Cell Research(1994),**4**, 163–172

Observation of nuclei reassembled from demembranated *Xenopus* sperm nuclei and analysis of their lamina components

QU JIAN, CHUANMAO ZHANG, ZHONGHE ZHAI.
College of Life Sciences, Beijing University, Beijing 100871, China.

ABSTRACT

A cell-free preparation obtained from extracts of activated *Xenopus laevis* eggs induced chromatin decondensation and nuclear formation from demembranated *Xenopus* sperm nuclei. Electron microscopy revealed that the reassembled nucleus had a double-layered nuclear membrane, nuclear pore complexes, and decondensed chromatin etc. Indirect immunofluorescence analysis demonstrated the presence of lamina in newly assembled nuclei. Western-blotting results showed that lamin L_{II} was present in egg extracts and in lamina of the reassembled nuclei which were previously reported to contain only egg derived lamin L_{III}.

Key words: *Nuclear assembly in vitro, Xenopus laevis, Lamina.*

INTRODUCTION

Following the pioneer work of Lokha and Masui[1, 2], studies using cell-free system have greatly enriched our knowledge about the events during cell cycle. Extracts derived from activated *Xenopus laevis* eggs able to asemble nuclei around exogeneous naked DNA, isolated chromosomes and demembranated sperm chromatin have been widely used to study the pathways and biochemistry of nuclear envelope and pore complexes assembly, lamina formation, DNA replication and nuclear cytoplasmic transportation[3-6]. Meanwhile, extracts prepared from mataphase eggs were adopted to study spindle organization and nuclear disassembly[7-10]. Plenty of information on cell cycle regulation concerning nuclear reassembly and mitosis has also been obtained from the studies using cell-free system[9, 10]. Since 1989, colleagues

in our laboratory have devoted to this field and achieved a number of results[11-14]. Recently we have successfully got the nuclei assembled *in vitro* around demembranated sperm chromatin in activated *Xenopus* egg extracts. The nuclei assembled in our system were morphologically similar to those of intact cells.

The nuclear lamina, a fibrous network located between nuclear envelope and chromatin, is composed of lamins. It has long been proposed that lamin proteins play important roles in nuclear assembly, chromatin topology and DNA replication and the interactions among membrane, lamins and chromatin bring about these processes[15-18]. Molecular and biochemical anaylyses have revealed that the lamin proteins are highly conserved and developmentally expressed in a tissue-specific manner. Based on comparison of their primary structure, biochemical features and expression pattern, two major lamin isotypes can be distinguished, A-type lamins (mammalian laminA and C, chicken and *Xenopus* lamin A) and B-type lamins which seem to be constitutively expressed and can be further classified into B1 (mammalian and chicken lamin B1 and *Xenopus* lamin L_I), B2 (mammalian and chicken B2 and *Xenopus* lamin L_{II}) and B3 *Xenopus* lamin L_{III}) type lamins. When the nuclear envelope breaks down during mitotic or meiotic cell division, lamin filaments are hyperphosphorylated by the protein kinase $P34^{cdc2}$ and depolymerized into oligomers. While A-type lamins are dispersed throughout the cytoplasm as soluble oligomers being not in contact with membranes, B-type lamins, though depolymerized, remain associated with the reminants of the nuclear envelope[19-22].

Lamin L_{III} is reported to be the single lamin isoform in *Xenopus* oocytes, eggs and early embryos and is present in embryos up to the tail bud stages, reappearing only in certain adult cells (neurons, muscle cells, and diplotene oocytes)[21]. Though classified as B-type lamin it is soluble during nuclear envelope break-down[19-21]. Lamin L_I and L_{II} appear at characteristic stages in development. Lamin L_I first appears at midblastula transition and L_{II} at the gastrula [21]. Considering the importance of lamin proteins in normal cells, we were prompted to examine whether the nuclei assemnled *in vitro* had lamina structure and what the lamin components were. Our results showed that the reassembled nuclei did have lamina and, interestingly, lamin L_{II} was found to be present in egg extracts and in nuclei assembled in vitro.

MATERIALS AND METHODS

Preparation of sperm nuclei

Sperm nuclei were prepared as described by Lohka and Masui[1] with some modifications. Testes were dissected from sexually mature *X. laevis* which had been injected with 100 I.U. human chorionic gonadotropin (HCG) and kept for 1 h at 22°C. The testes were washed free of blood and incubated overnight at 18°C in 200 % Steinberg's solution containing antibiotics and HCG(10 I.U./ml). Sperms were released by gently squeezing the testes, harvested by centrifugation at 1,500g for 10 min and washed 3 times with nuclear isolation buffer (NIB, 15 mmol/L NaCl, 60 mmol/L KCl, 15 mmol/L Tris, pH 7.4, 1 mmol/L DTT, 0.5 mmol/L spermine, 0.15 mmol/L spermidine). Sperms

were treated with NIB containing lysolecithin at 330 μg/ml and proteinase inhibitor for 10 min at 22 ℃. Lysolecithin -treated sperms were washed once with ice-cold NIB/3% bovine serum albumin (BSA), 3 times with NIB/0.4% BSA and finally resuspended in NIB/30% glycerol. They were counted with a hemocytometer, and stored at -70℃.

Preparation of HeLa cell chromosome clusters

As previously described[14], HeLa cells were cultured in E-MEM (Eagle's modified essential medium) and synchronized at metaphase by treatment with TdR and nocodazole. Cells were swelled in 1/2 Hank's solution for 2 h and then homogenized. Chromosome clusters were pelleted by centrifugation at 1,000g for 10 min, resuspended in E-MEM containing 20% newborn calf serum and 10% glycerol and stored at -70℃.

Preparation of egg extracts

Egg extracts were prepared as previously described[11]. Briefly, unfertilized eggs were collected from HCG-treated mature female *X. laevis* and dejellied with 2% cysteine. After activated by Ca^{2+} ionophore A23187, they were washed with buffer containing cycloheximide and cytochalasin B. Then the eggs were crushed at 10,000 g, 4℃ for 15 min. The material between the lipid top layer and yolk pellet was removed and centrifuged again at 10,000 g, 4℃ for 15 min. The supernatant was used as egg extracts.

Nuclear assembly in vitro

Demembranated sperm nuclei and clusters of HeLa cell chromosomes were respectively mixed with freshly prepared egg extracts at a concentration of 10^3 nuclei or chromosome clusters/ μl egg extracts and then the ATP regenerating system (2 mmol/L ATP, 50 μg/ml creatine kinase, 2 mmol/L phosphocreatine) was added. The mixture was incubated at 22℃.

Antibodies

All antibodies used here were antilamin monoclonal antibodies generously donated by Dr. Krohne. They were antilamin L_{II}/ L_{III} mAb X155, antilamin L_{II} mAb X223 and antilamin L_I/ L_{II} / lamin A mAb X167[26].

Immunofluorescent microscopy

Incubated mixtures were smeared onto glass slide, air-dried for 5 min and fixed for 30 min in mixture of acetone and methanol (1:1) at -20℃. After incubated in PBS containing 1% BSA for 10 min, the specimen were incubated with mAb X155 for 1 h, and washed 3 times with PBS. Then FITC labeled goat anti-mouse IgG was added and the specimen were incubated for 30 min and washed 3 times with PBS. Chromatin was stained by DAPI (DNA dye). The specimen were mounted with a coverslip and examined by fluorescence microscope.

Electron microscopy

Aliquots of the incubation mixture were fixed in ice-cold 2% glutaraldehyde and osmium tetroxide successively, dehydrated through a graded concentration series of acetone and then embedded in Epon 812. Ultrathin sections were cut, stainned with uranyl acetate and lead citrate, and finally examined under JEM-100CX transmission electron microscope.

Gel electrophoresis and immunoblotting

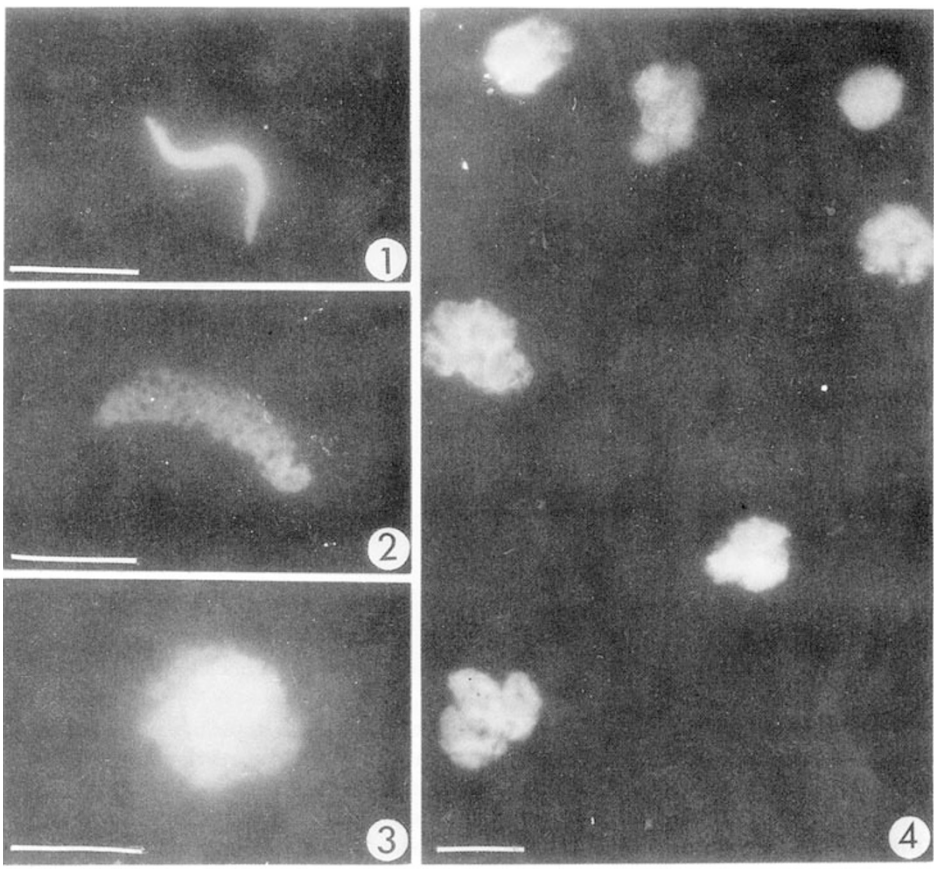
After diluted 10 times with NIB, the incubation mixture was centrifuged at 3,000g for 10 min. The pelleted nuclei were washed 3 times with NIB and then suspended in SDS sample buffer. Proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose filters. Filters were blocked

(37℃) in 3% BSA/ TBS (200 mmol/L Tris, 500 mmol/L NaCl, pH 7.5) for 2 h and then incubated (37℃) in 1% BSA /TTBS (200 mmol/L Tris, 500 mmol/L NaCl, pH 7.5, 0.05% Tween20) containing antilamin antibody mAb X155 or mAb X223 or mAb X167 for 3.5 h. After washed with TTBS and incubated in 1% BSA /TTBS containing Biotin labeled goat anti-mouse IgG for 2 h, filters were washed with TTBS and then blocked in 3% BSA / TBS for 1.5 h. Filters were incubated in buffer 1 (100 mmol /L Tris, 1 mol/L NaCl, 2 mmol /L $MgCl_2$, 0.05% TritonX-100, pH 7.5) containing Avidin-AP (akaline phosphotase) for 15 min and then washed successively with buffer 1 and buffer 2 (100 mmol/L Tris, 1 mol/L NaCl, 5 mmol/L $MgCl_2$, pH 9.5). Filters were stained in buffer 3 (100 mmol/L Tris, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, pH 9.5) containing NBT and BCIP.

RESULTS AND DISSCUSSION

Incubation of demembranated *Xenopus* sperm nuclei in egg extracts resulted in rapid chromatin decondensation followed by nuclear formation. The demembranated sperm chromatin was in a long, thin and condensed form which was the characteristic of the sperm nucleus and could be stained strongly with DAPI[Fig 1]. When it was added to egg extracts and incubated at certain temperature (22℃), the sperm chromatin began to elongate and swell, showing discernible decondensation, in the first 10 min of incubation[Fig 2]. After 60-90 min, nearly all of the originally sharp-contoured demembranated sperm nuclei had decondensed and changed into spherical shape like a pronucleus[Fig 3], while less than 5% of them remained in their initial form. Most of the added sperm chromatin followed the same steps in assuming morphological alteration[Fig 4]. Lysolecithin was effective in removing cell membrane and nuclear envelope of sperm. At the ultrastructral level, we observed that after it was treated with lysolecithin, sperm chromatin was devoid of double-layered nuclear envelope and was rather condensed[Fig 5]; After incubation for about 1 h, the decondensed chromatin gradually acquired a continuous nuclear envelope with pores. The newly assembled nuclei were morphologically indistinguishable from those in intact interphase cells[Fig 6].

Nuclei assembled *in vitro* were assessed for the presence of lamina by indirect immunofluorescent microscopy. When incubated with mAb X155 (specific for lamin L_{II} /L_{III}), the demembranated sperm nuclei had no positive staining while the newly assembled nuclei exhibited positive rim-staining[Fig 7]. It was reported that only lamin L_{IV} was found in spermatids and spermatozoa[27]. Due to the lack of antibody specific for lamin L_{Iv}, we could not make sure whether the demembranated sperm nuclei still had some attached lamin L_{Iv} protein, although they had no intact lamina structure after demembranation. However, even though we could not conclude from the negative result that there was not any type of lamin protein around the periphery of demembranated sperm nuclei, we surely knew that there was no lamin L_{II} or L_{III} protein; and then from the positive result we knew that during incubation, lamin L_{II} or/and L_{III} which were previously stored in egg extracts participated in the formation of lamina structure of the newly assembled nuclei. Our previous work had demonstrated that nuclei assembled around clusters of HeLa cell chromosomes in egg extracts also had lamina [14].



Fluorescence microscopy (DAPI stain) showing the decondensation of demembranated sperm nuclei in egg extracts. Bars: 10 μm.

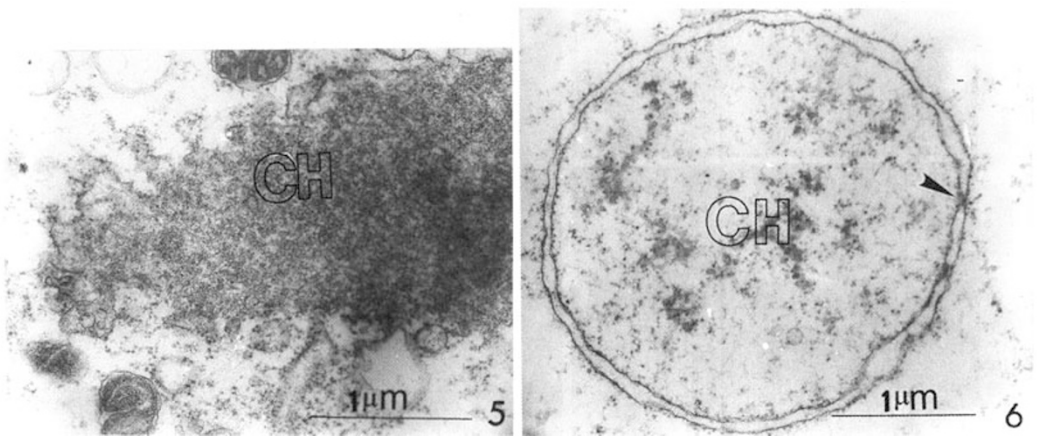
Fig 1. The highly condensed sperm chromatin before incubation.

Fig 2. Elongated and swelled sperm chromatin after incubation of 10 min.

Fig 3. Highly decondensed sperm chromatin taking a spherical shape after incubation of 60 min.

Fig 4. Most of the added demembranated sperm nuclei followed the same steps to decondense to a spherical shape after incubation of 60 min.

Western-blotting analysis was used to detect the lamina components of nuclei assembled in vitro. Nuclei were isolated from the incubation mixture by centrifugation and proteins were separated by gel-electrophoresis and then transferred to nitrocellulose filters. The fiters were probed with antilamin L_{II} / L_{III} mAb X155, antilamin L_{II} mAb X223, or antilamin L_{I} /L_{II} /lamin A mAb X167 respectively. Using mAb X155 we found that both demembranated sperm nuclei and chromosomes of HeLa cells had no egg-derived lamin, whereas nuclei assembled in vitro from both of them



Ultrathin section of demembranated sperm chromatin and nuclei assembled *in vitro*. Bars: 1 μm

Fig 5. Demembranated sperm chromatin devoid of nuclear envelope.

Fig 6. Typical nuclei assembled *in vitro* with continuous nuclear envelope, pores(↑) and well-decondensed chromatin.

contained the same lamin components as those found in egg extracts[Fig 8]. This confirmed our observation by indirect immunofluorescent microscopy that the lamins in the newly formed nuclei were derived from egg extracts. It is widely believed that *X. laevis* oocytes, eggs and early embryos contain only one lamin isoform, the lamin L_{III}, which serves as a maternal pool for the formation of lamina in the pronuclei and early cleavage nuclei. During development lamin L_{III} is replaced in embryonic nuclei by lamin L_I and L_{II} at the midblastula and gastrula stages respectively[21]. Considering that lamin L_{II} and L_{III} had the same molecular weight of 68kD and mAb X155 recognized both lamin L_{II} and L_{III}, we then used mAb X223 and mAb X167 which both reacted with lamin L_{II} but not with lamin L_{III}[26]. To our interest, we found that both egg extracts and the nuclei assembled in the egg extracts reacted positively with the two antibodies. These results unequivocally suggested that besides lamin L_{III}, lamin L_{II} was also present in egg extracts and was involved in lamina formation of the nuclei assmbled *in vitro* in egg extacts[Fig 9, 10]. When we are preparing this paper, it comes to our notice that the recent work of Lourim and Krohne also confirmed the existence of lamin L_{II} in *Xenopus* oocytes, eggs and embryos[26].

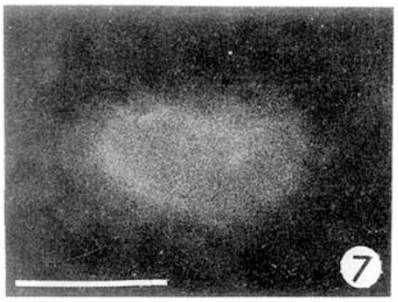


Fig 7. Indirect immunofluorescent staining of lamina in the nucleus assembled *in vitro* with antilamin mAb X155. Bars: 10μm.

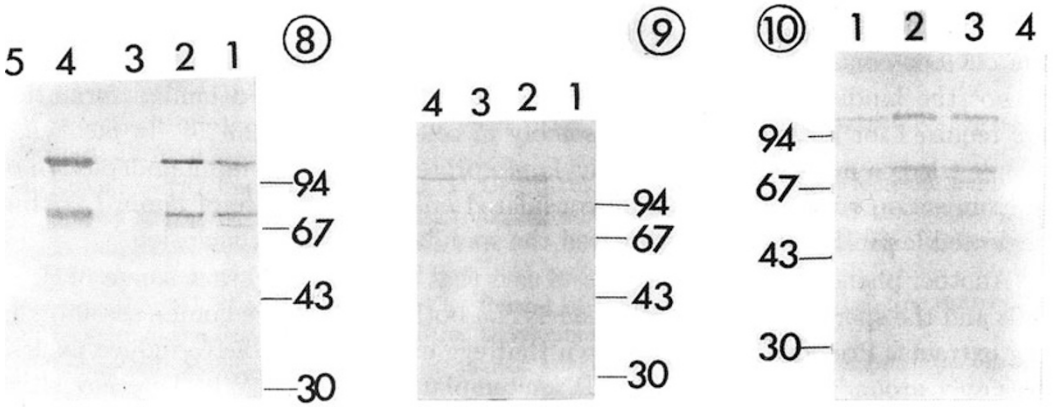


Fig 8. Both the nuclei assembled *in vitro* around sperm chromatin and chromosomes of HeLa cells had the same lamin peptides as those in egg extracts. Proteins were separated by SDS-PAGE and transferred to NC filters and then probed with mAb X155 in combination with ABC-Western-blotting technique. 1. egg extracts. 2.nuclei assembled around demembranated sperm nuclei. 3.demembranated sperm nuclei. 4. nuclei reassembled around chromosomes of HeLa cells. 5. chromosomes of HeLa cells. Molecular weight was showed at right in kD.

Fig 9. Lamin L_{II} was present in egg extracts and nuclei assembled *in vitro* detected by mAb X167. 1. egg extracts. 2.nuclei assembled around chromosomes of HeLa cells. 3. chromosomes of HeLa cells. 4.nuclei assembled around demembranated sperm nuclei.

Fig 10. Lamin L_{II} was present in egg extracts and nuclei assembled *in vitro* detected by mAb X223. 1. egg extracts. 2. nuclei assembled around demembranated sperm nuclei. 3. nuclei assembled around chromosomes of HeLa cells. 4. chromosomes of HeLa cells.

In discussing lamina formation of mammalian cells, Gerace and Blobel proposed a different functional specialization for lamin A and B in terms of chromatin and membrane binding respectively[22]. Since only lamin L_{III} is found in *X. laevis* oocytes, eggs and early embryos, it has been suggested that lamin L_{III} alone can build up a lamina structure in early cleavage nuclei and carry out both functions of chromatin-binding and membrane-binding in lamina formation through the mediation of some kinds of membrane or chromatin associated receptors. It has been

hypothesized that different lamins are functionally equivalent during the developmental change of lamina composition in *Xenopus* embryogenesis and the change may be resulted from gene regulation[21]. This is somewhat interesting compared with the situations in mammalian and bird embryos. Now that lamin L_{II} is detected in egg extracts and nuclei reassembled in vitro, questions concerning lamin function and expression turn out even more complicated than previously presumed. How do these two polypeptides function and what is their relationship? Though both classified as B-type lamin, do lamin L_{II} and L_{III} interact during lamina formation in a manner similar to lamin A and B in mammalian cells? Moreover, the finding of lamin L_{II} in egg extracts and nuclei assembled in vitro will help us to explain the obvious contradiction among observations concerning the problem of whether or not the binding of lamin proteins to chromatin surface and lamina formation are required for nuclear envelope assembly in cell-free system[23-25]. In fact when people tried to inactivate endogeneous lamin protein in eggs by immunodepletion or microinjection with antibody, they considered only the presence of lamin L_{III} but neglected lamin L_{II} and thus impaired the soundness of their conclusion.

Another phenomenon which interests us is that the somatic chromosomes of HeLa cells and the sperm chromatin of *X.* laevis can both demonstrate lamina assembly in egg extracts. Previous work has shown that egg extracts are able to induce nuclear assembly around a wide variety of DNA templates[1, 3, 14]. Whether they share the same lamina formation pattern and whether developmentally controlled factors in egg extracts are required for the lamina formation remain unresolved and need further study.

It has been reported that soon after their synthesis in the cytoplasm the lamin proteins, including lamin L_{II} and L_{III}, dimerize and are transported to the nucleus. A nuclear localization signal and a CaaX box at the carboxyl terminus are necessary to the latter process[15, 16, 28, 29]. The CaaX motif (C: cysteine, a: an aliphatic amino acid, X: any amino acid) is a substrate for 3 successive post-translational modifications (isoprenylation of the cysteine, proteolytic removal of three carboxyl terminal acids; and carboxyl-methylation of the isoprenylated cysteine). Another kind of modification of lamin proteins is phosphorylation [10]. In all our Western-blotting positive lanes there were two bands at the position of 68 kD. Because mAb X223 is specific for only lamin L_{II}, we regard that the doublets in Fig 10 may be resulted from different migrating abilty of two forms of lamin L_{II} with different modification. As for the doublets in Fig 8, there may be two possibilities: 1)Each band contains both lamin L_{II} and L_{III} and either one of lamin L_{II} and L_{III} is of different modification form in the two bands. Because lamin L_{II} and L_{III} have similar molecular weight and are both B-type lamin, it is quite possible that after being modified, either in the same manner or not, they have the same mobile activity in our gel system. 2) While both of the two bands contain lamin L_{II}, only one band contains lamin L_{III}; in other words, there is only one form of lamin L_{III} which has the same migrating pattern as that of one of the two forms of lamin L_{II}. Since mAb X167

is specific for lamin L_{II} and two other lamin isoforms but not for lamin L_{III}, there is one possibility that the situation in Fig 9 is just like that in Fig 10: each band represents a modification form of lamin L_{II}. The much slower migrating band in the three figures at the position of approximately 150 kD may be the dimer of lamin proteins, either homodimers of lamin L_{II} or L_{III} or heterodimers of lamin L_{II} and L_{III}.

REFERENCES

[1] Lokha MJ, Masui Y. Formation *in vitro* of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. Science 1983; **220:**719-21.

[2] Lokha MJ, Masui Y. Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparation from amphibian eggs. J Cell Biol 1984; **98:**1222-30.

[3] Newport J. Nuclear reconstitution *in vitro*: Stages of assembly around protein-free DNA. Cell 1987; **48:**205-17.

[4] Newmeyer DD, Lucocq JM, Burglin TR, Derobertis EM. Asssembly in vitro of nuclei and nuclear protein transport: ATP is required for nucleoplasmic accumulation. EMBO 1986; **5:**501-10.

[5] Wilson KL, Newport J. A trypsin-sensitive receptor on membrane vesicles is required for nuclear envelope formation *in vitro*. J Cell Biol 1988; **107:**57-68.

[6] Hutchison D J, Cox R, Ford CC. The control of DNA replication in a cell-free extract that recapitulates a basic cell-cycle *in vitro*. Development 1988; **103:**553-66.

[7] Lohka M J, Maller JL. Induction of nuclear envelope breakdown, chromosome condensation, spindle formation in cell-free extracts. J Cell Biol 1985; **101:**518-22.

[8] Sawin KE. Mitchison TJ. Mitotic spindle assembly by two different pathways *in vitro*. J Cell Biol 1991; **112:**925-40.

[9] Luscher B, Brizuela L, Beach D, Eiseman RN. A role for the $p34^{cdc2}$ kinase and phosphotases in the regulation of phosphorylation and disassembly of lamin B2 during the cell cycle. EMBO 1991; **10:**865-75.

[10] Peter M, Nakagawa J, Doree M, Labbe JC. Nigg EA. *In vitro* disassembly of the nuclear lamina and M Phase-specific phosphorylation of lamins by cdc2 kinase. Cell 1990; **61:**591-602.

[11] Cai ST, Zhang B, Qing J, Zhang CM, Zhai ZH. Nuclear assembly *in vitro* using Xenopus egg extracts and Lambda DNA. Chinese Sci Bulletin 1990; **35(16):**1261-4.

[12] Zhang CM, Zhang B, Zhai ZH. Chromatin and nucleosome organization and DNA replication of nucleus reassembled *in vitro* using purified exogenous DNA and *Xenopus* egg extracts. Sci China(Series B) 1994; **37(6):**677-88.

[13] Zhang CM, Qu J, Zhai ZH. Biological activity assays of cell-free reassembled nuclei. Sci China (Series B) 1994; **24(2):**165-70.

[14] Zhang CM, Qu J, Zhai ZH. Nuclear reconstitution around purified HeLa chromosomes in a cell-free system from *Xenopus* eggs. Acta Biol Exp Sin 1994; **27(1):**51-9.

[15] Nigg EA. Assembly-disassembly of the nuclear lamina. Cur Opin Cell Biol 1992; **4:**105-9.

[16] Mckeon F. Nuclear lamin proteins: domains required for nuclear targeting assembly and cell-cycle regulated dynamics. Cur Opin Cell Biol 1991; **3:**82-6.

[17] Aebi U, Cohn J, Buhle L, Gerace L. The nuclear lamina is a meshwork of intermediate filaments. Nature 1986; **323:**560-4.

[18] Nigg EA. The nuclear envelope. Cur Opin Cell Biol 1989; **1:**438-40.

[19] Stick R. cDNA cloning of the developmentally regulated lamin L_{III} of Xenopus laevis. EMBO 1988; **7:**3189-97.

[20] Hoger TH, Krohne G, Franke WW. Amino acid sequence and molecular characterization of murine lamin B as deduced from cDNA clones. Eu J Cell Biol 1988; **47:**283-90.

[21] Benavente R, Krohne G, Franke W. Cell type-specific expression of nuclear lamina proteins during development of *Xenopus* laevis. Cell 1985; **41:**177-90.
[22] Gerace L, Blobel G. The nuclear envelope is reversibly depolymerized during mitosis. Cell 1980; **19:**277-87.
[23] Dabauvalle MC, Loos K, Merkert H, Scheer U. Spontaneous assembly of pore complex-containing membranes ("Annulate Lamellae") in Xenopus egg extracts in the absence of chromatin. J Cell Biol 1991; **112:**1073-82.
[24] Newport J, Wilson K, Dunphy W. A lamin-independent pathway for nuclear envelope assembly. J Cell Biol 1990; **111:**2247-59.
[25] Burke B, Gerace L. A cell-free system to study reassembly of the nuclear envelope at the end of mitosis. Cell 1986; **44:**639-52.
[26] Lourim D, Krohne G. Membrane-associated lamins in *Xenopus* egg extracts: Identification of two vesicle populations. J Cell Biol 1993; **123:**501-12.
[27] Benavente R, Krohne G. Change of karyoskeleton during spermatogenesis of *Xenopus:* expression of lamin L_I, a nuclear lamina protein specific for the male germ line. Proc Natl Sci 1985; **82:**6176-80.
[28] Kitten GT, Nigg EA. The CaaX motif is required for isoprenylation, carboxyl methylation and nuclear membrane association of lamin B2. J Cell Biol 1991; **113:**13-23.
[29] Firmbach-Kraft, Stick R. The role of CaaX-dependent modifications in membrane association of *Xenopus* nuclear lamin B3 during meiosis and the fate of lamin B3 in transfected mitotic cells. J Cell Biol 1993; **123:**1661-70.

Received 2-7-1994. Revised 2-11-1994. Accepted 7-11-1994.