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In vitro nuclear reconstitution could be induced in a plant cell-free system

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Abstract A cell-free system derived from carrot cell cytosol extract has been developed for reassembling nuclear structure around the added demembranated sperm chromatin of *Xenopus*. Morphological evidence suggests that reassembled nuclei display the typical characteristics of normal eukaryotic nuclei, such as double-layered nuclear membrane and nuclear pores. Micrococcal nuclease treatment indicates that remodeling of the demembranated sperm chromatin has occurred and the structure of nucleosome is formed during nuclear reconstitution. These data indicate that the nuclear reconstitution can be induced in cell-free systems from plants, and the self-assembly of the nucleus is ubiquitous in both animal and plant cells.

Key words: Carrot cell; Cell-free system from plant; Demembranated sperm chromatin; Nuclear reconstitution; Nucleosome

1. Introduction

Nuclear reassembly or reconstitution in cell-free systems has been used to investigate the mechanism of the nuclear assembly occurring in the cell cycle, and to describe the process of assembled nucleus when the raw and processed materials of nuclear reconstitution are mixed and induced in vitro. In the cell-free system from *Xenopus* eggs, typical structural nuclei can be formed when induced by the sperm chromatin or isolated DNA [1–10]. And the reassembled nuclei have double-layered nuclear membrane with nuclear pore complexes, lamina formation, DNA replication and nuclear cytoplasmic transportation. The results of the following experiments indicate that the induction of nuclear reconstitution in *Xenopus* extract is independent of the source and length of exterior DNA [11,12]. In contrast, the typical reconstituted nucleus also can be formed in cell-free systems derived from other kinds of cell extracts [13,14,24,25].

Since nuclear reconstitution in a cell-free system can imitate the process of nuclear reconstitution in the normal intact cell and can be controlled at some level by adding certain materials that take part in reconstitution, it becomes a nice system to study the process and mechanism of nuclear reconstitution. Plenty of information on cell cycle regulation concerning nuclear reconstitution, DNA replication and mitosis have also been obtained from the studies on the nuclear reconstitution in cell-free systems [6,7,15–20]. Except for the cell-free system based on *Xenopus* eggs extract, the cytosol extracts of *Drosophila* embryo [24,25], CHO cells [13] and HeLa cells [14] are all perfect cell-free systems for studying nuclear reconstitution.

While there has been no plant cell-free system reported for the study of nuclear reconstitution so far, several questions arise. Does the cell-free system based on plant cells extract have the ability to induce nuclear reconstitution? And is the process of nuclear reconstitution in plant cell-free system similar to the process in animal cell extracts? To address these questions, we employed a cell-free system based on the cytosol extracts from carrot cells to induce in vitro nuclear reconstitution.

The cytosol extract, based on the carrot cell suspension cultures, is a well-established cell-free system. Nuclei could be efficiently induced to undergo apoptosis [21,22]. In the present work, however, we have applied this system to inducing nuclear reconstitution in vitro. This kind of cell-free system has some obvious advantages: (1) It is made based on the synchronized carrot cell suspension cultures and large amounts of cell extract are easily obtained. (2) It is easy to control and analyze single factors, and can be used to address what kind of factor is involved in nuclear reconstitution. The demembranated sperm chromatin and membrane vesicle from *Xenopus* eggs were added to this kind of cell-free system, and an ATP-regenerating system was then introduced into it at the proper concentration. Following incubation at 23°C, the demembranated sperm chromatin changed from the condensed to the decondensed form during the process of nuclear reconstitution, followed by a reassembled nucleus with double-layered nuclear membrane and nuclear pore complexes. Micrococcal nuclease digestion showed that remodeling of the demembranated *Xenopus* sperm chromatin had occurred and the structure of the nucleosome had formed. All of these results indicated that nuclear reconstitution could be induced in this kind of cell-free system based on plant cell extracts, which suggested that the self-assembly of the nucleus is ubiquitous in nature.

2. Materials and methods

2.1. The synchronized carrot cell suspension cultures and preparation of cell-free system

The synchronized carrot cell suspension cultures were prepared as described by Mathews [23]. After culturing for 4 days carrot cells were transferred into the fresh culture medium containing hydroxycarbamide (0.1 mmol/l), then cultured in the dark for 16 h at 27°C. After washing three times with fresh culture medium, carrot cells were incubated for 8 h at 27°C. Colchicine (0.01%) was then introduced into

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Abbreviations: EGTA, ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*′-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HCG, human chorionic gonadotropin; XN buffer, *Xenopus* sperm buffer; BSA, bovine serum albumin; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; DAPI, 4′6-diamidino-2-phenylindole

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the culture medium. Carrot cells were harvested 12 h later. About 5 g carrot cells (gross weight) were added into 10 ml enzymatic buffer (2% (w/v) cellulase, 0.5% Macerozyme, 5 mmol/l 4-morpholinethansulfonic acid, 6.8 mmol/l $CaCl_2$, 11 mmol/l KH_2PO_4, 0.6 mol/l mannitol, 0.4% polyvinylpyrrolidone, pH 5.8), treated with an orbiting speed of 50–80 rpm in the dark for 2 h at 28°C. After filtering through a 200# nylon meshwork, the protoplasts were harvested by centrifugation for 5 min at 120×*g*. The protoplast sediment was re-suspended in a 0.6 mol/l sucrose buffer, and purified protoplasts (located at the upper layer) were collected by centrifugation for 5 min at 120×*g*. The purified protoplasts were suspended in buffer A (20 mmol/l *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES)-KOH, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l disodium ethylenediaminetetraacetate, 1 mmol/l ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 1 mmol/l dithiothreitol (DTT)) and homogenized appropriately until about 70% protoplasts were broken. Aprotinin, phenylmethylsulfonyl fluoride (PMSF), and leupeptinin were introduced into the buffer at a final concentration of 6 mg/ml, 0.1 mmol/l, and 8 mg/ml respectively. The soluble cytosol extracts were collected and used as the nuclear reassembled cell-free system after centrifugation for 2 h at 100 000×*g* (Beckman TLS-55) at 4°C. The result of quantitative determination suggested that the protein concentration in the extract was 160 μg/ml.

2.2. Preparation of demembranated Xenopus sperm

Sperm was prepared as described by Lohka and Masui [1] with some modifications. The spermary was dissected from sexually matured *Xenopus laevis*, which had been injected with 100 units human chorionic gonadotropin (HCG, Sigma) and kept for 1 h at 22°C. The spermary was washed free of blood and incubated overnight at 18°C in 200% Steinberg's solution containing antibiotics and HCG (10 units/ml). By gently squeezing the spermary, sperm was released and harvested by centrifugation for 10 min at 4°C, 1500×*g* and washed three times with *Xenopus* sperm buffer (XN buffer) (15 mmol/l NaCl, 60 mmol/l KCl, 15 mmol/l Tris–HCl, pH 7.4, 1 mmol/l DTT, 0.5 mmol/l spermine, 0.15 mmol/l spermidine). Sperm was treated with XN buffer containing lysolecithin at 330 μg/ml and proteinase inhibitor (6 mg/ml Aprotinin, 0.1 mmol/L PMSF and 8 mg/ml leupeptinin) for 10 min at 22°C. Lysolecithin-treated sperm was washed once with ice-chilled XN/3% bovine serum albumin (BSA), three times with XN/0.4% BSA and finally resuspended in XN/30% glycerol. Sperm was counted with a hemocytometer, and stored at −70°C.

2.3. Preparation of membrane vesicle

Healthy *Xenopus laevis* females were injected with 600 units of HCG (Sigma) on the night before use and were placed in 5 l of 100 mM NaCl. Eggs were dejellied with 2% cysteine (pH 7.8), rinsed three times in MMR buffer (50 mmol/l HEPES–KOH, pH 7.4, 0.1 mol/l NaCl, 2 mmol/l KCl, 1 mmol/l $MgSO_4$, 2 mmol/l $CaCl_2$, 2 mmol/l mercaptoethanol), and activated by incubation in a solution containing MMR, 5% Ficoll, and 0.2 μg/ml of the Ca^{2+} ionophore A23187. Eggs were rinsed three times with 1/4 volumes of MMR plus 5% Ficoll. After incubation at room temperature for 20 min, the eggs were rinsed three times with three volumes of a solution containing 250 mmol/l sucrose, 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 100 mg/ml cycloheximide, 5 μg/ml cytochalasin B, and 1 mmol/l DTT. Eggs were then packed via a 30 s centrifugation at 100×*g*. Thereafter the eggs were crushed for 15 min at 10 000×*g*, 4°C, the soluble cytosol could be collected. The cytosol was centrifuged for 75 min at 200 000×*g*, 4°C, the white membrane vesicle could be carefully collected at the top of the centrifugal tube. Then the white membrane vesicle was carefully put on the surface of the sucrose solution (0.5 mol/l) as the superstratum. Following this procedure, the purified membrane vesicle could be obtained after centrifugation for 30 min at 26 000×*g*, 4°C.

2.4. Nuclear reconstitution

The demembranated sperm (500 per μl cytosol extract) was incubated at 23°C in 50 μl of reconstitution extract containing membrane vesicle (0.08 μl/μl cytosol extract) and an ATP regenerating system containing 2 mM ATP, 20 mM phosphocreatine, and 50 μg/ml creatine kinase. To monitor the process of nuclear reconstitution in the incubation system, 2 μl aliquots of the reaction mixture were sampled at different time intervals and mixed with 2 μl glutaraldehyde (2.5% (v/v)), and 1 μl of the DNA-specific fluorescent stain 4′6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml). After blocking with glycerol and PBS (1:1, v/v), samples were observed under the fluorescent microscope (Leica DMRB), and photos were taken.

2.5. Electron microscope

To monitor the process of nuclear reconstitution by using electron microscope, aliquots of the incubation mixture were sampled at different time intervals and were fixed in ice-chilled 0.5% glutaraldehyde for 1 h. After fixation, the samples were precipitated in an Eppendorf centrifuge for 10 min. Washed three times (5–10 min/time) using 0.1 mol/l PBS (pH 7.2), the pellets were then post-fixed in 1% OsO_4 for 2 h at 4°C. Samples were then dehydrated through a graded concentration series of acetone and embedded in Epon 812. Ultrathin sections were cut under Leica Ultracut R Cutter and stained with uranyl acetate and lead citrate. Finally, sections were examined under JEM-1010 transmission electron microscope.

2.6. Digestion of micrococcal nuclease

To examine if the nucleosome could be formed during the nuclear reconstitution in the plant cell-free system, two samples were taken, one was incubated for 0.5 h and the other was incubated for 1 h. Micrococcal nuclease (0.01 units/μl, Sigma) was then added into the reaction mixtures, and incubated for 5 min at 37°C. At the same time, demembranated sperm was added into the XN buffer as control 1. After incubation for 1 h, it was digested with micrococcal nuclease. For control 2, about 1×10^5 mouse liver nuclei were added into nuclear storage buffer (10 mmol/l PIPES, 80 mmol/l KCl, 20 mmol/l NaCl, 250 mmol/l sucrose, 5 mmol/l EGTA, 0.5 mmol/l spermidine, 0.2 mmol/l spermine). After freezing and thawing three times, micrococcal nuclease was added to the buffer and incubated for 5 min at 37°C. All samples were extracted with 1:1 phenol–chloroform, precipitated by two volumes of ethanol. Mouse liver nuclei were prepared as described by Zhao [22]. DNA extracts were electrophoresed in 1% agarose gels in TAE buffer. DNA was visualized by ethidium bromide staining, observed and photographed.

3. Results

3.1. Nuclear reconstitution occurred in the plant cell-free system

Treated with lysolecithin, nuclear and cellular membranes of *Xenopus* sperm were effectively removed. The demembranated sperm chromatin evolved into a long, thin and condensed style. After the demembranated sperm and membrane vesicle were introduced into the carrot cell cytosol extract, which contained an ATP regenerating system, nuclear reconstitution could be induced. Samples were taken at different time intervals and observed under transmission electron microscope or fluorescent microscope after staining with DAPI. When introduced into carrot cell extract and observed immediately under fluorescent microscope, the demembranated sperm displayed a long, thin and highly condensed form and could be strongly stained with DAPI (Fig. 1a). Results of observation under transmission electron microscope suggested that the sperm was in a strongly condensed form at this time (Fig. 2a). When incubated at a certain temperature (23°C), the demembranated sperm exhibited a series of structural and morphological changes. Over time, the sperm chromatin began to elongate and swell, and showed discernible decondensation during the first 5 min (Fig. 1b). During the incubation from 5 to 20 min, the sperm chromatin gradually changed to a rounded shape and displayed a nucleus-like structure (Fig. 1c,d). Results of observation at the ultrastructural level suggested that the condensed sperm began to swell and decondense at the initial stages of the formation process of reconstituted nuclei. However, there was no nuclear membrane around the newly formed nucleus (Fig. 2b). Along with more decondensation and swelling, the sperm chromatin

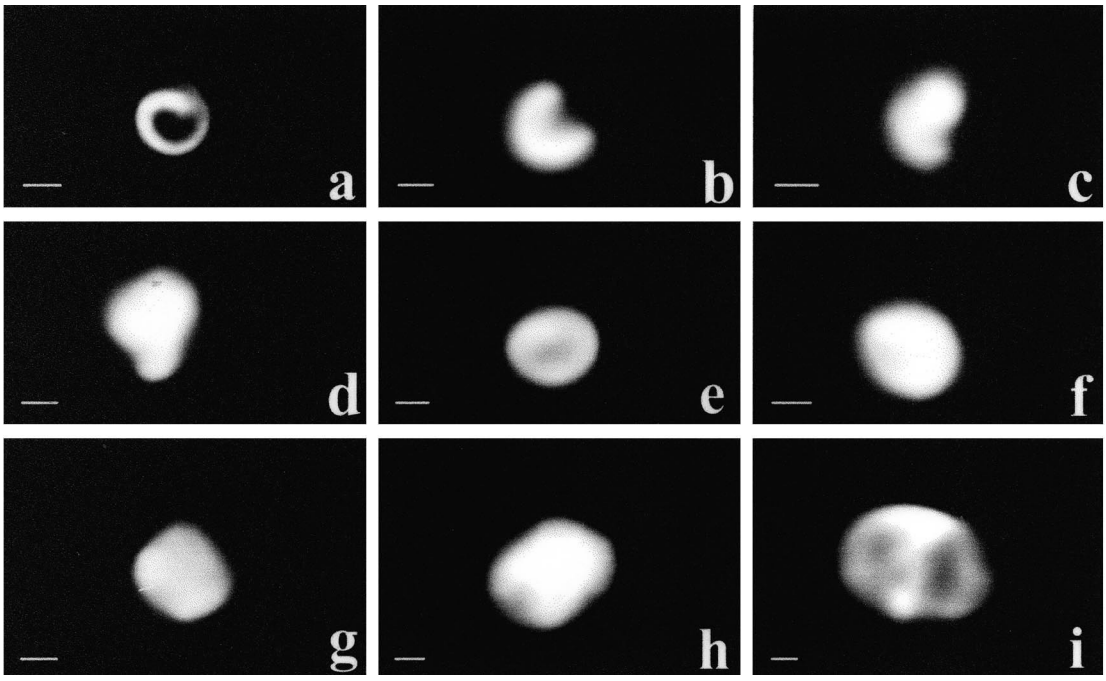

Fig. 1. Stage of nuclear reconstitution in a cell-free system based on plant cells. A mixture containing 50 μl of carrot cell cytosol extracts, 2 mM ATP, 20 mM creatine phosphate, 50 μg/ml creatine kinase, membrane vesicle (0.08 μl per μl cytosol extracts), and the demembranated sperm (500 per μl cytosol extract) was incubated at 23°C. At 0 min (a), the demembranated sperm displayed a thin and highly condensed form. After incubation for 5 min in a cell-free system, the demembranated sperm began to elongate and swell (b). After incubation from 5 to 20 min, the sperm chromatin displayed a round shape in this process and the nucleus-like structure appeared (c,d). From 30 min to 1 h, the reconstituted nuclei could be induced to form in the cell-free system (e,f,g). Two hours after incubation, the newly assembled nuclei began to swell (h). Four hours after incubation, the newly assembled nuclei swelled remarkably (i). Bar = 1 μm.

gradually acquired a continuous double-layered nuclear envelope (Fig. 2c, arrows indicate the double-layered nuclear envelope). After longer incubation, the newly assembled nucleus appeared in the plant cell-free system (Fig. 2d). From 30 min to 1 h, reconstituted nuclei, which were morphologically indistinguishable from those in intact interphase cells, could be induced to form in a cell-free system (Fig. 1e,f,g). Observed under transmission electron microscope, the newly formed nucleus displayed the typical characteristics of normal nuclei. The sperm chromatin were further swollen and evenly distributed in the reassembled nucleus (Fig. 2e). The high magnification electron micrographs showed that there were typical double-layered nuclear envelopes with nuclear pores on the surface of the assembled nucleus (Fig. 2f, arrows indicate the nuclear pores). After incubation for 2 h, the newly assembled nuclei began to swell (Fig. 1h). After incubation for 4 h, the newly assembled nuclei swelled remarkably (Fig. 1i).

The statistical data showed that, after incubation for 1 h, about 20% demembranated *Xenopus* sperm could be induced to form assembled nuclei. We also noticed that the swelling of sperm in this kind of cell-free system were almost synchronous. The formation of newly assembled nuclei, however, was not completely synchronous. Observation after incubation for 8 h indicated that the newly assembled nuclei could be cleaved again, implying that they could not maintain their structural integrity in this system all the time. In the control of XN incubation buffer, the above mentioned phenomenon was not observed.

3.2. Remodeling of the demembranated sperm chromatin

Micrococcal nuclease possesses the ability of cutting the chromatin DNA fibers between nucleosomes. Digested with micrococcal nuclease, the chromatin DNA, which has the nucleosome structure, can form a DNA ladder after electrophoresis. This would help us to identify the nucleosome structures existing in chromatin. The results suggested that the chromatin remodeling of the sperm occurred in the reassembled nuclei to form the nucleosome. After incubation at 23°C for 0.5 h or 1 h in the cell-free system and treated with micrococcal nuclease for 5 min at 37°C, the typical DNA ladder appeared after electrophoresis (Fig. 3, lane 2, incubated for 0.5 h at 23°C; lane 3, incubated for 1 h). This result verified that the chromatin remodeling of the sperm occurred and the nucleosome structures were assembled during nuclear reconstitution. Similarly, after digestion for 5 min, the positive control (freezing and thawing mouse liver nuclei) showed the typical DNA ladder after electrophoresis (Fig. 3, lane 4). As a negative control, lane 1 was loaded with sperm chromatin in XN buffer.

4. Discussion

As the material used to reassemble the nuclei in the cell-free system, the demembranated sperm was widely applied to the study of the mechanism of nuclear reconstitution [1,3,5,7,10]. The demembranated sperm chromatin was in a long, thin and condensed form, and could undergo the obvious course of elongating and swelling during the process of nuclear reconstitution. This was convenient for monitoring the process of reassembly. The sperm, purified from *Xenopus*, was highly purified for the experiment. Since it had been proven that the induction of reassembled nuclei in an animal cell-free sys-

tem was independent of the source and length of exterior DNA [9–12], the nuclear reconstitution in a carrot cell-free system using highly purified *Xenopus* sperm chromatin could partially answer the question of whether a cell-free system based on plant cell extract could induce nuclear reconstitution, and if the process of nuclear reconstitution in a plant cell-free system was similar to the process in the animal cell extracts.

The newly reassembled nuclei in the plant cell-free system had the typical characteristics of normal nuclei, such as a double-layered nuclear envelope, nuclear pores and a nucleosome. However, only about 20% of the sperm could be induced to undergo the nuclear reconstitution in this cell-free system, which differs from *Xenopus* extract, in which about 40% or more sperm formed new nuclei [10]. The reason might be that there are much more nutrients (such as proteins) stored in *Xenopus* egg extract than in carrot cell extract, which only could support a small amount of sperm undergoing nuclear reconstitution. In *Xenopus* egg extract, the newly reassembled nuclei could maintain the conformation for more than 8 h [10]. However, we did not observe intact newly reassembled nuclei in carrot cell extract after incubation for 8 h. This result also indicated that there is a correspondingly small quantity of storage nutrition in the plant cell-free system, and there was a contrast between its holding ability to reassembled nuclei and *Xenopus* extract.

Micrococcal nuclease can cut the chromatin between nucleosomes to form a DNA ladder. Since no H1 histone or nu-

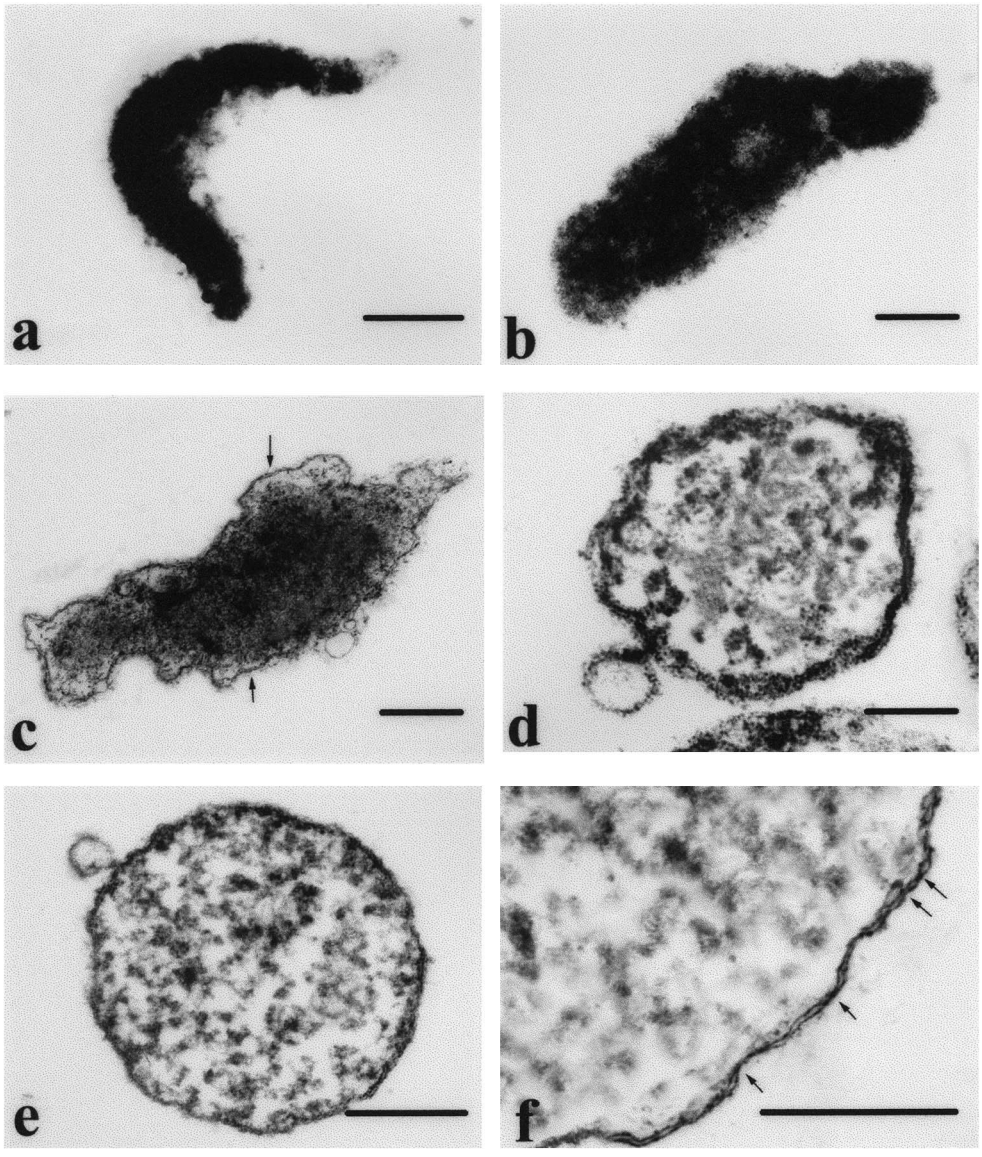

Fig. 2. Observation of nuclear reconstitution at the ultrastructural level. Transmission electron microscope results showed that the sperm, which did not undergo nuclear reconstitution, was in a strongly condensed form (a). After incubation at 23°C, the condensed sperm began to decondense at the initial stages of the formation process of nuclear reconstitution, and there was no nuclear membrane around the newly formed nucleus at this time (b). Compared with the more decondensed and swollen sperm, the sperm chromatin gradually obtained a continuous double-layered nuclear envelope (c, arrows indicate the double-layered nuclear envelope). After a long incubation the newly formed and assembled nucleus with double-layered appeared in the plant cell-free system (d). After incubation for 1 h, the newly formed nucleus displayed the typical nucleus and chromatin equally distributed (e). The high magnification electron micrographs showed that there were typical double-layered nuclear envelopes with nuclear pores on the surface of the assembled nucleus (f, arrows indicate the nuclear pores). Bar = 1 μm.

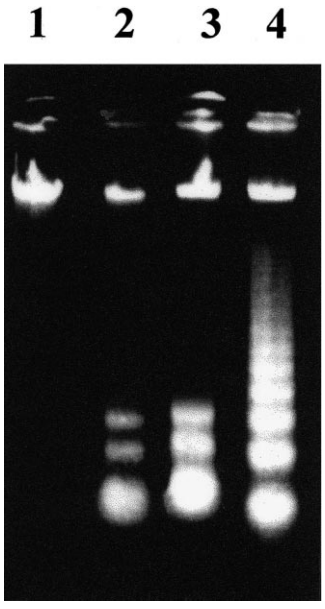

Fig. 3. The newly assembled nuclei formed the nucleosome structure. After treatment with micrococcal nuclease, chromatin remodeling and the formation of nucleosomes were observed in newly assembled nuclei. Being treated for 5 min with micrococcal nuclease, no digestion and DNA ladder appeared with the sperm chromatin in XN buffer (lane 1, negative control). Incubation was at 23°C for 0.5 h (lane 2) or 1 h (lane 3) in a cell-free system and treated with micrococcal nuclease for 5 min, the typical DNA ladder appeared. As the positive control, the mouse liver nuclei were frozen, thawed and digested for 5 min by micrococcal nuclease from which a DNA ladder was formed (lane 4).

cleosome structure are observed in *Xenopus* sperm [26–28], we did not observe a DNA ladder after treatment by micrococcal nuclease. The typical DNA ladder appeared in the carrot cell extract after micrococcal nuclease treatment (Fig. 3). This result indicated that the obvious remodeling process occurred on the sperm chromatin and the new nucleosome was formed during the nuclear reconstitution in this cell-free system. Whether advanced structures occurred, such as solenoid, remains unresolved and needs to be further examined.

It has been reported that the cytosol extract system is not only based on mammiferous cells (CHO cells) [13], but also amphibian cells such as *Xenopus* eggs. Even the system based on the *Drosophila* embryo cell-free extract has the ability to induce nuclear reconstitution [24,25]. Up to now, however, there was no report on nuclear reconstitution in a cell-free system based on plant cells. Our data demonstrated that the nuclear reconstitution can be successfully induced in a plant cell-free system, which implies the general mechanism or process of the nuclear self-assembly in both animal and plant cells. In addition, the nuclear reconstitution in the plant cell-free system provides an in vitro experiment model for studying the cell cycle in plant cells.

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References

[1] Lohka, M.J. and Masui, Y. (1983) Science 220, 719–721.
[2] Forbes, D.J., Kirschner, M.W. and Newport, J.W. (1983) Cell 34, 13–23.
[3] Blow, J.J. and Laskey, R.A. (1986) Cell 47, 577–587.
[4] Newport, J. (1987) Cell 48, 205–217.
[5] Sheehan, M.A., Mills, A.D., Sleeman, A.M., Laskey, R.A. and Blow, J.J. (1988) J. Cell Biol. 106, 1–12.
[6] Blow, J.J. and Sleeman, A.M. (1990) J. Cell Sci. 95, 383–391.
[7] Leno, G.H. and Laskey, R.A. (1991) J. Cell Biol. 112, 557–566.
[8] Vigers, G.P. and Lohka, M.J. (1991) J. Cell Biol. 112, 545–556.
[9] Chen, Y., Zhang, B., Li, X. and Zhai, Z. (1997) FEBS Lett. 413, 449–452.
[10] Qu, J., Zhang, C. and Zhai, Z. (1994) Cell Res. 4, 163–172.
[11] Zhang, B., Chen, Y. and Zhai, Z. (1995) Sci. China 25, 623–630.
[12] Jiang, Z.F. and Zhai, Z. (1996) Mol. Biol. Cell 7, 578.
[13] Burke, B. and Gerace, L. (1986) Cell 44, 639–652.
[14] Zhang, C., Luo, W. and Zhai, Z. (1993) Jiepou Bull. 24, 158–162.
[15] Cox, L.S. (1992) J. Cell Sci. 101, 43–53.
[16] Finlay, D.R. and Forbes, D.J. (1990) Cell 60, 17–29.
[17] Gant, T.M., Harris, C.A. and Wilson, K.L. (1999) J. Cell Biol. 144, 1083–1096.
[18] Finlay, D.R., Meier, E., Bradley, P., Horecka, J. and Forbes, D.J. (1991) J. Cell Biol. 114, 169–183.
[19] Nicolas, F.J., Zhang, C., Hughes, M., Goldberg, M.W., Watton, S.J. and Clarke, P.R. (1997) J. Cell Sci. 110, 3019–3030.
[20] Smythe, C. and Newport, J.W. (1992) Cell 68, 787–797.
[21] Zhao, Y., Sun, Y., Jiang, Z. and Zhai, Z. (1999) Chin. Sci. Bull. 44, 1497–1501.
[22] Zhao, Y., Jiang, Z., Sun, Y. and Zhai, Z. (1999) FEBS Lett. 448, 197–200.
[23] Mathews, B.F. (1983) Plant Sci. Lett. 31, 165–172.
[24] Steller, H. and Pirrotta, V. (1985) Dev. Biol. 109, 54–62.
[25] Ulitzur, N., Harel, A., Feinstein, N. and Gruenbaum, Y. (1992) J. Cell Biol. 119, 17–25.
[26] Risley, M.S. (1983) Genet. Res. 4, 331–346.
[27] Abe, S.I. (1987) Int. Rev. Cytol. 109, 159–209.
[28] Philpott, A. and Leno, G.H. (1992) Cell 69, 759–767.