation, since our observations indicate that, unlike the case in intact cells, chromosome condensation in the ooplasmic preparations is induced without breakdown of the nuclear envelope. Therefore, it may be that the factors responsible for chromosome condensation and for nuclear envelope breakdown during mitosis are different molecular entities that could act independently.

The results we present here suggest the possibility of analysing nuclear-cytoplasmic interactions during the cell cycle by using a cell-free preparation from amphibian eggs. Since this in vitro preparation is amenable to a wider range of manipulations than intact cells, it would be particularly useful for the biochemical investigation of the cell cycle.

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