ISOLATION OF NUCLEI FROM SEA URCHIN EGGS AND EMBRYOS

M. M. THALER, M. C. L. COX, and C. A. VILLEE, JR. From the Department of Pediatrics, University of California, San Francisco Medical Center, San Francisco, California 94122, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02113. Dr. Cox's and Dr. Villee's address is the Harvard Medical School

INTRODUCTION

In the course of investigations of nucleoprotein synthesis in young embryos,[1] we developed a simple, readily reproducible procedure for the isolation of clean nuclei in relatively high yields from sea urchin eggs and embryos. In this communication, we present the isolation procedure and describe various ultrastructural features of nuclei isolated from unfertilized eggs and growing embryos.

MATERIALS AND METHODS

Eggs of the sea urchin *Arbacia punctulata* were collected by the electroshock method (1), washed in artificial seawater, and sedimented by centrifugation at 500 *g* for 3 min. Embryos were obtained by fertilizing eggs in artificial seawater with a drop of fresh dilute sperm suspension. Only those cultures in which fertilization was 95–100% effective were used for the isolation of nuclei. The sedimented cells were resuspended in 9 volumes of a 1 M sucrose solution containing 2% (w/v) of the nonionic detergent Triton X-100 and 2 mM $MgCl_2$. Subsequent steps were monitored by light microscopy. The suspension was homogenized in a Potter-Elvehjem homogenizer with 15 slow up-and-down strokes of a loose Teflon pestle. The homogenate contained cytoplasmic debris, occasional unbroken cells, and fragments of fertilization membrane when unhatched embryos were used.

The homogenate was next centrifuged at 2000 *g* for 30 min, the supernatant discarded, and the detergent wash repeated. The sediment, containing nuclei and membranes, was then suspended in 3 volumes of 1.4 M sucrose, and layered on a discontinuous gradient composed of 1 ml of 2.5 M sucrose layered on the bottom, 1 ml of 2.2 M sucrose in the middle layer, and 1 ml of 1.9 M sucrose on the top. The preparation was then centrifuged at 25,000 rpm for 45 min at 4°C in a Spinco SW-39 swinging bucket rotor.

The layers containing lysed cytoplasm were carefully removed from the top, with a pasteur pipette. The bulk of the nuclei were arranged in a ring between the middle and bottom layers. The only contaminants present were membrane fragments, when nuclei from unhatched embryos were used. Most of the membranous material was deposited, along with some nuclei, at the bottom of the tube. In order to eliminate the remaining contaminants, an aliquot of nuclear suspension was diluted with 1 volume of 2 mM $MgCl_2$, centrifuged at 1000 *g* for 15 min, and resuspended in 1 volume of 1.4 M sucrose. The high-speed gradient centrifugation was then repeated. The final nuclear suspension was collected as before and diluted with 1 volume of 2 mM $MgCl_2$ to yield a concentration of approximately 1.2 M sucrose. For preparing larger amounts of nuclei, a Spinco SW-25.1 head was used for the gradient separations, with 10 ml of sucrose solution in each layer of the gradient.

Nuclear pellets were prepared for electron microscopy by centrifugation of the nuclear suspension at 25,000 rpm for 45 min in the Spinco SW-39 rotor. The pellets were sectioned into small pieces, fixed in 2.8% glutaraldehyde for 75 min at room temperature, and postfixed in 2% osmium tetroxide for 45 min at room temperature. The vehicle for both fixatives was hyperosmotic (1500 milliosmols) sodium cacodylate buffer, pH 7.3. The pellet was further sectioned and dehydrated through a graded acetone series and embedded in Araldite (Fluka AG, Basel, Switzerland). Thin sections were cut at different levels of each pellet on a Porter Blum MT2 microtome, mounted on carbon-coated grids, and stained with uranyl acetate and lead citrate. Electron micrographs were taken with an RCA EMU 3F microscope.

RESULTS AND COMMENTS

Interference-contrast photomicrographs of a nuclear suspension in 1.2 M sucrose obtained by this method are shown in Figs. 1 and 2. No whole or broken cells, cytoplasmic aggregates, or membranous fragments are evident. The nuclei are spherical, uniform in size, and are not clumped. The nuclear pellet isolated from early gastrulae was found to be free of contamination when photographed at low magnification in the electron microscope (Fig. 3). Dense aggregates of chromatin

[1] M. M. Thaler, M. C. L. Cox, and C. A. Villee. 1969. Nucleoprotein synthesis in sea urchin embryos. In preparation.

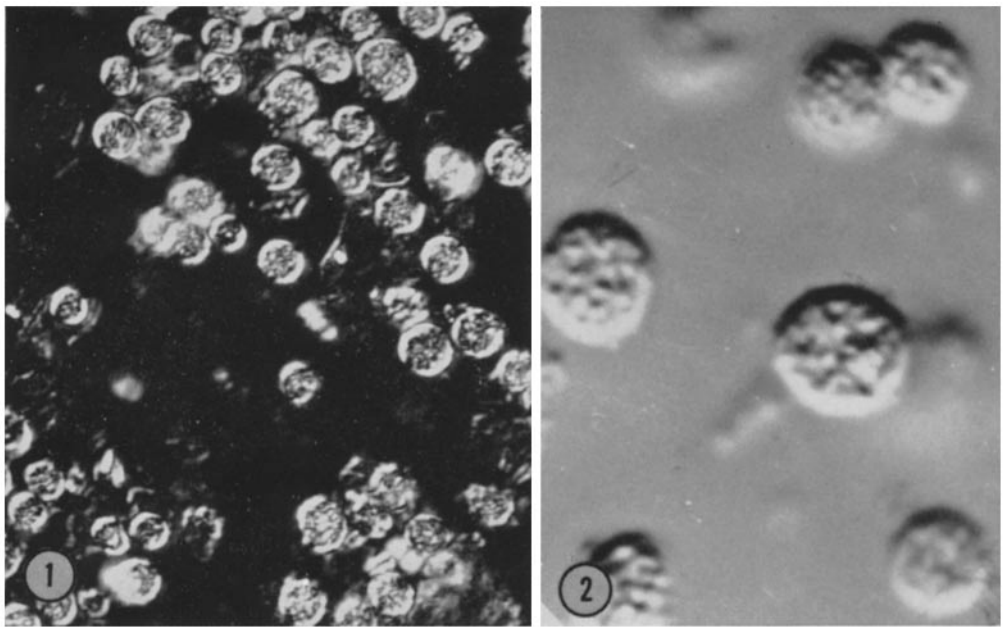

FIGURE 1 Isolated nuclei suspended in 1.2 M sucrose. No whole cells or large debris particles are present. Interference-contrast, darkfield illumination. × 1250.

FIGURE 2 Higher power, showing regular spherical shape of detergent-treated nuclei. Interference-contrast, brightfield illumination. × 3500.

make up the major content of the nucleus; smaller patches of chromatin are arranged intermittently around the periphery of the nucleus. The nucleoli are large and granular.

For comparison with nuclei from embryos, a nucleus isolated from an unfertilized egg is shown in Fig. 4. The nuclear membrane is not clearly visible. Dense chromatin formations and nucleoli are absent, but the network of loose strands appears condensed in regions which contain electron-opaque granules about 200 A in diameter (Fig. 5).

For comparing the appearance of isolated nuclei with nuclei *in situ*, the nuclear suspension in 1.2 M sucrose–2 mM $MgCl_2$ solution was mixed with fragments of gastrula cells and prepared for electron microscopic examination. The nuclei which retained the cytoplasmic envelope closely resembled the nuclei isolated by detergent treatment (Fig. 6), but the outer nuclear membrane was no longer present in the latter.

Hinegardner's method for isolating nuclei from eggs and embryos of the sea urchin *Strongylocentrotus purpuratus*, published in 1962 (2), involves numerous washes with dextrose, followed by osmotic cytolysis in the presence of divalent cations, and centrifugation through five layers of sucrose solution. In our hands, this procedure resulted in excessive losses of nuclei, especially from unfertilized eggs. Lysis of whole cells without damage to nuclei, and separation of the thick upper layers of contaminants from the nuclear layer in the dense sucrose solution, were accomplished only with great difficulty. There was considerable variability in yield and purity with different batches of cells. The nuclei isolated by this means retained their outer membranes with tags of endoplasmic reticulum and ribosomes adhering to them. Another procedure, utilizing a modification of Hinegardner's method for unfertilized eggs and a combination of the ionic detergent sodium lauryl sulfate with a buffered solution containing magnesium and mercaptoethanol, was described as part of a study of RNA synthesis in developing embryos of the sea urchin *Lytechinus variegatus* (3). Nuclei obtained in this manner were grossly contaminated with fragments of fertilization membrane, cytoplasmic debris, and externally attached ribosomes.

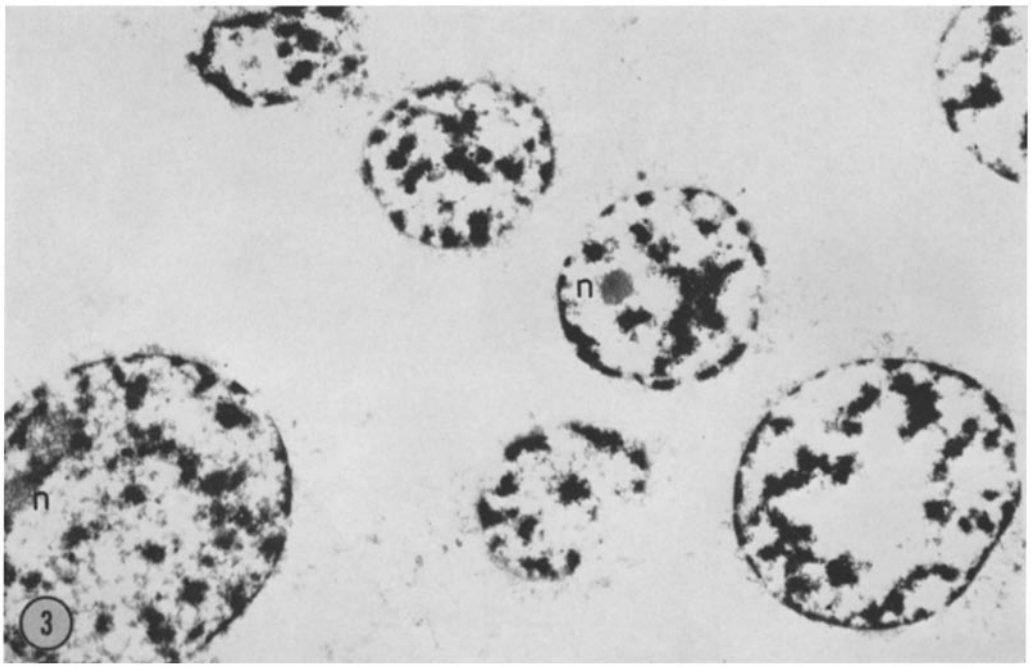

FIGURE 3 Nuclear pellet. Minimal cytoplasmic contamination. Outer nuclear membrane has been removed by detergent treatment. The nuclear periphery is outlined by thin interrupted patches of condensed chromatin. Other electron-opaque chromatin aggregates are distributed within the nucleus. *n*, granular nucleoli, less opaque than the condensed chromatin. Note broken nucleus which still retains its spherical outline. It was probably ruptured during preparation for electron microscopy. × 10,700.

The use of nonionic detergents in combination with divalent cations has led to a number of simple and rapid methods for the isolation of intact mammalian nuclei free of cytoplasmic contamination (4-7). The detergents lyse whole cells and solubilize cytoplasmic structures. We tested the completeness of this solubilizing effect of Triton X-100 by comparing homogenates prepared by Hinegardner's (2) and our procedures, and centrifuged on a five-layer sucrose gradient according to the former procedure. Homogenates prepared without detergent yielded viscous, deeply pigmented collections of whole eggs, yolk granules, broken eggs, and debris distributed among the three upper layers of sucrose. In contrast, the initial homogenate prepared with Triton X-100 and centrifuged on such a gradient produced three clear layers, shading in color from yellow to pink, above the ring of nuclei. Microscopic examination confirmed the absence of formed elements, cytoplasmic clumps, or debris in these layers.

Ionic detergents, or combinations of nonionic and ionic detergents, have been utilized for the isolation of nuclei from mammalian cells (5, 6). We examined the effects of the ionic detergent sodium dodecyl sulfate and the nonionic detergent Tween-80, used singly or in combination, on sea urchin nuclei. Concentrations of these detergents from 0.5 to 2% (w/v) in the homogenizing medium, or in the final nuclear suspension after Triton X-100 had been removed by extensive washing, resulted in excessive breakown of nuclei, especially from unfertilized eggs. Sodium dodecyl sulfate alone induced complete lysis of all structural elements, while Tween-80 alone was ineffective in separating the nuclei completely from cytoplasmic debris. Nuclei from sea urchin eggs appear to be more sensitive than mammalian nuclei to the action of ionic detergents.

Structural changes in the nuclei of intact embryogenic cells have been observed with the light microscope (8) and recently confirmed with the electron microscope (9). The appearance of nuclei isolated from unfertilized eggs differs from such nuclei in gastrulae. The egg nucleus contains a loose network of chromatin strands which are

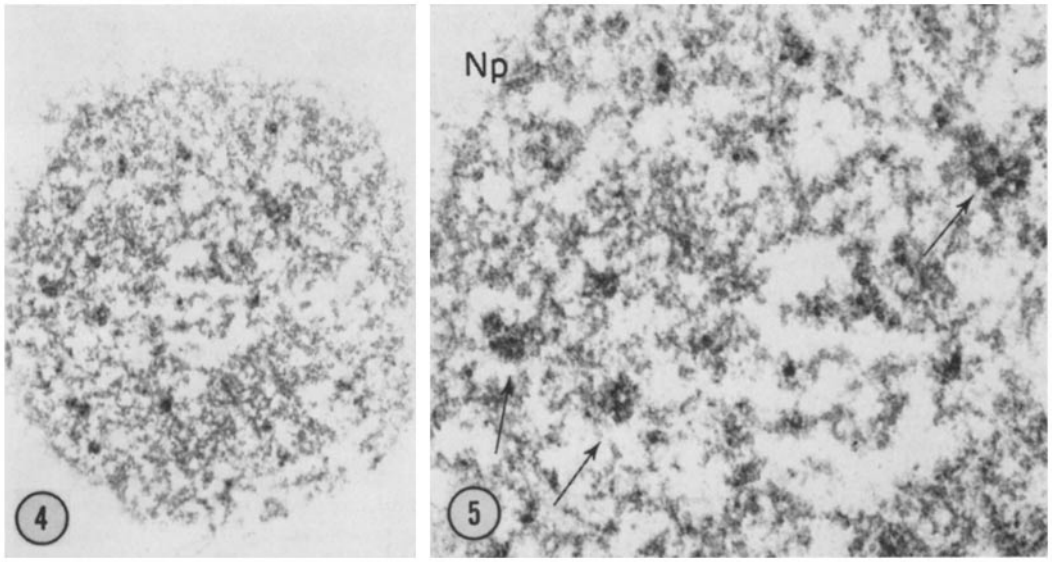

FIGURE 4 Unfertilized egg nucleus. Strands of chromatin and clusters of dark granules form a loose network which fills the nucleus. No nucleoli are seen. × 24,000.

FIGURE 5 Detail of Fig. 4. Thin chromatin fibrils are associated with condensed patches within which dense granules approximately 200 A in diameter are embedded (arrows). *Np*, nuclear periphery. × 50,300.

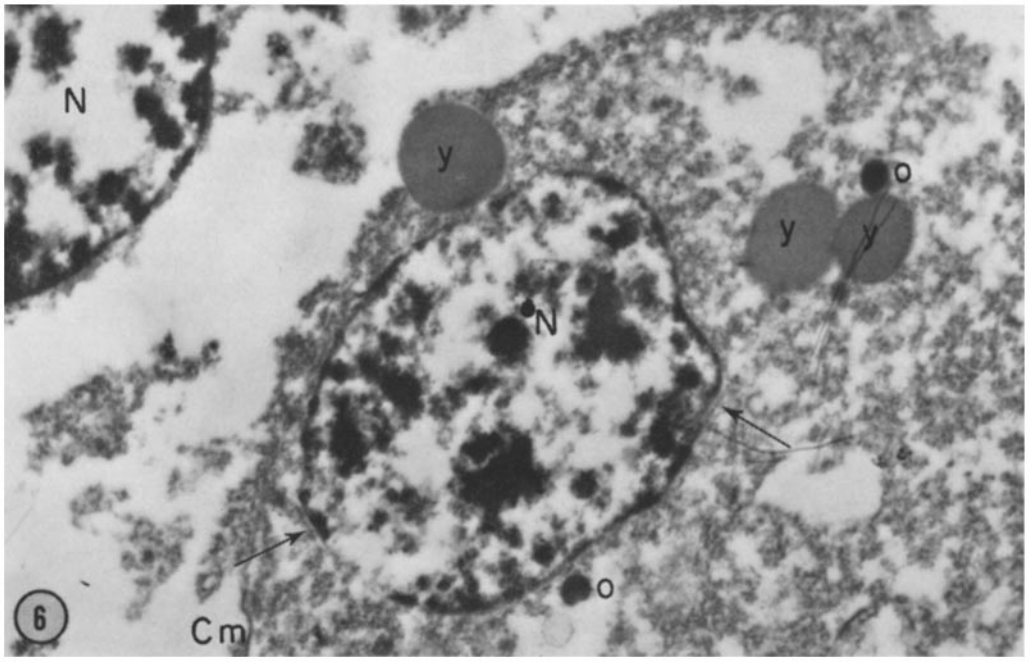

FIGURE 6 Fragment of gastrula and nucleus isolated from gastrula cell. The nucleus within the cell is similar in appearance to the isolated nucleus (upper left corner). The outer layer of the nuclear membrane which surrounds the nucleus *in situ* (arrows) has been removed from the isolated nucleus by detergent treatment. *N*, nuclei; *Cm*, cell membrane; *y*, yolk granules; *o*, oil droplets. × 21,200.

continuous with condensed patches. Embedded within these patches are clusters of dense granules, each about 200 A in diameter. In their size and appearance these granules correspond to ribosomal particles. The absence of similar configurations in gastrula nuclei raises the possibility that these structures with the appearance of nucleoprotein-ribosomal complexes may play a role in translational activation at fertilization. Results of fractionation studies by Spirin and Nemer (10) and by Stavy and Gross (11) appear to support the existence of such functional aggregates in unfertilized eggs.

The much denser electron-opaque masses of chromatin within the gastrula nucleus tend to maintain a close relationship with the inner nuclear membrane throughout most of the DNA replicative cycle. Similar observations in bacterial (12), plant (13), and mammalian (14, 15) cells have led to theories which implicate the nuclear membrane in the control of chromosomal replication (16, 17). The peripheral distribution of chromosomal aggregates and their gradual extension into the nuclear interior during interphase are consistent with these proposals.

SUMMARY

A simple, improved procedure for the isolation of clean nuclei in high yield from sea urchin eggs and embryos combines treatment with the nonionic detergent Triton X-100 with high-speed centrifugation on a discontinuous sucrose gradient. Nuclei isolated from unfertilized eggs contain a fine mesh of chromatin strands which are continuous with concentrated regions filled with dense granules. Nuclei isolated from gastrulae display a peripheral arrangement of very condensed chromatin and granular nucleoli. Nuclei isolated by this method appear morphologically very similar to untreated intracellular nuclei.

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REFERENCES

1. HARVEY, E. B. 1956. The American *Arbacia* and Other Sea Urchins. Princeton University Press, Princeton, N. J.
2. HINEGARDNER, R. T. 1962. The isolation of nuclei from eggs and embryos of the sea urchin. *J. Cell Biol.* **15**:503.
3. COMB, D. G., S. KATZ, R. BRANDA, and C. J. PINSINO. 1965. Characterization of RNA species synthesized during early development of sea urchins. *J. Mol. Biol.* **14**:195.
4. HYMER, W. C., and E. L. KUFF. 1964. Isolation of nuclei from mammalian tissues through the use of Triton X-100. *J. Histochem. Cytochem.* **12**:359.
5. TRAUB, A., E. KAUFMANN, and Y. GINZBERG-TIETZ. 1964. Studies on nuclear ribosomes. I. Association of DPN-pyrophosphorylase with nuclear ribosomes in normal and neoplastic tissues. *Exp. Cell Res.* **34**:371.
6. PENMAN, S. 1966. RNA metabolism in the HeLa cell nucleus. *J. Mol. Biol.* **17**:117.
7. BLOBEL, G., and V. R. POTTER. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science.* **154**:1662.
8. IMMERS, J. M., B. MARKMAN, and J. RUNNSTRÖM. 1967. Nuclear changes in the course of development of the sea urchin studied by means of Hale staining. *Exp. Cell Res.* **47**:425.
9. RUNNSTRÖM, J. 1968. Structural changes of the chromatin in early development of sea urchins. *Exp. Cell Res.* **48**:691.
10. SPIRIN, A. S., and M. NEMER. 1965. Messenger RNA in early sea urchin embryos: cytoplasmic particles. *Science.* **150**:214.
11. STAVY, L., and P. R. GROSS. 1967. The protein-synthetic lesion in unfertilized eggs. *Proc. Nat. Acad. Sci. U.S.A.* **57**:735.
12. GANESAN, A. T., and J. LEDERBERG. 1965. A cell-membrane bound fraction of bacterial DNA. *Biochem. Biophys. Res. Commun.* **18**:824.
13. PUSA, K. 1963. Some new principles governing chromosome pairing - the spatial relations of chromosomes and nuclear membrane. Proceedings of the 11th International Congress for Genetics held in The Hague, Sept. 1963. Excerpta Medica Foundation, Publishers, Amsterdam, The Netherlands. **1**:110.
14. WOOLAM, D.M.M., J. W. MILLEN, and D.H.R. FORD. 1967. Points of attachment of pachytene chromosomes to the nuclear membrane in mouse spermatocytes. *Nature.* **213**:298.
15. COMINGS, D.E., and T. KAKEFUDA. 1968. Initiation of DNA replication at the nuclear membrane in human cells. *J. Mol. Biol.* **33**:225.
16. COMINGS, D.E. 1968. The rationale for an ordered arrangement of chromatin in the interphase nucleus. *Amer. J. Hum. Genet.* **20**:440.
17. MARVIN, D.A. 1968. Control of DNA replication by membrane. *Nature.* **219**:485.