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DEPARTMENT OF BIOLOGY, GRADUATE SCHOOL OF SCIENCE

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CONTENTS

Part I

GENERAL INTRODUCTION

The structure of nuclear membrane

The nuclei of higher eukaryotic cells are surrounded by nuclear envelope (NE), that consists of two phospholipid-containing membrane bilayers, the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). They are joined at the site of nuclear pore complex (NPC). The membrane in this region is called pore nuclear membrane (PNM) (Franke et. al., 1981, for a review, see Goldberg and Allen, 1995).

The INM is a nucleoplasmic side of the NE. Using scanning electron microscopy (EM), several filamentous structures have been observed on the surface of the INM (Goldberg and Allen, 1992, 1993). The major filamentous structure is a nuclear lamina (Aebi et al., 1986; Goldberg and Allen, 1993). The component of nuclear lamina was identified as a set of intermediate filament protein family, named as lamin (Gerace and Blobel, 1980). Several integral membrane proteins on INM were identified. These are thought to be attached with nuclear lamina and also with chromatin (for a review see Georgatos et. al., 1994). In addition, thin-filamentous structure, which was different from nuclear lamina, was also observed by EM. This structure is called as nuclear envelope lattice (NEL) (Goldberg and Allen, 1992, 1993). The precise protein components of this structure have not yet been identified. The ONM is a cytoplasmically opposed side of the NE. The ONM is connected to and resembles the rough endoplasmic reticulum (ER). The structural feature of the ONM is the presence of ribosome particles. The PNM is the part of the NE

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(a)

Nuclear Pore Complex

Nucleolei

Nuclear Matrix

Nuclear Envelope

Chromatin

Nuclear Lamina

(b)

Cytoplasm

Ribosome

ONM

ONM

Nuclear Lumen   PNM

PNM   Nuclar Lumen

INM

INM

Nuclear Lamina
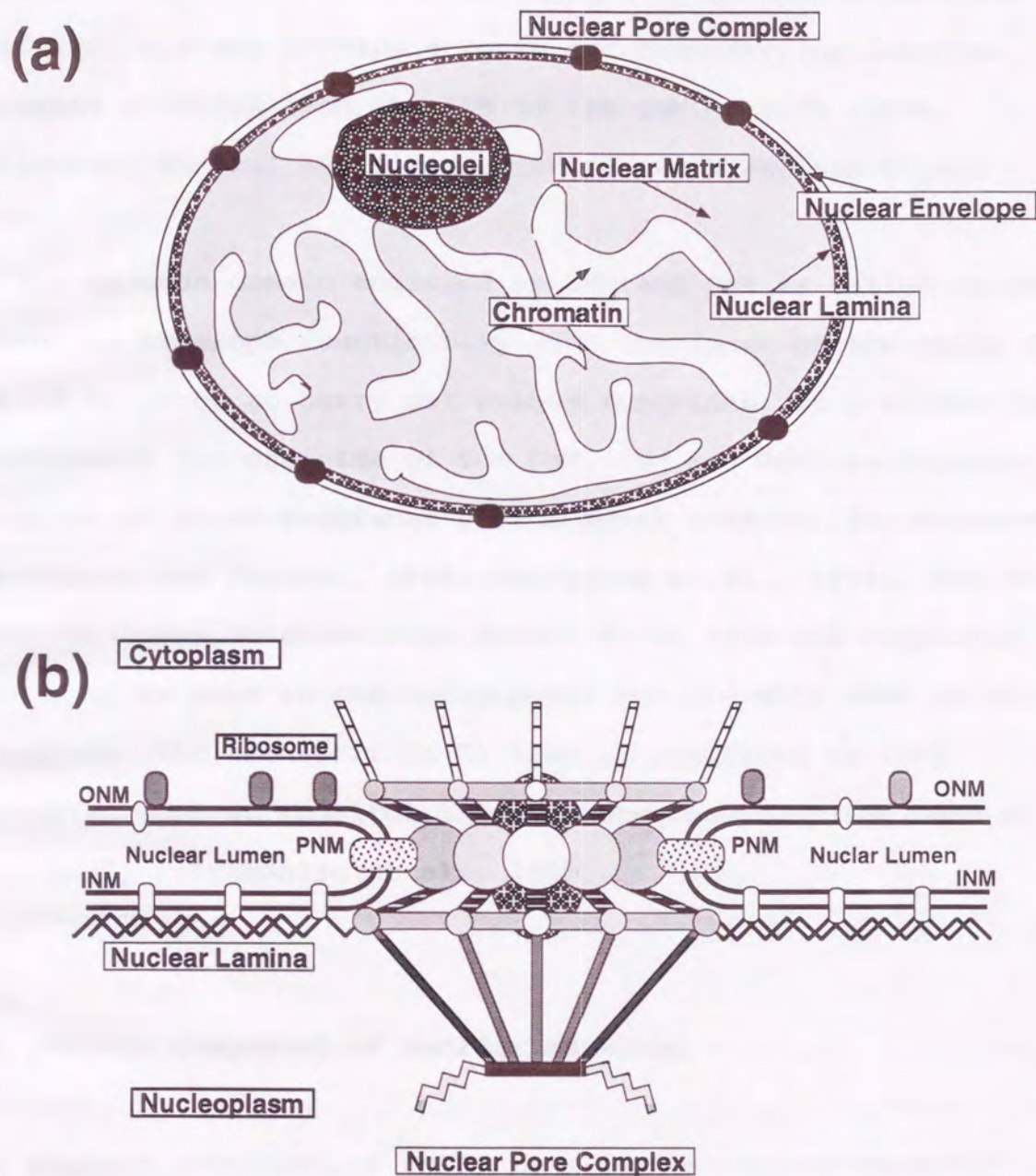
Nucleoplasm

Nuclear Pore Complex

Fig. 1. Schematic models of nuclear structure.
(a) Structure of nucleus.
(b) Structure of nuclear envelope.

present in the NPC. The PNM is thought to be associated with NPC proteins, and may provide a route for transferring integral membrane proteins from the INM to the ONM or vice versa (Ellenberg et al., 1997; for a review see Wiese and Wilson, 1993).

The aqueous domain enclosed by INM and ONM is called as NE lumen. It overlaps functionally with the lumen of the rough ER, but is believed to carry out unique functions. It provides the environment for one side of the ONM, INM and PNM, so integral proteins of these membranes have lumenal domains (for reviews, see Gerace and Foisner, 1994; Georgatos et al., 1994). The NE lumen is known to store high levels of Ca ions and regulates the levels of Ca ions in the nucleoplasm and provably that in the cytoplasm. The transport of Ca ions is regulated by some proteins, such as inositol triphosphate receptors (Guihard et al., 1997; Subramanian et al., 1997).

The protein component of nuclear envelope

The nuclear pore complex (NPC) is a supramolecular assembly embedded in the NE, and mediates molecular trafficking between the cytoplasm and the nucleus in interphase eukaryotic cells. NPC of vertebrate cells is estimated to be 120 MD, and is believed to be composed of about 100 different proteins. The structure of NPC has been extensively investigated by electron microscopy, and 3D reconstructions (Unwin and Milligan, 1982; Reichelt et al., 1990;

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Hinshaw et al., 1992; Akey and Radermacher, 1993). The NPC accommodates both "passive diffusion" and "active transport". Whereas ions and small metabolites diffuse freely though 10 nm diameter aqueous channels in the NPC, most of macromolecules are transported though a gated channel by a signal- and energy-dependent mechanism. Recently, several factors, that govern the active transport, have been identified (for reviews, see Melchior and Gerace, 1995; Hurt et al., 1996; Ullman et al., 1997).

The component of NPC is grouped into two categories. The first is a group of integral membrane proteins, that is proposed to be NPC anchoring proteins. The second is a group of peripheral membrane proteins that have been called nucleoporins. Some nuclear pore proteins have a characteristic amino acids repeats structure, such as FXFG repeats or GLFG repeats. These repeat region is thought to be the interaction site with soluble transport factors. In addition, some nuclear pore proteins possess glycosilation site, but the function of this modification has not been revealed (for reviews, see Pante and Aebi, 1993, 1994; Davis, 1995; Doye and Hurt, 1997). p62 is one of the most abundant nucleoporin. p62 contains FXFG repeat and *O*-linked *N*-acetylglucosamine residues, and is essential for nuclear protein import (Starr et al., 1990; Carmo-Fonseca et al., 1991; Cordes et al., 1991; Finlay et al., 1991; Buss and Stewart, 1995).

Nuclear lamina is composed of intermediate filament (IF) proteins called lamins which are identified from nematode to mammals (Gerace and Blobel, 1980; Mckeon et al., 1986; Aebi et al., 1986; for a review, see Georgatos et al., 1994). Lamins fall

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into two sub-group, the A-type and B-type lamins, based on primary sequence, expression pattern and behavior at mitosis (for review, see McKeon, 1991). On mammals, A-type lamin gene is translated into two alternatively spliced products, called lamin A and lamin C. On the other hand, two independent genes of B-type lamin are known to be exist on mammal, and called as lamin B1 and lamin B2. Recently, another tissue and stage specific splicing variants of A-type and B-type lamin were identified. Lamin C2 is a splicing variant of lamin A (Furukawa et al., 1994; Alsheimer and Benavente, 1996) and lamin B3 is a splicing variant of lamin B2 (Furukawa and Hotta, 1993). On *Xenopus*, four lamin cDNAs were isolated, those are lamin A, lamin B1 (LI), lamin B2 (LII) and lamin B3 (LIII). *Xenopus* lamin B3, which is not a splicing product as mammal, is a unique lamin and expressed only in amphibian embryonic stages (Krohne et al., 1987; Wolin et al., 1987; Stick, 1988; Doring and Stick. 1990; Hoger et al., 1990).

Like other IF, lamins possess two non-helical NH_2- and COOH-terminal regions (head and tail, respectively) and a coiled-coil middle domain (rod). The rod domain comprises three subdomains (coils 1a, 1b and 2) connected by non-helical linkers. Apart from these features, the lamin molecules show certain unique characteristics, the nuclear localization signal (NLS) in the tail domain, and a COOH-terminal site ('CaaX box') for post-translational isoprenylation and carboxymetylation, which governs the interaction with the surface of INM lipid bilayer. The CaaX box occurs in all type B lamins and in a short-lived lamin A precursor, but not exist in mature lamin A or in lamin C (for

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reviews, see Nigg, 1992; Georgatos et al., 1994).

Some integral membrane proteins have been identified on INM and are suggested to be involved in nuclear lamina formation. p58 is one of such proteins, and also named as "lamin B receptor" (LBR). p58/LBR contains eight putative transmembrane domain and NH_2-terminal chromatin binding domain exposed into nucleoplasmic side. p58/LBR is known to form a complex with lamin B, integral membrane protein p18, splicing factor (SF2) p32, LBR kinase, and p150 (Worman et al., 1988, 1990; Sioms and Georgatos, 1992; Ye and Worman, 1994, 1996; Pyrpasopoulou et al., 1996; Simos et al., 1996). LAP1 (lamina associated polypeptide 1) and LAP2/thymopoietin are integral membrane proteins. They contain one transmembrane domain and are interact with lamin B (Foisner and Gerace, 1993; Furukawa et al., 1995; Martin et al., 1995). Otefin is an integral membrane protein identified only on *Drosophila* (Harel et al., 1989; Pandan et al., 1990; Ashery-Pandan et al., 1997).

Dynamics of nuclear membrane and nuclear lamina during mitosis

In higher eukaryotic cells, the NE is disassembled completely during mitosis. This process is called as "nuclear envelope breakdown (NEBD)", and the nuclear membrane dissociation and the nuclear lamina depolymerization is involved. NE dynamics were studied by several methods. The common method is the observation of mitotic cells by immunofluorescence microscopy with labeled
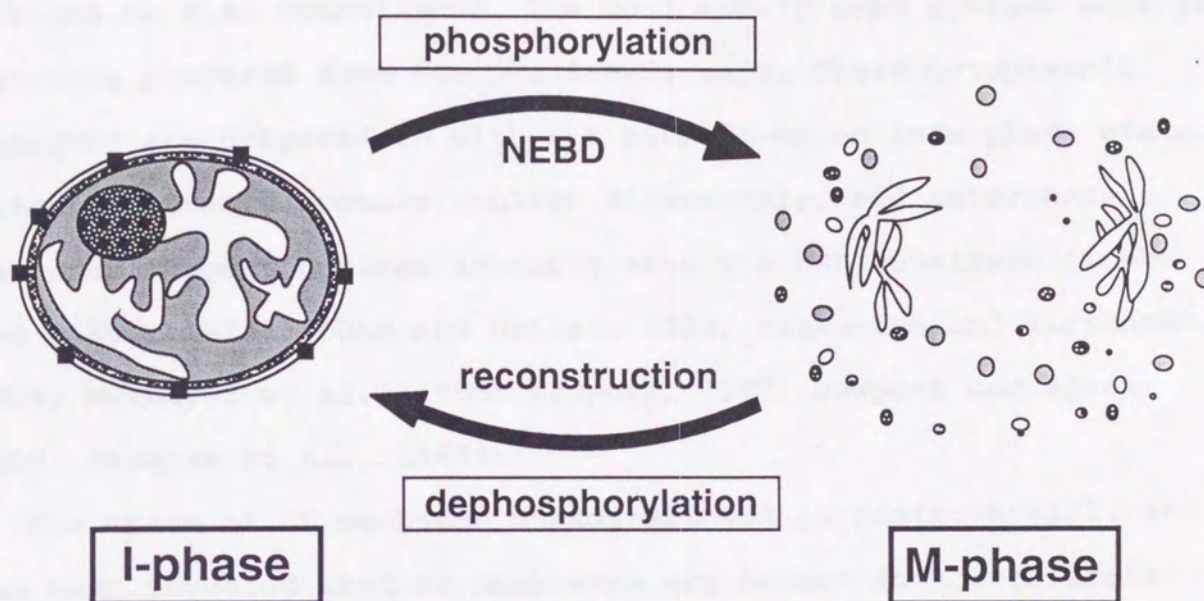
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Fig. 2. Nuclear dynamics during cell cycle.

antibody or a combination with ectopic gene expression system (Chaudhary and Courvalin, 1993; Buendia and Courvalin, 1997; Ellenberg et al., 1997; Maison et al., 1997; Yang et al., 1997). In addition, the biochemical approach that employs *in vitro* systems is also established. The most widely used systems employs extracts prepared from *Xenopus laevis* eggs. These cytoplasmic extracts are prepared in either a mitotic or an interphase state. Mitotic extracts promote nuclear disassembly, and interphase extracts support nuclear assembly around a DNA substrate (Lohka and Masui, 1983; Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Newmeyer et al., 1986; Newport, 1987; Newport and Spann, 1987; Sheehan et al., 1988).

The state of NE membrane during mitosis is controversial. It has been revealed that NE membranes are broken down into small vesicles during mitosis. The nuclear mitotic vesicles were fractionated biochemically and their features and protein components have been studied on *Xenopus* eggs (Vigers and Lohka, 1991, 1992; Lourium and Krohne, 1993; Pfaller and Newport, 1995; Lourim et al., 1996; Weise et al., 1997) sea urchin eggs (Collas et al., 1996; Collas and Poccia, 1996), and normal cells (Maison et al., 1993, 1995; Meier and Georgatos, 1994; Buendia and Courvalin, 1997). The two models have been proposed about NE membrane vesiculation. The first model is that INM, ONM or ER membrane is disassemble independently, resulting in functionally different vesicles, that contain different protein components. The second model is that INM, ONM or ER membrane is disassemble as a fused manner, resulting in a single population of vesicles

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and no functional difference exists on each vesicle (Yang et al., 1997). In addition, recent reports suggested that at the time when NEBD occurs at prometaphase, NE membrane is fused to ER membrane and incorporated into mitotic ER/NE membrane network (Ellenberg et al., 1997). Following this hypothesis, NE and ER membrane vesiculation dose not occur during mitosis.

The nuclear lamina is disassembled at prometaphase and reassembled at telophase. This behavior is parallel with nuclear envelope dynamics (Chaudhary and Courvalin, 1993; Georgatos et al., 1997). The process of assembly and disassembly of lamin filaments are regulated through reversible phosphorylation. cdc2/cyclin B kinase and protein kinase C are known to phosphorylate lamins (Heald and McKeon, 1990; Peter et al., 1991; Dessev et al., 1991; Hocevar et al., 1993; Collas et al., 1997). Other NE proteins are also known to be phosphorylated at the onset of mitosis. These are p58/LBR (Simos et al., 1992; Nikolakaki et al., 1996, 1997), LAP1, LAP2 (Foisner and Gerace, 1993), and some nucleoporins (Macaulay et al., 1995; Favreau et al., 1996). The phosphorylation of these proteins may be important for NEBD and disassembly of nuclear structures, such as nuclear lamina and NPC (Dabauvalle et al., 1990; Finlay et al., 1991;).

The key factor that induces NEBD and another mitotic event was first identified as an activity that induce meiotic maturation of *Xenopus* oocyte, and named as MPF (maturation promoting factor or M-phase promoting factor) (Masui and Markert, 1971). This factor was purified and characterized later as an cdc2/cyclin B kinase

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(for reviews, see Maller, 1991; Coleman and Dunphy, 1994; Stern and Nurse, 1996). When the cdc2/cyclin B kinase is once activated, the most nuclear mitotic events are induced. So cdc2/cyclin B kinase is thought to be as a master regulator for mitosis. In addition to cdc2/cyclin B, another factor that participates in mitotic regulation was also identified. One of the candidate regulator for nuclear mitotic events is nimA kinase family (Fry and Nigg, 1995). Lu and Hunter (1995) reported that when *Aspergillus* derived NIMA proteins were injected into *Xenopus* immature oocyte, the NEBD and chromosome condensation were induced without the increase of cdc2/cyclin B activity. It seems that many regulatory factors are involved in NEBD, but these factors and the regulation are poorly understood.

In the first part of this report, I investigated the B-type lamin dynamics during cell cycle on *Xenopus* A6 culture cells. In the second part I investigated the nuclear envelope breakdown of isolated nucleus in *Xenopus* egg mitotic extracts.

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Part II

Dynamics of B-type lamins during the cell cycle of *Xenopus* A6 cells

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SUMMARY

Lamin is a member of intermediate filament proteins and a major
component of nuclear lamina structure. Lamin possesses non-
helical N-terminal and C-terminal domains and an α-helical rod
domain. We isolated lamin B1 and lamin B2 cDNAs from *Xenopus*
oocyte cDNA library, and constructed recombinant lamin proteins
using lamin B1 N-terminal (N), lamin B1 C-terminal (C) and lamin
B2 rod (R) domains. We prepared domain-specific anti-lamin B
antibodies from these constructs, and studied the subcellular
distribution during the cell cycle of *Xenopus* A6 cell. In
interphase cell, antibodies against lamin B1(C) and lamin B2(R)
gave nuclear rim staining. In contrast, the antibody against
lamin B1(N) gave weak nuclear rim staining but strong
intranuclear staining. Similar nucleoplasmic signals were
detected within a nucleus of *Xenopus* liver. However on
prophase cell, anti-lamin B1(N) antibody stained the nuclear rim
strongly, and the intensity of rim staining of other antibodies
also became strong. At telophase when the nuclear envelope was
formed around chromosome, anti-lamin B1(N) antibody also gave
strong rim staining. This rim staining were disappeared at the
onset of interphase. These results suggest that the nuclear
lamina structure and the intranuclear lamin structure are
differently aligned and the conformation change of nuclear lamina
occurs at prophase and telophase.

The nuclear lamina is a filamentous meshwork structure on the nucleoplasmic surface of the inner nuclear membrane. The major structural protein of nuclear lamina is a nuclear lamin (Gerace and Blobel, 1980; McKeon et al., 1986; Aebi et al., 1986; for a review, see Georgatos et al., 1994). Lamin is a member of intermediate filament (IF) superfamily. Lamins fall into two sub-groups as A-type and B-type, based on primary sequence, expression pattern and behavior at mitosis (for a review, see McKeon, 1991). In *Xenopus laevis*, four different lamin cDNAs were identified. These are lamin A, lamin B1 (LI), lamin B2 (LII), and lamin B3 (LIII). Furthermore, two isoforms were reported on lamin B3 (Krohne et al., 1987; Wolin et al., 1987; Stick, 1988; Doring and Stick, 1990; Hoger et al. 1990).

The expression of lamin proteins is regulated during development and cell differentiation on *Xenopus*. In oocytes and eggs, lamin B3 is a major subtype, and lamin B1 and B2 are rare. During development, lamin B3 is gradually replaced by lamin B1 and lamin B2 (Benavente et al., 1985; Stick and Hausen, 1985; Lourim and Krohne, 1993; Lourim et al., 1996).

Lamins and IF proteins have a remarkable structural similarities. As cytoplasmic IF, lamins have three separated domains, a central α-helical rod domain and non-α-helical head and tail domains. The rod domain is further subdivided into three α-helical coiled coils characterized by a heptad repeat of

hydrophobic amino acid. The rod domains are primarily responsible for lamin-lamin interactions that govern the formation of parallel aligned dimer. The head and tail domains are involved in the assembly of dimmers into head-to-tail aligned polymers (for reviews, see McKeon, 1991; Heins and Aebi, 1994).

Lamins are found not only on nuclear envelope but in foci within the nucleoplasm. Lamin A and lamin A precursor were found at the intranuclear foci of G1-phase cells (Goldman et al., 1992; Bridger et al., 1993; Sasseville and Raymond, 1995). Lamin B was detected on DNA replication foci and suggested to be involved in DNA replication (Moir et al., 1994). The involvement of lamin B on DNA replication also comes from the experiments using *Xenopus* egg cell-free system (Newport et al., 1990; Meier et al., 1991; Jenkins et al., 1993; Goldberg et al., 1995; Span et al., 1997; Ellis et al., 1997).

During mitosis the distribution of lamins change dynamically parallel with the behavior of nuclear envelope. The nuclear lamina is disassembled at prometaphase and reassembled at telophase (Chaudhary and Courvalin, 1993; Georgatos et al., 1997;). The assembly and disassembly of lamin filaments are regulated through reversible phosphorylation. It was reported that cdc2/cyclin B kinase and protein kinase C phosphorylate lamins and disassemble the nuclear lamina structure (Heald and McKeon, 1990; Peter et al., 1991; Dessev et al., 1991; Hocevar et al., 1993; Collas et al., 1997).

In this study, we examined the subcellular distribution of B-type lamins of *Xenopus* A6 cells at interphase and mitosis using

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domain-specific antibodies. The results obtained here suggest
that B-type lamins form not only a nuclear lamina structure but
intranuclear structure, and conformation transition of nuclear
lamina occurs at prophase and telophase, respectively.

MATERIALS AND METHODS

Preparation of anti-Xenopus lamin B antiserum

As a souse of antigen, we prepared nuclear skeletal fractions of *Xenopus* erythrocytes. *Xenopus laevis* were purchased from San-ai Shoji. *Xenopus* blood cells were washed with the EDTA buffer (25 mM EDTA, 75 mM NaCl, pH 7.5), then washed with STM buffer (0.6 M sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF). Prepared *Xenopus* erythrocytes were treated with 100 µg/ml saponin and homogenized by teflon homogenizer. The pellet was collected by centrifugation and was used as a nuclear fraction. This fraction was treated with Micrococcal nuclease (Worthington Biochemical Co.) at 1 unit/ml and washed with the washing buffer (0.4 M NaCl, 20 mM Tris, pH 7.5, 1 mM PMSF) for several times. The precipitate was collected by centrifugation and was used as a nuclear skeletal fraction. These precipitates were boiled in the sample buffer (Laemmli, 1970), and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels. The doublet major bands with a molecular weight of 70 and 68 kD, which were identical to lamin B1 and B2, respectively (Krohne and Franke, 1983) were excised from gels. Rabbits and mice were immunized using these bands by the standard protocols. The produced rabbit anti-lamin antiserum was used to screen lamin cDNAs.

cDNA cloning of Xenopus lamins

A lambda ZAP-derived cDNA library of *Xenopus* oocytes mRNAs,
provided by Dr. H. Takisawa (Kubota et al., 1995, 1997), was
screened using anti-*Xenopus* lamin B antisera (described above).
From 5 X 10^5 plaques, we isolated 17 positive clones. Each clone
was sequenced partially for both strands using an automatic DNA
sequencer (ABI, 373A), and compared them with the sequences in
the database. Six clones had the sequence identical to that
reported previously as *Xenopus* lamin B1 (accession number X06344,
Krohne et al., 1987). Six clones had similar but different
sequence from lamin B1 and one clone had similar but different
sequence from lamin B2. We determined full nucleotide sequences
of these clones. We named these isoforms as *Xenopus* lamin B1 type
2 (B1-2) and *Xenopus* lamin B2 type 2 (B2-2). Two lamin B3 clones
were also obtained, which were identical to the clone isolated by
Stick (accession number X13169, Stick, 1988).

Expression and purification of recombinant proteins

Recombinant lamin proteins were constructed as GST-fusion
proteins, and expressed in bacteria cells (see Fig. 1). The
NH_2-terminal fragment of lamin B1-1, that encodes 7-173 amino
acid region (lamin B1(N)), was constructed using EcoRI-BamHI
fragment of 5' region deleted clone, and ligated into expression
vector pGEX-4T-3 (Pharmacia LKB Biotechnology Inc.), using

standard protocols. The COOH-terminal fragment of lamin B1-1, that encodes 365-583 amino acid region (lamin B1(C)), was constructed using XbaI-XhoI fragment and ligated into pGEX-4T-2 vector. The lamin B2-2 rod, that encodes 63-418 amino acid region lamin B2(R), was constructed using EcoRI-XbaI fragment of lamin B2, and ligated into pGEX-4T-3 vector. Each vectors were transformed into *Escherichia coli* cells (strain PR745). The transformed cells were grown at 25°C, and GST-fusion proteins were expressed by addition of 0.1 mM isopropyl β-thiogalactopyranoside (IPTG) (Nacalai Tesque). The cells were lysed using French pressure cell, and GST fusion proteins were affinity purified using glutathione-Sepharose 4B beads (Pharmacia LKB Biotechnology).

Antibody production and purification

The mouse polyclonal anti-lamin B2(R) antibody was affinity purified with recombinant GST-lamin B2 proteins immobilized on CNBr-activated sepharose-4B beads (Pharmacia) from mouse antiserum (see above). The rabbit polyclonal antibody against lamin B1(N) was obtained by immunization with the recombinant lamin B1 protein that was eluted from the column by thrombin, and affinity-purification with the same lamin B1 constructs immobilized on sepharose beads. The rabbit polyclonal antibody against lamin B1(C) was obtained generated by immunization with the recombinant GST-lamin B1(C) fusion protein, and affinity-
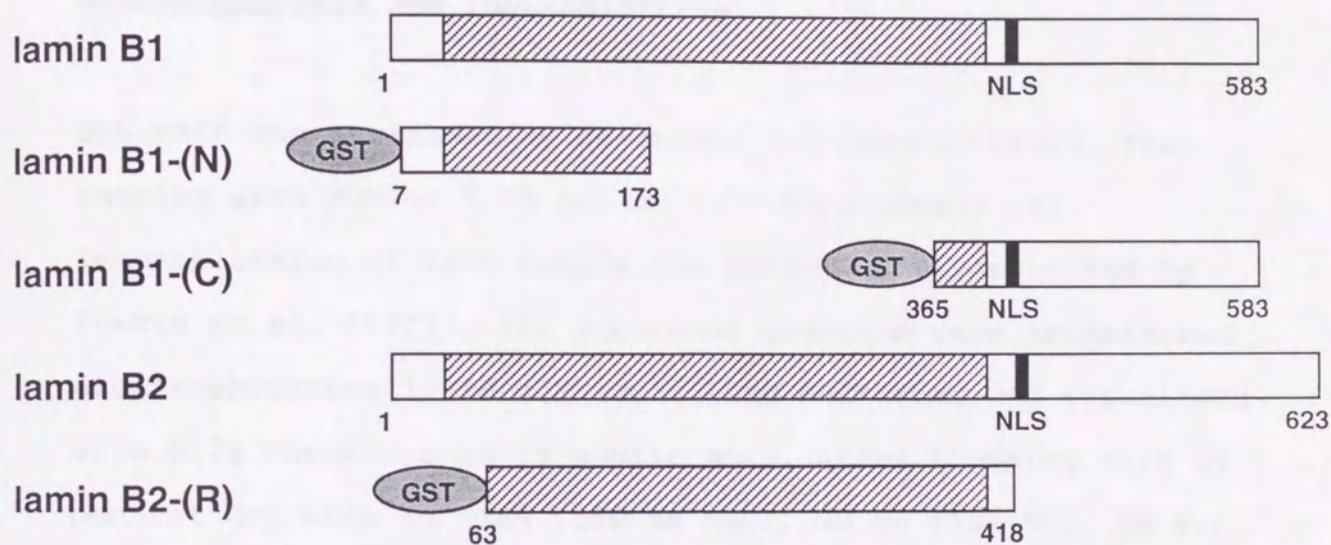
Figure 1. Schematic models of lamin constructs. Whole proteins of lamin B1 (type 1) and lamin B2 (type 2) are shown in a and d. Recombinant GST-lamin B1 N-terminal (N), lamin B1 C-terminal (C) and lamin B2-rod (R) fusion proteins are shown in b, c, and e. These constructs were used as antigens and used for antibody purification by affinity column chromatography.

purification purified with the same constructs immobilized on sepharose beads.

Electrophoresis and Immunoblotting

SDS-PAGE was performed as described by Laemmli (1970). The samples were run on 7.5% gel or 7.5-15% gradient gel. Immunoblotting of each sample was performed as described by Towbin et al. (1979). The separated proteins were transferred electrophoretically to nitrocellulose membranes and visualized with 0.2% Ponceau-S in 1% acetic acid. After blocking with 2% non-fat dry milk in tTBS (150 mM NaCl, 50 mM TRIS-HCl, pH 8.0, 0.1% Tween-20), the blots were incubated with first antibodies at 1:500 dilution for over night, followed by goat anti-rabbit or goat anti-mouse IgG coupled to horse radish peroxidase (Bio-Rad) for 1 hour. The blots were visualized by Konica Immunostaining kit (Konica Co., Tokyo, Japan).

Immunofluorescence microscopy

For indirect immunofluorescence microscopy, Xenopus A6 cells were cultured on coverslips. Cells were fixed in 3.7% formaldehyde in PBS for 10 minutes at room temperature, and treated with 0.2% NP-40 in PBS for 10 minutes at room temperature. After blocking with 2% non-fat dry milk, these cells were incubated with affinity

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purified primary antibodies at dilutions 1:20 in tTBS containing
2% non-fat dry milk and 20% goat serum for over night at 4°C.
After washing with PBS, these cells were incubated with a mixture
of Texas Red-labeled donkey anti-rabbit IgG (1:50; Amersham) and
Cye-2-labeled donkey anti-mouse IgG (1:50; Amersham) in tTBS
containing 2% non fat dry milk for 1 hour at 4°C. After washing
with PBS, these cells were stained with Hoechst dye 33258 and
viewed with a fluorescent microscopy (Orympath BH-2). The triple
staining images were taken by cooled CCD camera (Photometrics,
PXL) with IP-Lab Spectrum system (Signal Analytics Corporation).
The confocal images were taken by a confocal microscopy (Bio-
Rad).

Preparation of Xenopus liver nucleus

Xenopus female liver was dissected into pieces and homogenized in
STM buffer using loose-fitting teflon homogenizer. To avoid the
contamination of granule, light-colored Xenopus female was used.
The cell homogenate was centrifuged at 3000 rpm (Hitachi) for 5
minutes, and the precipitates were used as crude liver nuclei
fraction. This nuclei fraction was fixed with 3.7% formaldehyde
in PBS for 5 minutes and centrifuged onto coverslip coated with
1% poly-L-Lysine at 1000 rpm for 1 min. After blocking with 2%
non-fat dry milk in tTBS, nuclei were observed by
immunofluorescence microscopy.

RESULTS

Isolation of Xenopus lamin B1 and lamin B2 cDNA clones

We prepared nuclear skeletal protein fraction from *Xenopus* erythrocytes. This fraction contained 70 and 68 kD doublet protein as major bands on SDS-PAGE. The molecular weights correspond to lamin B1 and lamin B2, respectively (Krohne G. and Franke W.W., 1983). We excised the 70-68 kD doublet bands from gel, and immunized a rabbit to prepare antiserum against lamin B. The prepared antiserum recognized the 70 and 68 kD protein bands from *Xenopus* erythrocyte, liver cells, A6 culture cells by immunoblotting. This antiserum also stained nuclear envelope strongly by immunofluorescence microscopy (data not shown).

To isolate *Xenopus* B-type lamin cDNAs, we performed immunoscreening of expression cDNA library from *Xenopus* oocytes with this antiserum. Seventeen independent clones were isolated, and each clone was partially sequenced. Compared with reported sequences on data base, twelve clones were identified as lamin B1 (also known to as lamin L1), one as lamin B2 (L2), two as lamin B3 (L3) and two as uncharacterized proteins (data not shown). Among them, six lamin B1 clones matched with that previously isolated by Krohne et al. (1987), and two lamin B3 clones with that isolated by Stick (1988) without several nucleotide differences (data not shown). Other six lamin B1 clones and one lamin B2 clone have similar sequences but were clearly different

from previously reported clones. We decided full sequence of another type of lamin B1, and partial sequence of that of lamin B2, and termed as lamin B1-type 2 and B2-type 2, respectively.

The *Xenopus* lamin B1-2 cDNA clone was 2461 nucleotide long and had an open reading frame of 1,752 nucleotides, a 5'UTR of 190 nucleotides, a 3'UTR of 518 nucleotides and a long poly-A tail sequence. The 3'UTR had AATAAA consensus hexanucleotide sequence that is required for mRNA polyadenylation (Wickens, 1990), so it seems that this clone had complete 3'UTR region. The open reading frame gave 91.6% identity with previously reported type 1 clone (accession number X06344; Krohne et al., 1989). The predicted protein was 584 amino acids long and gave 91.6% identity compared with 583 amino acids long lamin B1-1 protein. The *Xenopus* lamin B2-2 partial cDNA clone, which lacks about 180 nucleotides of 5'region of open reading frame, gave 93.2% identity with type 1 clone (accession number X54099; Hoger et al., 1990). The predicted lamin B2-2 protein fragments gave 94.1% identity compared with lamin B2-1 protein.

Preparation of domain specific anti-lamin antibodies

We prepared rabbit polyclonal antibodies against lamin B1 NH_2-terminus (N) and COOH-terminus (C), and mouse polyclonal antibodies against lamin B2 rod domain (R). The constructs used for the preparation of the antibodies were shown in Fig. 1. The specificity of these antibodies was determined by immunoblotting

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of whole *Xenopus* A6 cell proteins (Fig. 2, a and b). Each antibodies reacted with single protein band. Anti-lamin B1(N) and anti-lamin B1(C) antibodies recognized 70 kD band, while anti-lamin B2(R) antibody recognized 68 kD band (Fig. 2 b). The molecular weights of these bands correspond those of lamin B1 and lamin B2, respectively. The specificity of two lamin B1 antibodies was further studied by Western blotting against bacterially expressed GST-fusion proteins (Fig. 2 c). Anti-NH2-terminus antibody only reacted with NH2-terminus fusion protein but not COOH-terminus fusion protein (Fig. 2, c lanes 3 and 4). The same result was obtained on anti-COOH-terminus antibody as well (Fig. 2, c lanes 5 and 6).

Antibodies against N-terminal and C-terminal of lamin B1 differentially stained nuclei of Xenopus A6 cell and liver cell

To determine the localization of lamin B in nucleus, immunofluorescence experiments were performed on *Xenopus* A6 cells using prepared antibodies (Fig. 3). Anti-lamin B1(C) antibody and anti-lamin B2(R) antibody gave strong nuclear rim staining. Furthermore, both antibodies gave a dot-like staining pattern in the nucleus. These dot-like structures were reported previously and suggested as 'nuclear envelope invaginations' (Fricker et al., 1997). Double staining showed that the two antibodies recognized the same nuclear compartment, the nuclear lamina and the dot-like structures (Fig. 3, d-e and d'-e').
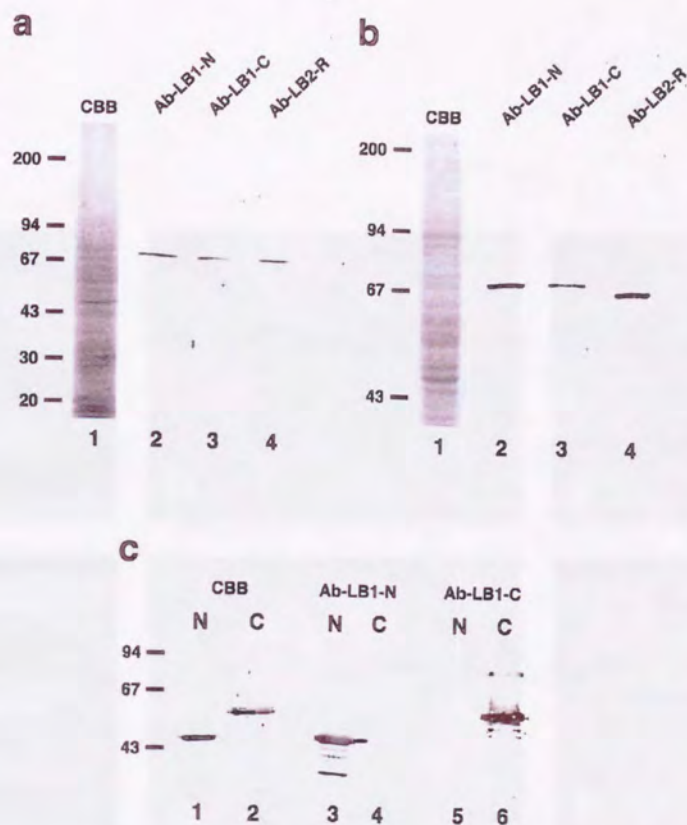
40

Figure 2. Characterization of specific antibodies against lamin
B1(N), lamin B1(C) and lamin B2(R). a and b, Immunoblotting of
total cell lysates from A6 cells with specific antibodies (a,
7.5-15% gradient gels. b, 7.5% gels). Proteins were visualized by
Coomassie blue staining (lanes 1). Immunoblottings were performed
using antibodies against lamin B1(N) (lanes 2), lamin B1(C)
(lanes 3) and lamin B2(R) (lanes 4), respectively. c, Bacterially
expressed GST-lamin B1(N) fusion proteins and GST-lamin B1(C)
fusion proteins were separated on SDS-PAGE (7.5-15% gradient
gels) and analyzed by Immunoblotting. Lane 1 and lane 2,
Coomassie blue staining. Immunoblottings were performed using
antibodies against lamin B1(N) (lanes 3 and 4) and lamin B1(C)
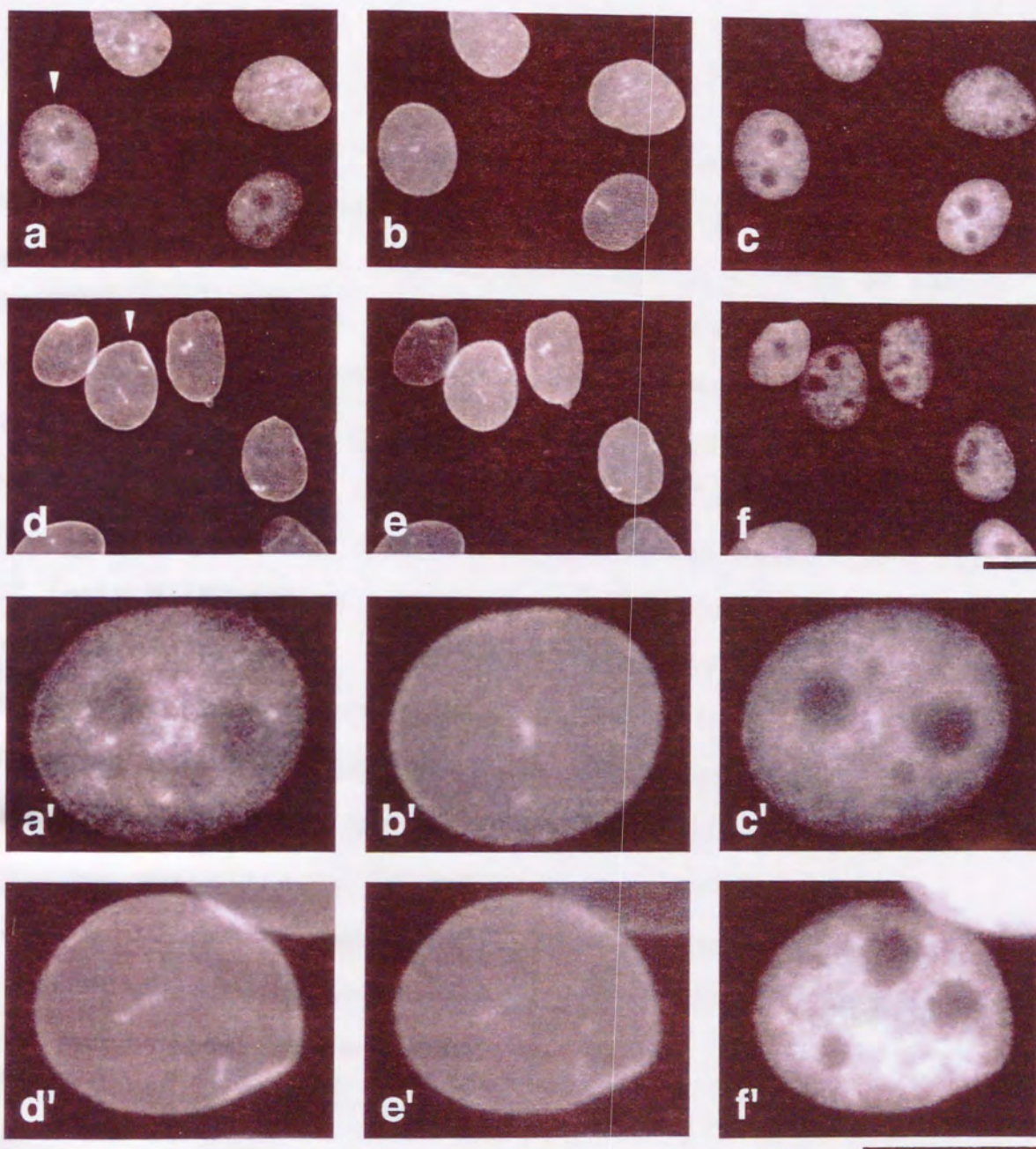(lanes 5 and 6), respectively.

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Figure 3. Immunofluorescence staining of *Xenopus* A6 interphase

cells with the antibodies against lamin B1(N), lamin B1(C) and

lamin B2(R). A6 cells were incubated with a mixture of rabbit

polyclonal antibodies against lamin B1(N) (a to c) or lamin B1(C)

(d to f) with mouse polyclonal antibodies against lamin B2(R)

antibodies, followed by incubation with a mixture of

Cye-2 anti-rabbit and Texas red anti-mouse secondary antibodies.

They were observed by fluorescent microscopy using Cye-2 filter

for lamin B1(N) (a) or lamin B1(C) (d) or Texas red filter for

lamin B2(R) (b and e). Cells were stained with Hoechst dye 33258

at the same time (c and f). Bar, 10 µm. a' to f' indicate

magnified images showed on (a) and (d) by arrowheads. a' and d'

show anti-lamin B1(N) and anti-lamin B1(C)) antibody

staining, respectively. b' and e' show anti-lamin B2(R)

staining. c' and f' show Hoechst staining. Nuclear envelope

invaginations and nucleoplasmic dots are indicated by large and

small arrowheads, respectively. Bar, 10 µm.

In contrast, anti-lamin B1(N) antibody gave weak nuclear rim staining but gave strong nucleoplasmic staining (Fig. 3, a-b and a'-b'). The staining pattern of this antibody was similar to the Hoechst pattern. This antibody stained not only the nuclear envelope invaginations which were recognized by anti-lamin B2(R) antibody simultaneously, but other nuclear dot-like structures which were not recognized by anti-lamin B2(R) antibody (Fig. 3, a' and b'). Confocal images supported the above results (Fig. 4, a and b). Anti-lamin B1(C) antibody stained the rim of nucleus strongly and also the invaginations, while anti-lamin B1(N) antibody stained nuclear rim faintly, but strongly the inside of nucleus.

Since both anti-lamin B1(C), and anti-lamin B2(R) antibodies stained the nuclear rim, lamin B molecule must exist at nuclear rim. If so, the special situation that anti-lamin B1(N) antibody also recognize lamin B on nuclear rim must exist. This idea was true. When the cells were fixed with cold methanol, anti-lamin B1(N) antibody showed nuclear rim staining as other antibodies (data not shown).

The different staining due to anti-lamin B1(N) and anti-lamin B2(R) antibodies was given not only on A6 cell nuclei but on isolated nuclei prepared from *Xenopus* liver (Fig. 5). As shown in the figure, anti-lamin B2(R) showed strong rim staining. However, anti-lamin B1(N) antibody showed week rim staining and strong intranuclear staining, as observed on A6 cells.
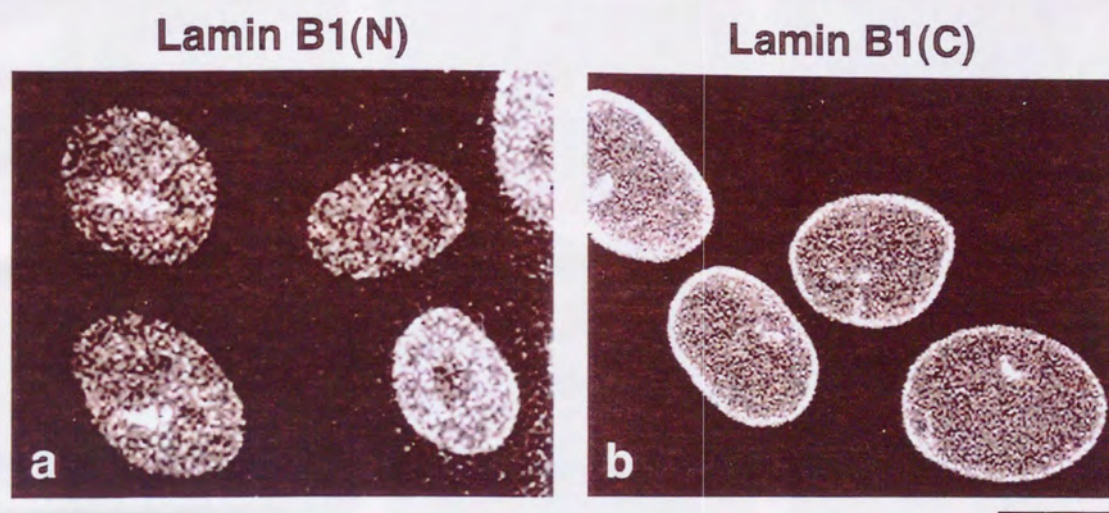
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Lamin B1(N) **Lamin B1(C)**

Figure 4. Confocal staining images of *Xenopus* A6 cell nucleus.
A6 cells were stained with rabbit polyclonal antibodies against
lamin B1(N) (a) or rabbit polyclonal antibodies against lamin B1
(C) (b). They were observed using a confocal microscopy.
Bar, 10 μm.
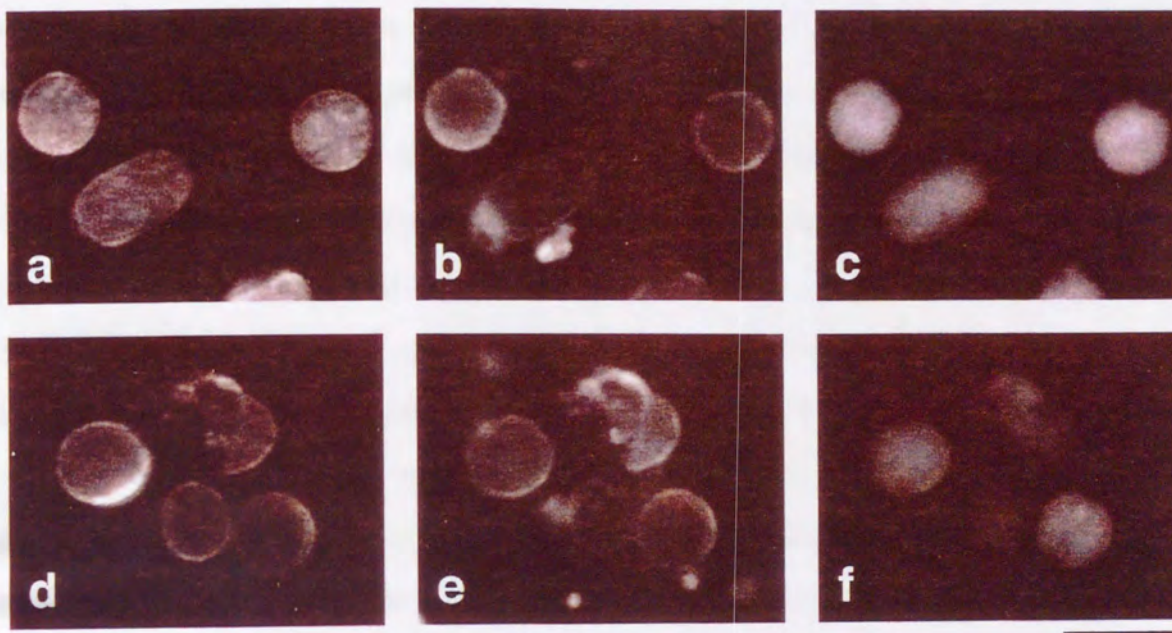
Figure 5. Immunofluorescence staining of *Xenopus* liver nucleus with the antibodies against lamin B1(N) and lamin B2(R). *Xenopus* liver nuclei were fixed onto cover slips and double-labeled with a mixture of rabbit anti-lamin B1(N) antibody (a) or anti lamin B1(C) antibody (d) with mouse anti-lamin B2(R) antibody (b and d). DNAs were visualized with Hoechst dye 33258 (c and f). Bar, 10 μm.

Different staining patterns with two anti-lamin antibodies on mitotic cells

Next, we examined the lamin B distribution on mitotic cell using these antibodies (Figs. 6, 7 and 8). As shown in Fig. 6 b, when chromatin started to condense at early prophase, the nuclear rim staining by anti-lamin B2(R) became stronger than that of interphase cells (Fig. 4b). At this stage, anti-lamin B1(N) antibody also showed gave nuclear rim staining (Fig. 6a). A dot-like staining of nuclear invagination was also observed even at this stage. However, the nucleoplasmic staining at this stage was different from that at interphase. On interphase cells nucleoplasmic lamin B signals seem to be colocalized with Hoechst staining, but at this phase lamin B1(N) signals seem to separate from DNA staining. Fig. 6, d to f show the staining image at later prophase. As the shape of nuclei was deformed, deep invaginations were began to be formed and strong lamin B signals were detected on invagination region as well as nuclear rim with anti-lamin B2(R) antibodies (Fig. 6, d and e). Staining pattern of anti-lamin B1(N) antibody became to colocalize well with that of anti-lamin B2(R) antibody (Fig. 6, d and e), but nucleoplasmic signals were still observed (Fig. 6 d). At late prophase, staining of nuclear rim and invaginations by either anti-lamin B1(N) and lamin B2(R) antibodies became diffused and two antibody signals were almost colocalized (Fig. 6, g and h).

At early metaphase, the two antibodies gave almost colocalized images (Fig. 7, a and b). The lamin B signals that had been

Lamin B1(N)   **Lamin B2(R)**   **Hoechst**

Figure 6. Immunofluorescence staining of prophase *Xenopus* A6 cells with anti-lamin B1(N) and lamin B2(R) antibodies. Unsynchronized A6 cells were fixed and double-labeled with a mixture of rabbit anti-lamin B1(N) antibody (a, d and g) and mouse anti-lamin B2(R) antibody (b, e and h). DNAs were visualized with Hoechst dye 33258 (c, f and i). Cells in early prophase (a to c), middle prophase (d to f) and late prophase (e to i) were shown. Bar, 10 μm.
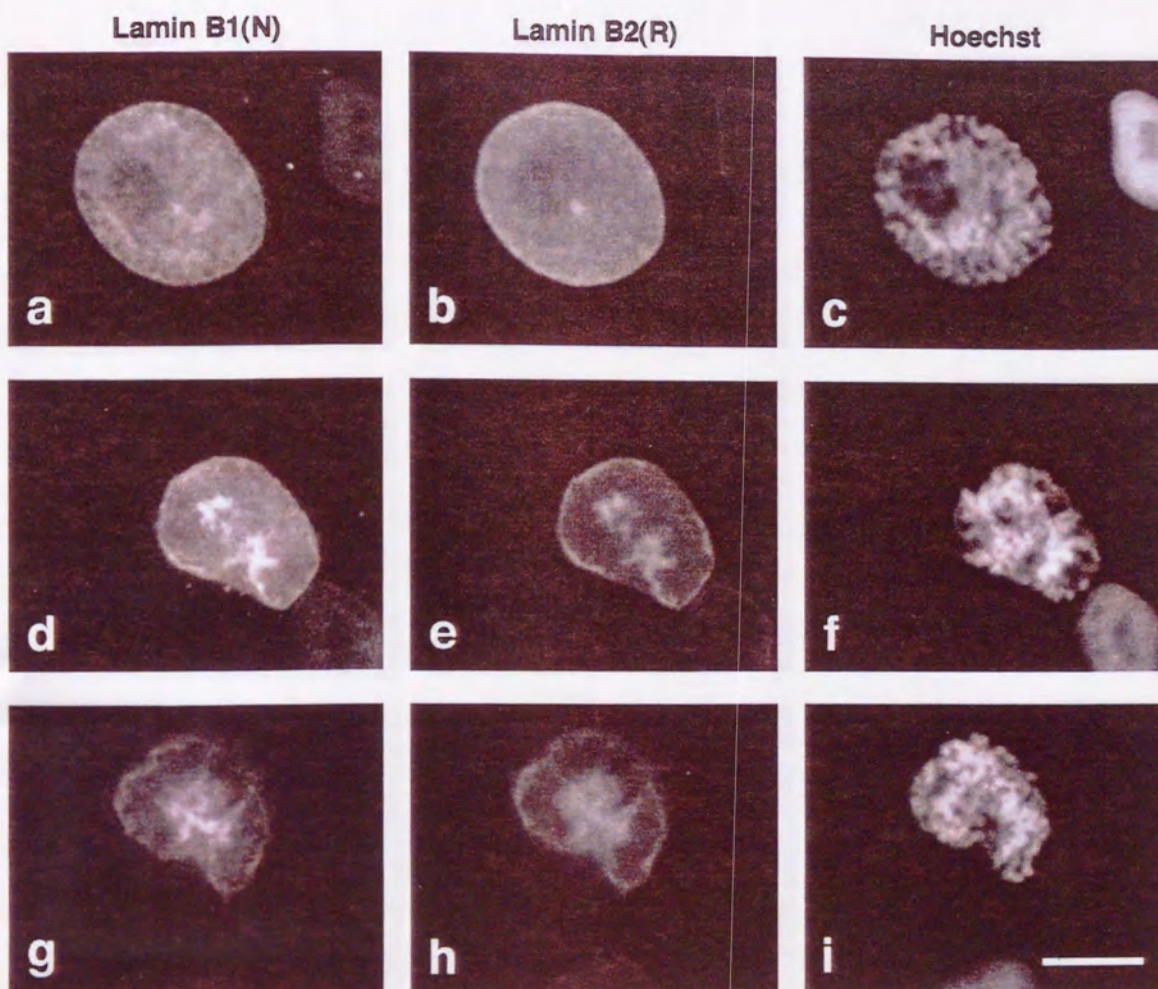
Figure 7. Immunofluorescence staining of metaphase and anaphase *Xenopus* A6 cells with anti-lamin B1(N) and lamin B2(R) antibodies. Unsynchronized A6 cells were fixed and double-labeled with a mixture of rabbit anti-lamin B1(N) antibody (a, d and g) and mouse anti-lamin B2(R) antibody (b, e and h). DNAs were visualized with Hoechst dye 33258 (c, f and i). Cells in early metaphase (a to c), metaphase (d to f) and anaphase (e to i) were shown. Bar,10 μ m.

observed on nuclear rim at prophase were disappeared and dispersed into cytoplasm except chromosomal region, but partial nuclear shape was still observed. The staining intensity of both antibodies at this stage was much stronger than that at interphase. From metaphase to anaphase both antibodies stained cytoplasm except chromosome (Fig. 7, d-e and g-h).

Staining patterns with two anti-lamin B antibodies during nuclear formation

At telophase, two antibodies gave similar images. The dispersed lamin B signals began to localize around chromosome (Fig. 8. a and b). The signal around chromosome was much stronger than that of interphase signal. Anti-lamin B1(N) also showed strong nuclear rim staining at this stage (Fig. 8a). At late telophase both antibodies gave signals not only on nuclear rim but on nuclear invagination or around partially decondensed chromatin (Fig. 8, d to f and g to i). After telophase, the signals of two antibodies became weak and showed different staining patterns as observed in interphase (Fig. 3).

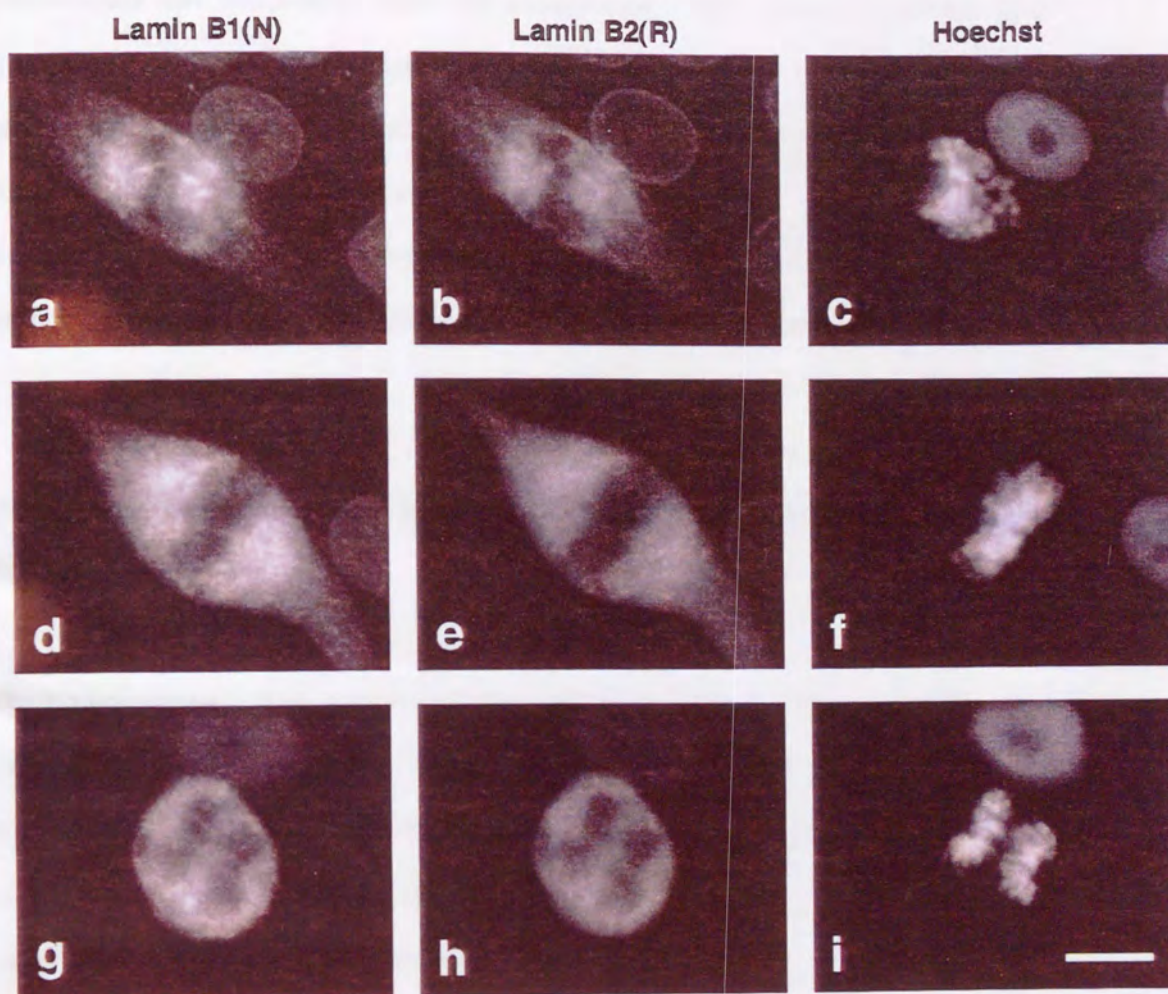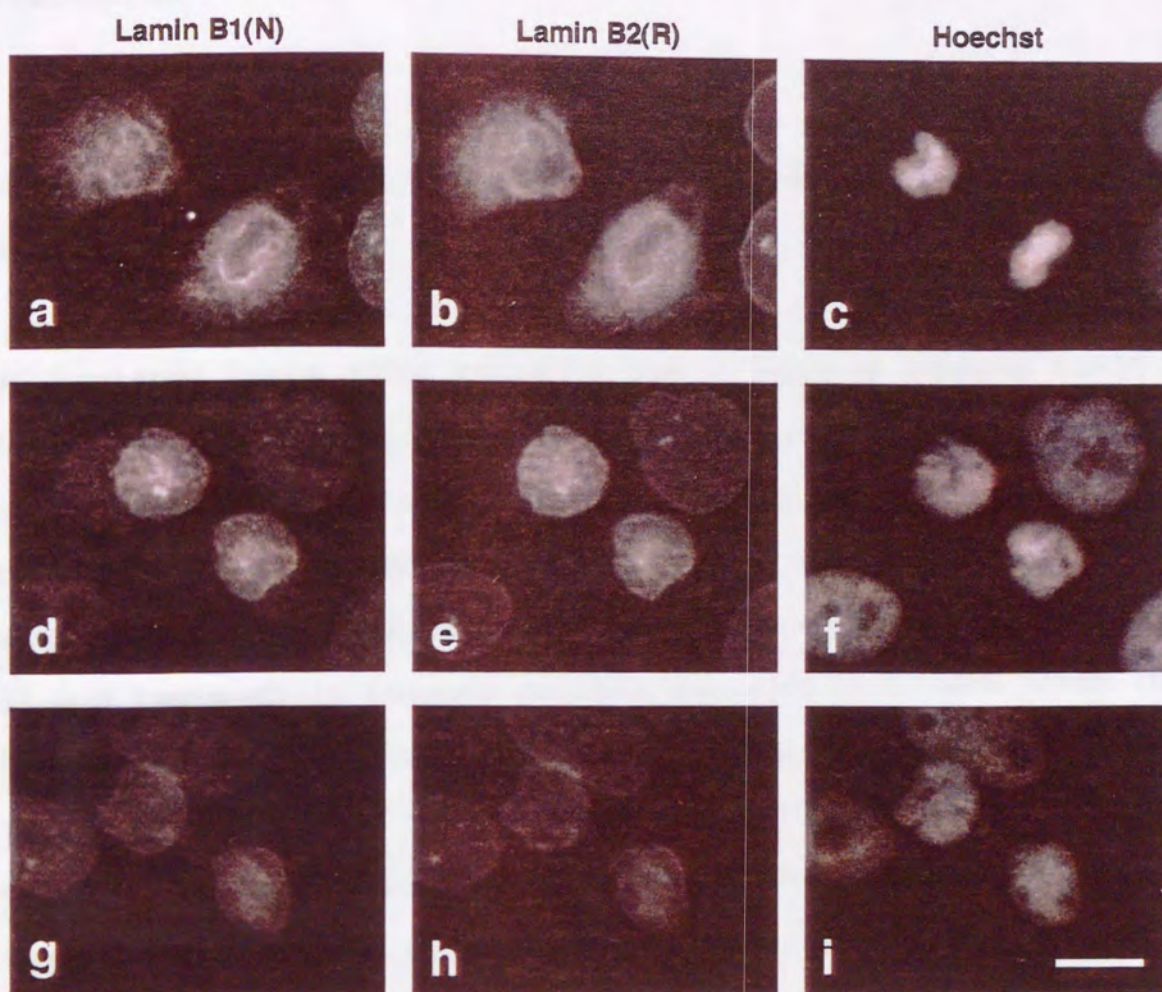Figure 8. Immunofluorescence staining of telophase *Xenopus* A6 cells with anti-lamin B1(N) and lamin B2(R) antibodies. Unsynchronized A6 cells were fixed and double-labeled with a mixture of rabbit anti-lamin B1(N) antibody (a, d and g) and mouse anti-lamin B2(R) antibody (b, e and h). DNAs were visualized with Hoechst dye 33258 (c, f and i). Cells in early telophase (a to c), late telophase (d to f) and interphase (e to i) were shown. Bar, 10 μ m.

DISCUSSION

cDNA cloning of three types of lamin B from Xenopus oocytes cDNA library

We isolated lamin cDNAs using antiserum against *Xenopus* lamin B by screening the cDNA library of *Xenopus* oocytes. We isolated three types of lamin cDNA, lamin B1, lamin B2 and lamin B3. This result shows that *Xenopus* oocytes contain three types of lamin mRNA. However, it was reported that the protein amounts of lamin B1 and lamin B2 in *Xenopus* oocytes and eggs were much lower than that of lamin B3 (Lourim and Krohne, 1993; Lourim et al., 1996). It was also reported that during oocyte maturation, the protein level of lamin B1 increased about ten times, probably by the result of activation of protein synthesis. In addition, the protein amount of lamin B1 increased further after the midblastula transition. The protein amount of lamin B2 also increased after gastrula stage (for a review see Krohne and Benavente, 1986). Therefore, it seems that translation of lamin B1 and lamin B2 is regulated during development. The mRNAs of lamin B1 and lamin B2 may be stored as an inactivated state in oocytes, and the translation of these mRNAs is activated at appropriate stages for protein synthesis (see Richter, 1991).

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Presence of two types of lamin B1 and B2 in Xenopus

We isolated two types of *Xenopus* lamin B1 and lamin B2
cDNAs, respectively (see RESULTS). The existence of several
isoforms of lamin B3 was reported previously by Doring and Stick
(1990). They showed by genomic southern blotting that two lamin
B3 genes exist in *Xenopus* genome. The partial sequence of
secondary isoform represented 90% identities on nucleotide level.
So, it can be said that in *Xenopus*, two types of lamin B1, lamin
B2 and lamin B3 genes are present in *Xenopus* genome,
respectively. In addition, two alternatively spliced products
were reported on one of lamin B3 gene (Doring and Stick, 1990).
The functional difference of these lamin isoforms on *Xenopus* is
not clear. It is proposed that *Xenopus laevis* species is
tetraploid with respect to DNA content. So most of the *Xenopus*
genes may be present in two copies in the haploid genome (see
discussion Doring and Stick, 1990). In fact, two sets of cDNA are
reported on various protein products, such as importin-α, MCM
family proteins, cyclin E, and so on (Gorlich et al., 1994;
Chevalier et al., 1997; Kubota et al., 1997).

Structure of lamins and the domain specific antibodies

It has been shown from primary sequence as well as structural
analysis that lamins has tripartite domain structure with a
central rod domain with α-helical coils and non-α-helical head

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and tail domains. It was suggested that filamentous structure is formed by the head to tail interaction. COOH-terminal tail portion contains nuclear localization signal, interaction site with DNA, and that with membrane. The NH_2-terminal truncated form of lamin induce aggregation of lamin proteins (Spann et al., 1997). In this study, we prepared antibodies against NH2-terminal region of lamin B1, COOH-terminal region of lamin B1 and rod region of lamin B2 (Fig. 1). These antibodies interact specifically with lamin B1 or lamin B2. Furthermore, they interact specially with NH_2-terminal and COOH-terminal domains of lamins (see Fig. 2).

Structure of intranuclear lamins in interphase cells

We performed indirect immunofluorescence microscopy studies on *Xenopus* A6 culture cells using three different antibodies (Fig. 3). Affinity purified rabbit antibody raised against lamin B1(C) and mouse antibody against lamin B2(R) gave typical nuclear rim staining (Fig. 3, d-d' and e-e'). These two antibodies also gave signals on nuclear envelope invaginations, which were observed on most of interphase nucleus as dot patterns. The positions of invaginations which were detected by each antibodies were completely matched on double immunostaining (Fig. 3, d' and e'). Therefore, lamin B1 and lamin B2 are colocalized on nuclear lamina in interphase cells.

In contrast, affinity purified rabbit antibody against lamin

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B1(N) gave weak nuclear rim staining. While this antibody gave relatively strong intranuclear staining (Fig. 3, a and a'). This antibody also recognized nuclear invagination which were detected by lamin B2(R) antibody, and showed the similar pattern to heterochromatin excluding nucleolus (Fig. 3, a' and b'). The similar results were obtained using *Xenopus* liver nucleus (Fig. 5). It is thought that lamin B1 and lamin B2 have similar properties. The function of intranuclear lamin B1 structure, which we observed, is not clear, but several speculations are available. These lamins may be excessively synthesized products for cell division or may be free molecules before targeted into nuclear lamina. The nucleoplasmic lamins may form a functional structure that is required for maintenance of nuclear matrix or chromatin, or involved in some nuclear events, such as DNA replication.

The existence of intranuclear lamin structures was reported by Fricker et al. (1997) using confocal microscopy and 3D visualization method. They showed two types of intranuclear dot-like lamin structures by anti-lamin antibody. The former structure was on nuclear envelope invaginations, since it colocalized with fluorochrome labeled Con A signals, that indicated membrane. While the latter was not colocalized with Con A signals. These results agree with our observation.

Moir et al. (1994) reported previously that the antibody against B-type lamins gave intranuclear foci staining. These B-type lamin foci were observed only at mid and late S-phase and were colocalized with PCNA, which is a component of DNA

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replication complex, and the sites of incorporation of bromodeoxyuridine (BrDU). So, it seems that intranuclear B-type lamin structures are involved in DNA replication. The relationship between B-type lamins and DNA replication was suggested by the experiments using *Xenopus* egg cell-free extracts (Meier et al., 1991; Goldberg et al., 1995; Ellis et al., 1997).

In addition to B-type lamins, A-type lamins were also reported to exist in intranuclear sites. Bridger et al. (1993) reported the intranuclear foci and fiber structures on human dermal fibroblasts by immunofluorescence using monoclonal antibodies against A-type lamin. These structures were detected only at G1 phase and were not detected at S phase. Sasseville and Raymond (1995) reported the intranuclear foci on human epitheloid carcinoma cells by immunofluorescence using anti-lamin A precursor specific antibody. The function of intranuclear lamin A and lamin A precursor is not yet clear, though it was proposed that these structures are the temporal stores for processing or modification of A-type lamins and the stored proteins are gradually translocated into nuclear lamina.

The antibody against lamin B1(N) gave weak nuclear rim staining compared with the other antibodies (Fig. 3, a' and b'). We speculate that this is a result of the difference in the accessibility of these antibodies. The antibodies against lamin B1(C) and lamin B2(R) may be able to access to the nuclear lamina structure easily and recognize the epitope. On the other hand, antibody against lamin B1(N) may not be able to access to the epitope easily. The epitope may be on the inside of the structure

and masked by another molecule.

The result showing epitope masking was reported by Buss and Stewart (1995), using antibodies against nucleoporin p62, which is a component of nuclear pore complex. They generated specific antibodies against p62 NH_2-terminal domain and p62 COOH-terminal rod domain. The anti-NH_2-terminal antibody gave strong nuclear rim staining but anti-COOH-terminal antibody gave weak nuclear rim staining by immunofluorescence staining. They showed the same results using dot blotting experiments using native p62 containing protein complex. They explained that the p62 rod epitope are masked when protein complex are formed. The result presented here also suggest that epitopes of anti-lamin B1(N) antibody is masked in the nuclear lamina in the interphase cells.

Subcellular dynamics of B-type lamins during mitosis

We performed immunofluorescence staining on mitotic A6 cells (Figs. 6 to 8). In interphase cells nucleoplasmic lamin signals that were detected by anti-lamin B1(N) antibody, coincide with chromatin-Hoechst signals (Fig. 6, a to c), but at early prophase, these two signals were separated (Fig. 6 f). It seems that lamin proteins were separated from partially condensed chromatin at this stage. At mid prophase, many deep invaginations were observed by either lamin B1(N) and lamin B2(R) antibodies (Fig. 6, d-e and g-h). These results show that B-type lamins were highly accumulated on deep invagination. Similar nuclear deep

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invagination structures were reported by Georgatos et al. (1997) on prophase human Ishikawa cells and mouse fibroblasts.

The most striking event at late prophase is that anti-lamin B1(N) antibody gave relatively strong nuclear rim staining (Fig. 6g). This result indicates that the lamin B1 N-terminal epitopes recognized by this antibody became unmasked at this phase. Therefore, a structural change such as loosening of nuclear lamina may occur and the accessibility of antibody is increased. These events may be induced by the phosphorylation of lamins or other lamina proteins, or the release of molecules that masked the epitopes. In addition, the signals of anti-lamin B2(R) antibody became stronger at prophase than that at interphase. This result also suggests that lamin proteins become depolymerized and protein-protein interaction of lamin is reduced during mitosis. So the accessibility of anti-lamin antibodies is increased at mitosis. At late prophase, nucleoplasmic lamin signals of anti-lamin B1(N) antibody became weak compared with early prophase (Fig. 6, a and g). It seems that nucleoplasmic lamins are dispersed earlier than nuclear lamina, or nuclear envelope invagination and the nucleoplasmic lamin proteins may be incorporated into these structures.

From metaphase to anaphase, lamin proteins are dispersed into cytoplasm (Fig. 7), as reported previously by Krohne and Benavente (1986). Both antibodies gave cytoplasmically-dispersed staining patterns that excluded only condensed chromosomes. Lourim and Krohne (1996) reported the similar staining patterns using specific antibodies against lamin B1 and B2 on metaphase

Xenopus A6 cells. We noticed that the intensity of signals is stronger than that of interphase cells (Fig. 7, a and b). This result may be the reflection of increased accessibility of antibodies, as discussed before.

At early telophase, dispersed lamins were accumulated around condensed chromosomes (Fig. 8, a to c). At late telophase, the cytoplasmically dispersed lamin signals became incorporated into nucleus probably by the import system through nuclear pore, but some disproportion of lamin signals was observed on nucleus (Fig. 8, d and e). This structure seems to be the nuclear envelope invaginations or the reflection of chromatin decondensation. Through the telophase of mitosis, both anti-lamin B1(N) and anti-lamin B2(R) antibodies colocalized, and the signals of these two antibodies were relatively stronger compared to that at interphase (Fig. 8, a-b and d-e).

At the end of telophase, the intensity of lamin signals was the same level as that of interphase cells (Fig. 8, g and h). These results indicate that the accessibility of antibody decreases at telophase, perhaps by the result of epitope masking. It seems that at early telophase the accumulated lamin structure around chromosomes is loose and immature, but it become tight and the matured structures are formed at the end of telophase. In addition, the difference on staining patterns between two antibodies became obvious at the end of telophase (Fig. 8, g and h).

Models of B-type lamin dynamics during cell cycle

We propose one hypothetical model shown in Fig. 9 for the dynamics of B-type lamins during cell cycle. The results obtained here can be explained by this model. In interphase cell, B-type lamins are incorporated into nuclei and form a nuclear lamina structure on nucleoplasmic side of the nuclear envelope. B-type lamins are also present in the nucleoplasm and probably constitute an intranuclear structure. B-type lamins may be aligned at different mode in these two structures. Our immunofluorescence data suggests that NH2-terminus of lamin B is masked in the nuclear lamina, but not in the intranuclear structure (see Fig. 3). At prophase, when chromosomes start to condense, B-type lamins are still present at the nuclear envelope, but the nuclear lamina structure are partially disrupted and loose. This conformation change was proposed by the increase intensity of immunofluorescence of anti-lamin B antibodies shown in Fig. 6. At metaphase and anaphase, B-type lamins are completely dispersed into cytoplasm. This disassembly of B-type lamins is thought to be caused by mitotic phosphorylation. At telophase, when nuclear envelope is formed around partially decondensed chromosome, B-type lamins become localized at the nucleoplasmic side of the nuclear envelope, forming a nuclear lamina structure, but the structure is not complete and immature. At late telophase, B-type lamins in nuclear lamina begin to form a tight lamina structure beneath the nuclear envelope.
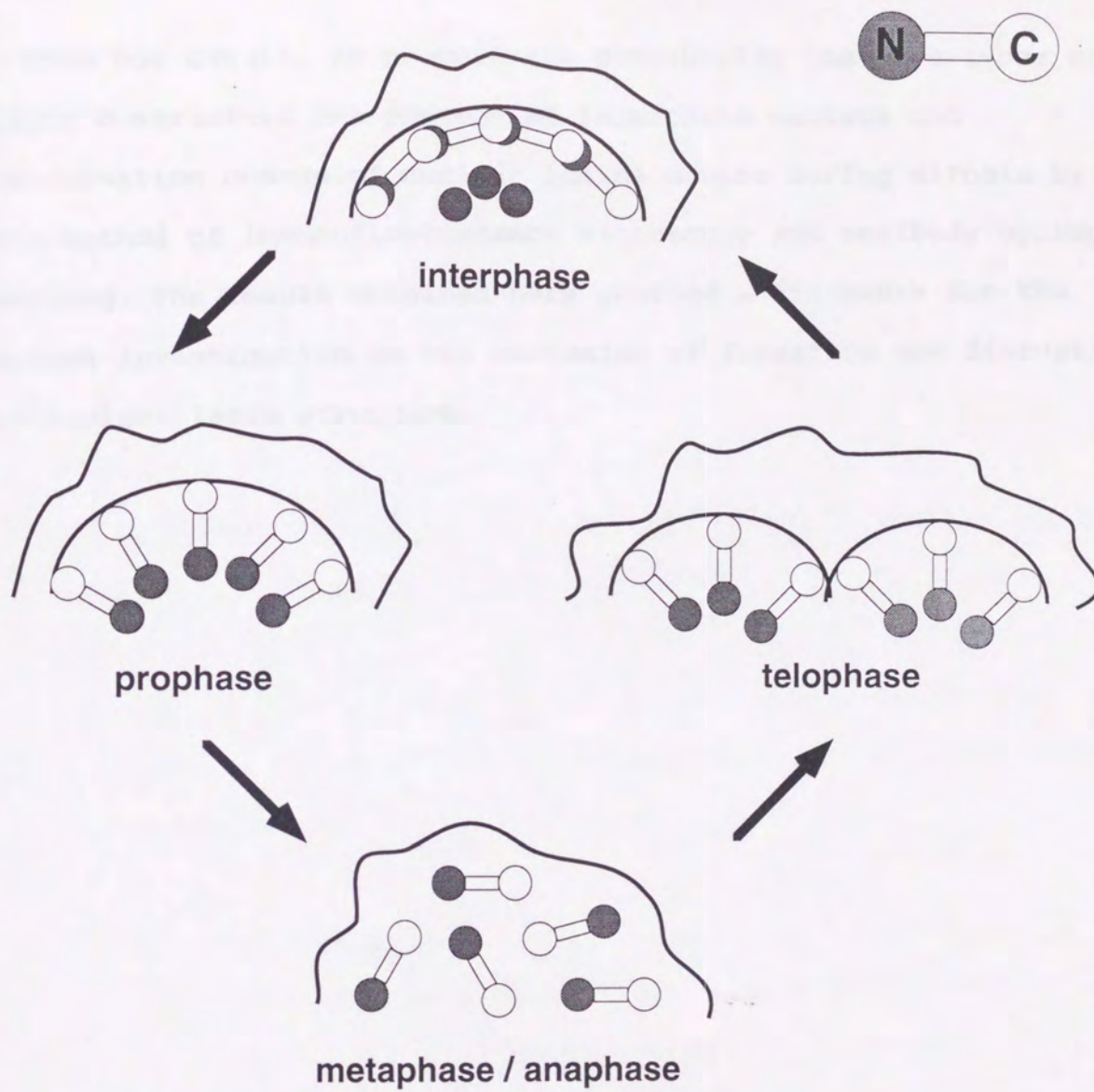
Figure 9. Schematic model for dynamics of B-type lamin during cell cycle.

From our result, we propose the possibility that two types of

lamin B structure are present at interphase nucleus and

conformation change of nuclear lamina occurs during mitosis by

the method of immunofluorescence microscopy and antibody epitope

masking. The result obtained here provide a framework for the

future investigation on the mechanism of formation and disruption

of nuclear lamin structure.

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Part III

In vitro nuclear envelope breakdown of isolated nucleus in
Xenopus egg mitotic extracts

SUMMARY

Xenopus egg cell-free extract is a useful system to study the nuclear dynamics during cell cycle. We found that when nuclei prepared from *Xenopus* erythrocyte or liver were incubated in *Xenopus* M-phase extracts at 25 °C in the presence of ATP, nuclei enlarged first, then elongated and condensed to a "worm-shape" structure within 60 min. Contrary to mitosis in usual cells, the nuclear membrane, observed by DHCC-staining, began to disperse before the chromosome condensation. Lamin B2 and nuclear pore complex protein p62, observed by immunofluorescence microscopy, also disappeared before the chromosome condensation. These results indicate that the regulatory mechanism of mitotic event observed in *Xenopus* egg extract is different from normal nuclear mitotic events in the cell, and that chromosome condensation and nuclear envelope break down are regulated by a different mechanism. This *in vitro* system is good tool to investigate the mechanism of nuclear envelope breakdown.

The nuclear envelope is composed of many proteinaceous structures, such as nuclear lamina and nuclear pore complex (NPC) (for reviews, see Georgatos et al., 1994; Goldberg and Allen, 1995). The nuclear lamina is a filamentous meshwork structure lying on the inner nuclear membrane, and consists of nuclear intermediate filament proteins called lamins (Gerace and Blobel, 1980; Aebi et al., 1986, McKeon et al., 1986). Lamins fall into two groups, A-type lamins and B-type lamins (for reviews, see McKeon, 1991; Nigg, 1992). Various lamina-associated proteins are also known to exist, such as p58/lamin B receptor (Worman et al., 1988, 1990; Ye and Worman, 1994), LAP1 (Martin et al., 1995), LAP2/thimopoietin (Furukawa et al., 1995) and otefin (Pandan et al., 1990).

Nuclear envelope shows dynamic rearrangement during mitosis. At prometaphase the nuclear envelope is broken down. The nuclear membrane and its protein components are dispersed into cytoplasm. At telophase mitotic nuclear membrane vesicles and the protein components are recruited to the surface of chromosome and form a nuclear envelope (Chaudhary and Courvalin, 1993; Buendia and Courvalin, 1997; Georgatos et al., 1997; Maison et al., 1997). Ellenberg et al. (1997) recently suggested that the nuclear membrane is not vesculized during mitosis and rearranged as a tubular network with ER membrane.

The dynamics of nuclear envelope are believed to be regulated

by reversible phosphorylation. Lamins are highly phosphorylated during mitosis. This phosphorylation is mediated by p34/cdc2 and other mitotic kinase, and this modification is involved in nuclear lamin dissociation. (Heald and McKeon, 1990; Peter et al., 1990; Dessev et al., 1991; Hocevar et al., 1993; Collas et al., 1997). During mitosis A-type lamins are dispersed as solubilized state, whereas B-type lamins are dispersed as membrane bound state. p58/LBR is also phosphorylated at mitosis, and this phosphorylation is also involved in the dissociation of nuclear envelope and nuclear lamina (Simos and Georgatos, 1992; Meier and Georgatos, 1994; Nikolakaki et al., 1996, 1997; Collas et al., 1996).

The NPC is a large protein complex with a molecular weights of about 120 MD, and composed of more than 100 proteins. The NPC is responsible for import and export of soluble molecules (for reviews, see Davis, 1995; Doye and Hurt, 1997). As a nuclear pore protein, p62 is well characterized (Starr et al., 1990; Cordes et al., 1991). The assembly and reassembly of nuclear pore complex are also caused at mitosis, and the processes are also regulated by a reversible phosphorylation (Macaulay et al., 1995; Favreau et al., 1996). p62 is known to be dispersed at mitosis as a multi-protein complex (Dabauvalle et al., 1990; Finlay et al., 1991; Buss and Stewart, 1995; Hu et al., 1996). The precise mechanism of nuclear envelope breakdown and reformation, and the recruitment of nuclear envelope proteins are not well understood.

The cell-free system is a good tool to investigate nuclear envelope dynamics, because biochemical procedures are easily

available. The most widely used system for cell-free analysis is *Xenopus* egg extracts (Lohka and Masui, 1983). The extract prepared from *Xenopus* M-phase eggs have an activities to induce mitotic events, such as nuclear envelope breakdown, chromosome condensation and mitotic spindle formation. As a souse of nuclei, *Xenopus* sperm pronucleus, and isolated nuclei from rat cells have been used well. The mitotic activity for nuclear envelope breakdown is due to MPF (cdc2/cyclin B) and other mitotic kinases (Miake-Lye and Kirschner, 1985; Vigers and Lohka, 1992: Pfaller and Newport, 1995).

In this report, we investigated nuclear mitotic events using *Xenopus* egg cell-free extracts and nuclei derived from *Xenopus* erythrocyte and liver. Nuclear envelope breakdown and chromosome condensation occurred rapidly in this combination. However, contrary to mitotic events in normal cell, the nucleus swelled first and nuclear the envelope breakdown occurred before the chromosome condensation.

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MATERIALS AND METHODS

Antibody preparation

To obtain rabbit polyclonal antibody against nuclear pore protein p62, we isolated Xenopus p62 cDNA. The lambda ZAP-derived cDNA library of Xenopus oocytes was provided by Dr. Takisawa (Kubota et al. 1995, 1997). Monoclonal antibody 414 (mAb 414, BAb Co, Richmond, CA; Davis and Blobel, 1986; Vigers and Lohka 1992) was used to screen Xenopus p62 cDNA . The isolated clones were identical to previously isolated Xenopus p62 (accession number S59344, Cordes et al., 1991). The DNA fragment, that encodes 103-433 amino acid region, was constructed using EcoRI-XhoI fragment of one of p62 clones. This fragment was ligated into expression vector, pGEX-4T-3 (Pharmacia), using standard protocol, and we obtained GST-fusion protein. Recombinant GST-p62 fusion protein was purified by trapping to glutathione-sepharose 4B beads (Pharmacia LKB Biotechnology Inc.) and elution with thrombin. The purified p62 fragments were separated by SDS-polyacrylamide-gel (PAGE) (Laemmli, 1970), and corresponding bands were excised and immunized onto rabbits by the standard protocols. The produced rabbit antiserum was affinity-purified with GST-p62 proteins immobilized on CNBr-activated Sepharose-4B beads (Pharmacia), and was used as rabbit polyclonal antibody against Xenopus p62. Rabbit anti-Xenopus lamin B antisera and mouse polyclonal antibody against Xenopus lamin B2 rod-domain (R) were prepared as

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described in the previous paper (Takamori and Inoue, submitted).

Electrophoresis and immunoblotting

SDS-PAGE was performed as described by Laemmli (1970). The
samples were run on to 7.5-15% gradient gel and the protein bands
were visualized by Coomassie-blue. Immunoblotting was performed
as described by Towbin et al. (1979). The separated proteins were
transferred electrophoretically to nitrocellulose membranes, and
visualized with 0.2% Ponceau-S in 1% acetic acid. After blocking
with 2% non-fat dry milk in tTBS (150 mM NaCl, 50 mM TRIS-HCl, pH
8.0, 0.1% Tween-20), the blots were incubated with first
antibodies at 1:500 dilution for over night, followed by goat
anti-rabbit or goat anti-mouse IgG coupled to horse radish
peroxidase (Bio-Rad) for 1 hour. The blots were visualized by
Konica Immunostaining kit (Konica Co., Tokyo, Japan).

Preparation of Xenopus egg extracts and in vitro reaction

Adult female *Xenopus laevis* were purchased from San-ai Shoji. Egg
extracts were prepared from unactivated *Xenopus* eggs. Ovulated
eggs were collected 12-13 hours after the injection with 700
units human chorionic gonadotropin (Sigma). Eggs were washed and
dejellied with 2 mM DTT in dejelly buffer (110 mM NaCl and 20 mM
Tris-HCl pH 8.8). The eggs were washed again with the extraction

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buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 20 mM Hepes-NaOH pH 7.0, 5 mM ATP, 1 mM DTT and 1 mM PMSF), packed by centrifugation at 2,000 g for 5 seconds, and then centrifuged at 15,000 g for 10 minutes. The resulting low-speed supernatant between the lipid cap and the pellet was collected. The supernatant was centrifuged again at 15,000 g for 10 minutes to remove residual lipid and pigment. The low speed supernatant was rapidly frozen with liquid N_2, and stored at -40°C for later experiments. This extract was used as *Xenopus* M-phase egg extract.

Xenopus erythrocyte and liver nuclei were prepared as described previously. (Takamori and Inoue, submitted). *Xenopus* erythrocytes were washed with EDTA buffer (25 mM EDTA, 75 mM NaCl, pH 7.5), then washed with STM buffer (0.6 M sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF). Prepared *Xenopus* erythrocyte were treated with 100 μg/ml saponin, and homogenized by teflon homogenizer. The pellet was collected by centrifugation, and was used as a nuclear fraction. *Xenopus* female (gray) liver was dissected into pieces and homogenized in STM buffer. The cell homogenate was centrifuged at 3000 rpm (Hitachi) for 5 minutes and the precipitates were used as a liver nuclei fraction.

The in vitro reaction was performed as follows. The nuclei were washed with the extraction buffer in 1.5 ml tube. After centrifugation at 300 g for 5 minutes, the supernatant was removed. The *Xenopus* M-phase extracts were added to the pellet, mixed and incubated at 25°C for various time.

Immunofluorescence microscopy

The nuclei were incubated in M-phase extracts for various time. The nuclear membrane was stained with 3,3'-dihexyloxacarbocyanine (DHCC) and DNAs with Hoechst dye 33258. For indirect immunofluorescence microscopy, samples were fixed with PBS containing 3.7% formaldehyde for 5 minutes at 25 °C. The nuclei were then centrifuged onto coverslips coated with 1% poly-L-Lysine at 1,000 rpm for 1 minutes. Samples were incubated with rabbit anti-*Xenopus* lamin B antisera (1:100) or a mixture of rabbit anti-p62 antibody (1:20) and mouse anti-*Xenopus* lamin B2(R) antibody (1:20) in tTBS containing 2% non-fat dry milk and 20% goat serum for over night at 4 °C. After washing with PBS, samples were incubated with Cye-2-labeled donkey anti-rabbit IgG (1:50; Amersham) or a mixture of Texas-Red-labeled donkey anti-rabbit IgG (1:50; Amersham) and Cye-2-labeled donkey anti-mouse IgG (1:50; Amersham) in tTBS containing 2% non-fat dry milk for 1 hour at 4 °C. After washing with PBS, the sample was further stained with Hoechst dye 33258. All Samples were viewed with a fluorescent microscopy (Orynpath BH-2). Staining images were taken by cooled CCD camera (Photometrics, PXL) with IP-Lab Spectrum system (Signal Analytics Corporation).

Preparation of antibodies

We prepared antibodies against B-type lamin and nuclear pore protein p62. The specificity of antibodies was determined by immunoblotting of proteins from *Xenopus* erythrocyte nucleus (Fig. 1). Rabbit antiserum against *Xenopus* B-type lamin recognized about 68 kD and 70 kD doublet proteins (data not shown). The molecular weights of these proteins were identical to those of lamin B1 (upper band) and lamin B2 (lower band) (Krohne and Franke, 1983; Krohne et al., 1987; Hoger et al., 1990). Mouse affinity-purified antibody against *Xenopus* lamin B2 rod region (see Takamori and Inoue, submitted) recognized only the lower band (data not shown). Rabbit affinity-purified antibody against *Xenopus* nuclear pore protein p62 recognized single 70 kD protein (Fig. 1, lane 2). Monoclonal antibody 414, which had been reported to react with *Xenopus* p62 (Viger and Lohka 1992), also recognized the same band (Fig. 1, lane 3).

Nuclear envelope break down of isolated erythrocyte nuclei in Xenopus egg M-phase extracts

Xenopus M-phase egg extract is a good system to investigate mitotic events in vitro (Lohka and Masui; 1983). To investigate
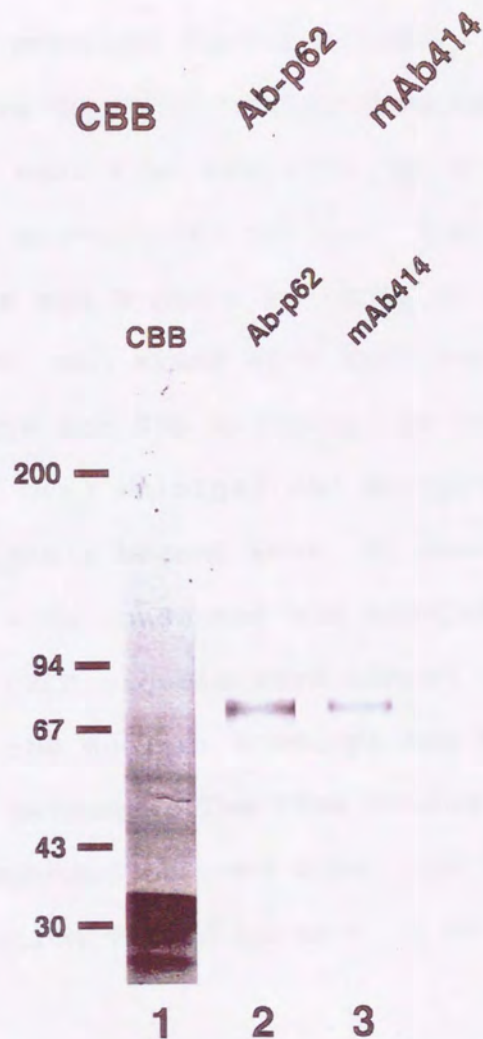
Figure 1. Specificity of antibodies against p62. *Xenopus*
erythrocyte nuclear fraction was separated on SDS-PAGE (7.5-15%
gradient gel) and proteins were visualized by Coomassie blue
staining (lane 1). Immunoblotting was performed using rabbit
polyclonal antibody against *Xenopus* p62 (lane 2) and monoclonal
antibody 414 (lane 2).

dynamics of nuclear envelope during mitosis, we tried to establish an in vitro nuclear envelope breakdown system using *Xenopus* egg M-phase cell free extracts. As a source of nucleus, we prepared *Xenopus* erythrocyte nucleus. The nuclei were incubated in *Xenopus* egg M-phase extracts at 25 °C in the presence of 5 mM ATP, and mixed with DHCC for membrane staining and Hoechst dye 33258 for DNA staining. By incubation for 30 minutes, the nuclei were enlarged and elongated (Fig. 2, c and d), and the DHCC signals became weak. By incubation for 60 minutes the nuclei were condensed and elongated to "worm shape" (Fig. 2, e and f). DHCC signals were almost undetectable. This result showed that the nuclear envelope was broken down and dispersed into the extracts. The time course of this reaction was not definite and depended on used eggs. The time necessary for chromosome condensation varied between 60 minutes and 120 minutes.

Next, we tried other nuclear envelope marker for the detection of nuclear envelope breakdown. Fig. 3 shows the result observed by indirect immunofluorescence microscopy using anti-*Xenopus* lamin B antiserum. At time 0, anti-lamin B antiserum stained the nuclear envelope (Fig. 3a). After 30 minutes the nuclei swelled and lamin B signal became weak (Fig. 3, c and d). The chromatin was not condensed at this time (Fig. 3 d), but at 60 minutes, the nucleus became elongated and condensed (Fig. 3, e and f). The B-type lamins became dispersed, but some signals remained on condensed chromosome surface and were seen as dot-like pattern.

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DHCC   **Hoechst**
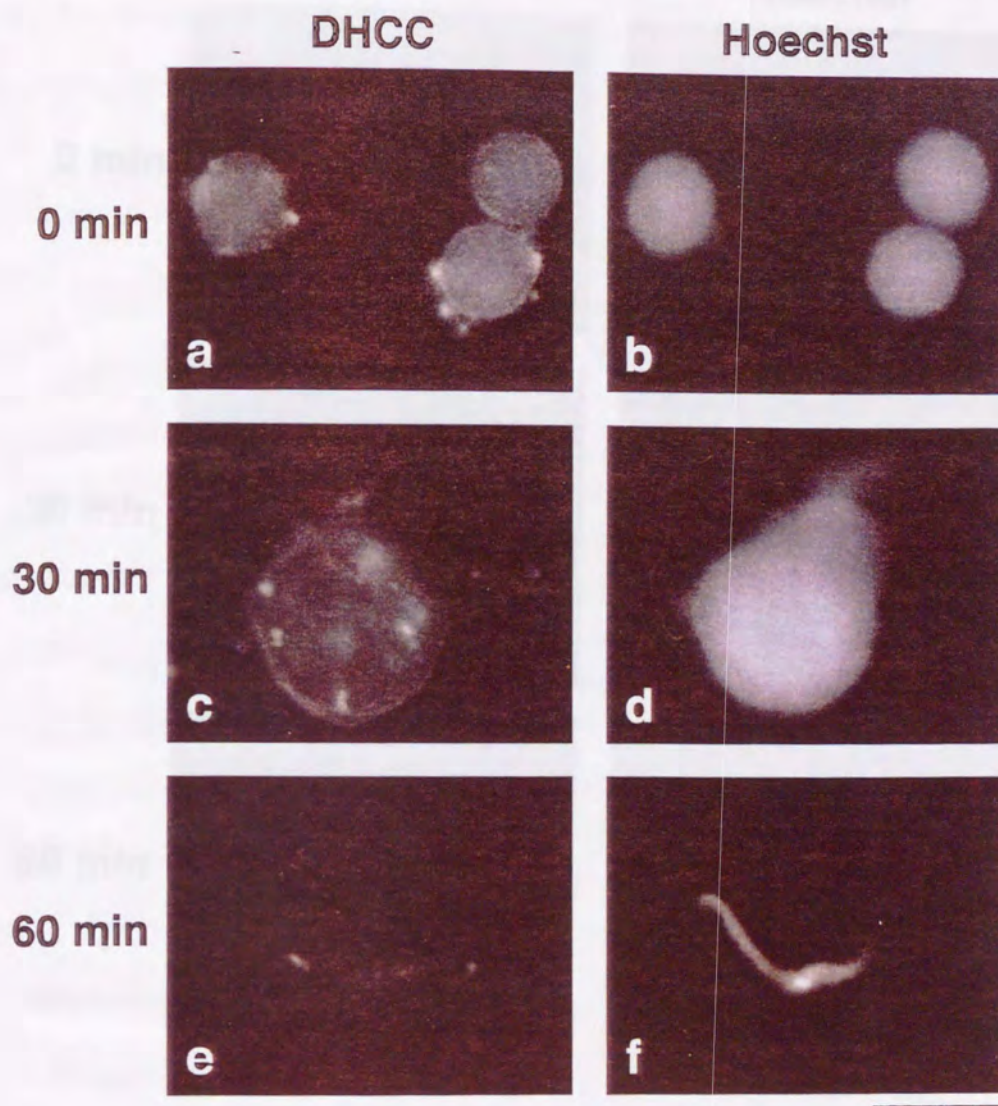
0 min

30 min

60 min

Figure 2. DHCC and Hoechst staining of *Xenopus* erythrocyte
nucleus in *Xenopus* M-phase egg extracts. Isolated *Xenopus*
erythrocyte nuclei were incubated in *Xenopus* egg M-phase extracts
for 0 min. (a and b), 30 min. (c and d), 60 min. (e and f), and
visualized with DHCC for membrane staining (a, c and e) and with
Hoechst dye 33258 for DNA staining (b, d and f). Bar, 10 μm.

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Lamin   **Hoechst**
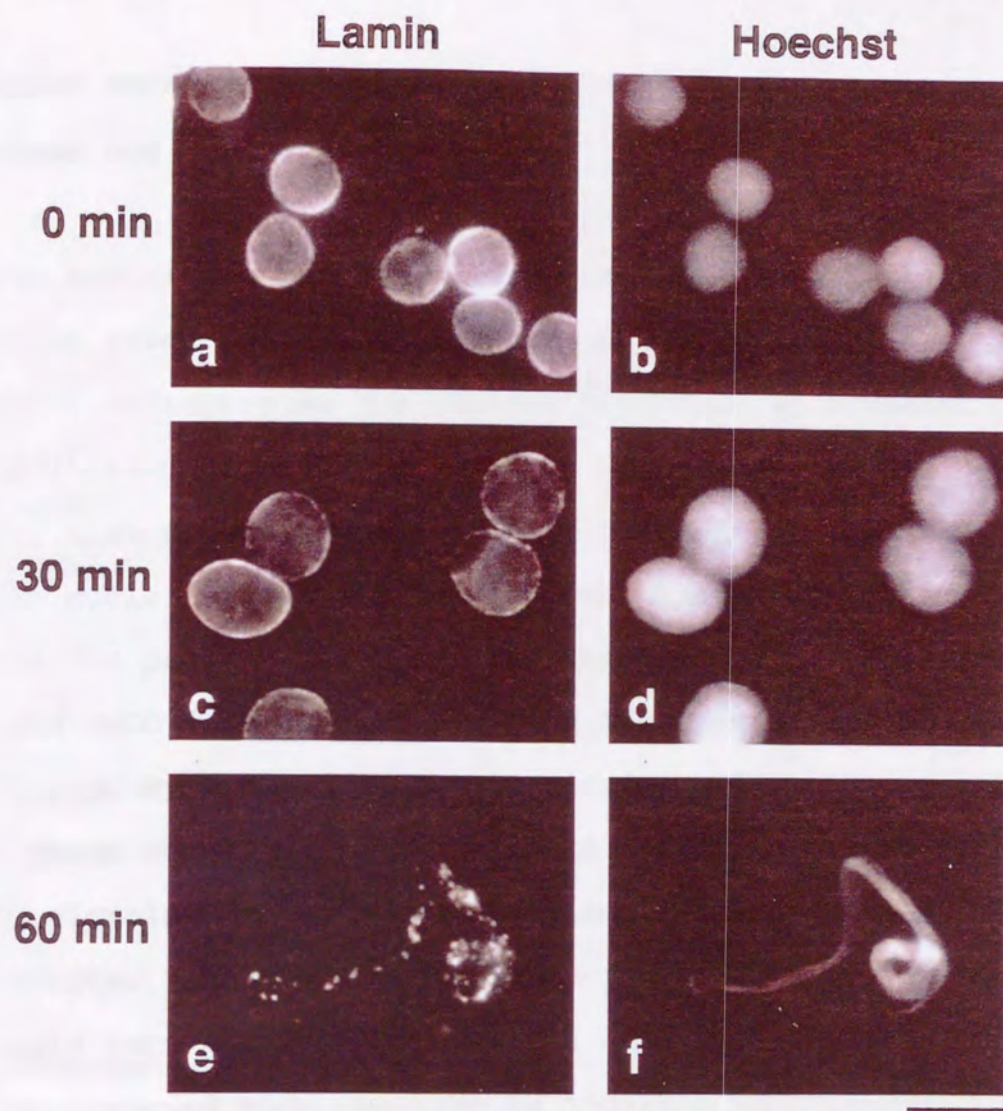
0 min

a   b

30 min

c   d

60 min

e   f

Figure 3. Immunofluorescence staining of *Xenopus* erythrocyte
nucleus in *Xenopus* M-phase egg extracts with anti-lamin B
antisera.   Isolated *Xenopus* erythrocyte nuclei were incubated in
Xenopus egg M-phase extracts for 0 min. (a and b), 30 min. (c and
d), 60 min. (e and f). Each nuclei were fixed onto coverslips and
stained with rabbit antisera against *Xenopus* B-type lamin (a, c
and e). DNAs were visualized with Hoechst dye 33258 (b, d and f).
Bar, 10 μm.

Nuclear envelope breakdown of isolated Xenopus liver nuclei in Xenopus egg M-phase extracts

Liver nuclei are frequently used for biochemical studies, however *Xenopus* liver contains a large amount of dark granule, and this granule contaminates the nuclear fraction. We prepared nuclei from light-colored *Xenopus* female, that contain less amount and small size of the granule. Fig. 4 shows the result when *Xenopus* liver nuclei were incubated in *Xenopus* egg M-phase extracts at 25 °C in the presence of 5 mM ATP. The nuclei was observed after mixing DHCC for membrane staining and Hoechst dye 33258 for DNA staining. By incubation for 30 minutes, the nuclei enlarged and the shape became elongated (Fig. 4 c), as erythrocyte nuclei. The DHCC signals were weak at this time (Fig. 4 d). By incubation for 60 minutes, the nuclei were condensed (Fig. 4 e), and DHCC signals were almost undetectable.

We observed this reaction by indirect immunofluorescence microscopy using antibody against *Xenopus* lamin B2 rod domain (Fig. 5). At time 0, anti-lamin B2 antibody stained strongly the nuclear rim (Fig. 5 c). After 30 minutes, the nuclei swelled, lamin B staining became weak, and non-stained region appeared on nuclear envelope (Fig. 5, d to f). At 60 minutes, the nucleus became elongated and condensed (Fig. 5, e and f). The B-type lamins became dispersed, but a part of signals remained on condensed chromosome.
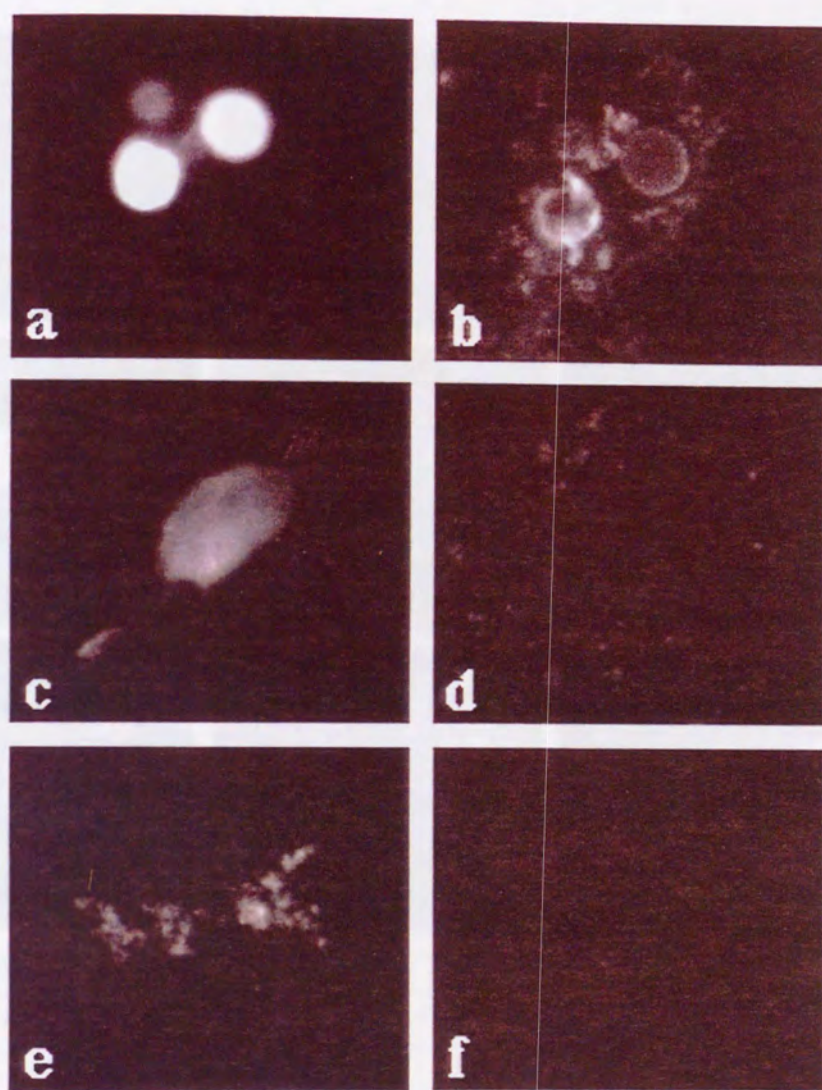
Figure 4. DHCC and Hoechst staining of *Xenopus* liver nucleus in *Xenopus* M-phase egg extracts. Isolated *Xenopus* liver nuclei were incubated in *Xenopus* egg M-phase extracts for 0 min. (a and b), 30 min. (c and d), 60 min. (e and f), and visualized with DHCC for membrane staining (b, d and f) and with Hoechst dye 33258 for DNA staining (a, c and e). Bar, 10 μm.
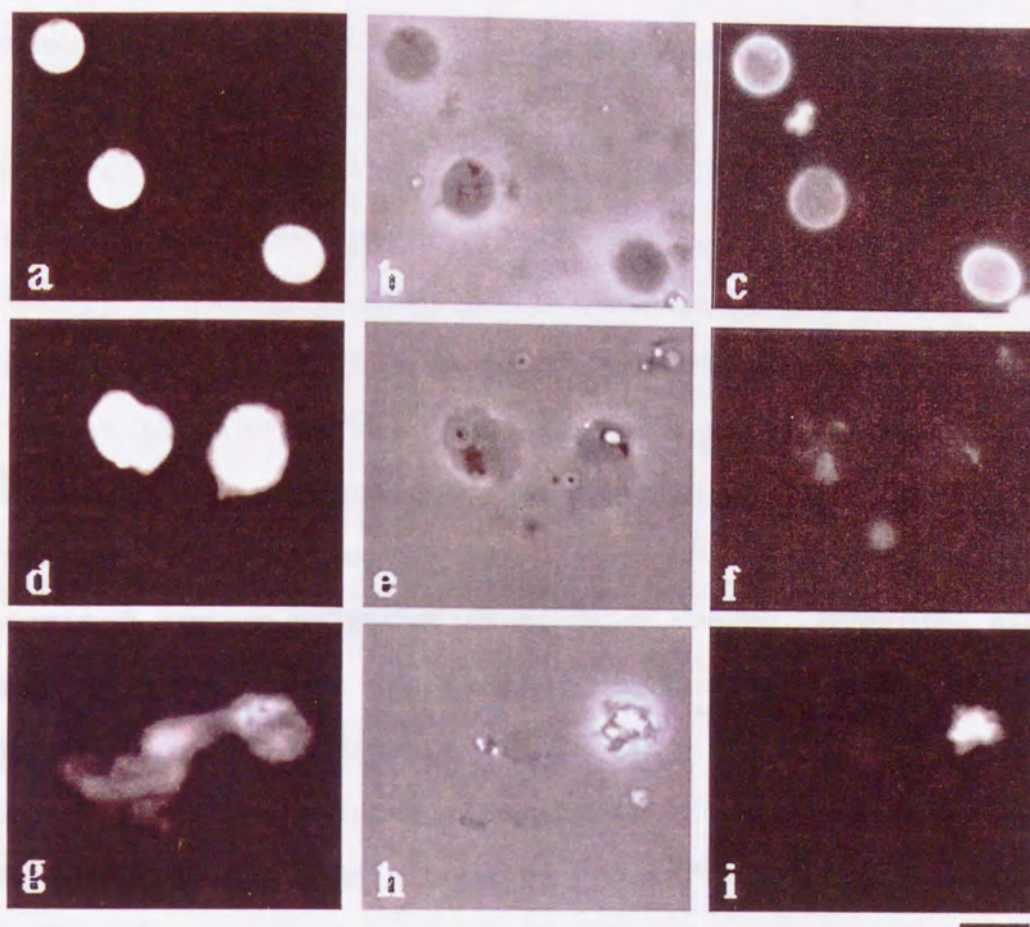
Figure 5. Immunofluorescence staining of *Xenopus* liver nucleus in M-phase egg extracts with anti-lamin B2 antibody. Isolated *Xenopus* liver nuclei were incubated in *Xenopus* egg M-phase extracts for 0 min.(a to c), 30 min. (d to f), 60 min. (g to i). After fixation, each nuclei were stained with anti-lamin B2 antibody (c, f, i). b, e, and h are the phase contrast images. DNAs were visualized with Hoechst dye 33258 (a, d, g). Bar, 10 μm.

Dynamics of B-type lamin and nuclear pore protein p62 of isolated
erythrocyte nucleus in Xenopus egg M-phase extracts

We compared the time course of dissociation of nuclear lamina and
nuclear pore complex. We performed indirect immunofluorescence
microscopy using antibodies against lamin B2 and p62 (Fig. 6). At
0 time, signals of either lamin B2 and p62 were strong (Fig. 6, a
and b). At 15 minutes when nucleus was enlarged, the signals
became week, and non-stained region appeared on nuclear envelope
for either lamin B2 and p62 (Fig. 6, d and e). After 30 minutes,
non-stained region extended (Fig. 6, g and h). However, staining
pattern of two markers was similar. By incubation for 15 minutes
to 30 minutes, the nuclear envelope proteins were dispersed, but
chromatin was not condensed (see Figs. 2, 3 and 6, d to j). At 60
minutes, nucleus became elongated and condensed (Fig. 6 l).
Swelled nucleus was also seen. At this time, both lamin B2 and
p62 signal were almost dispersed (Fig 6, j and k).

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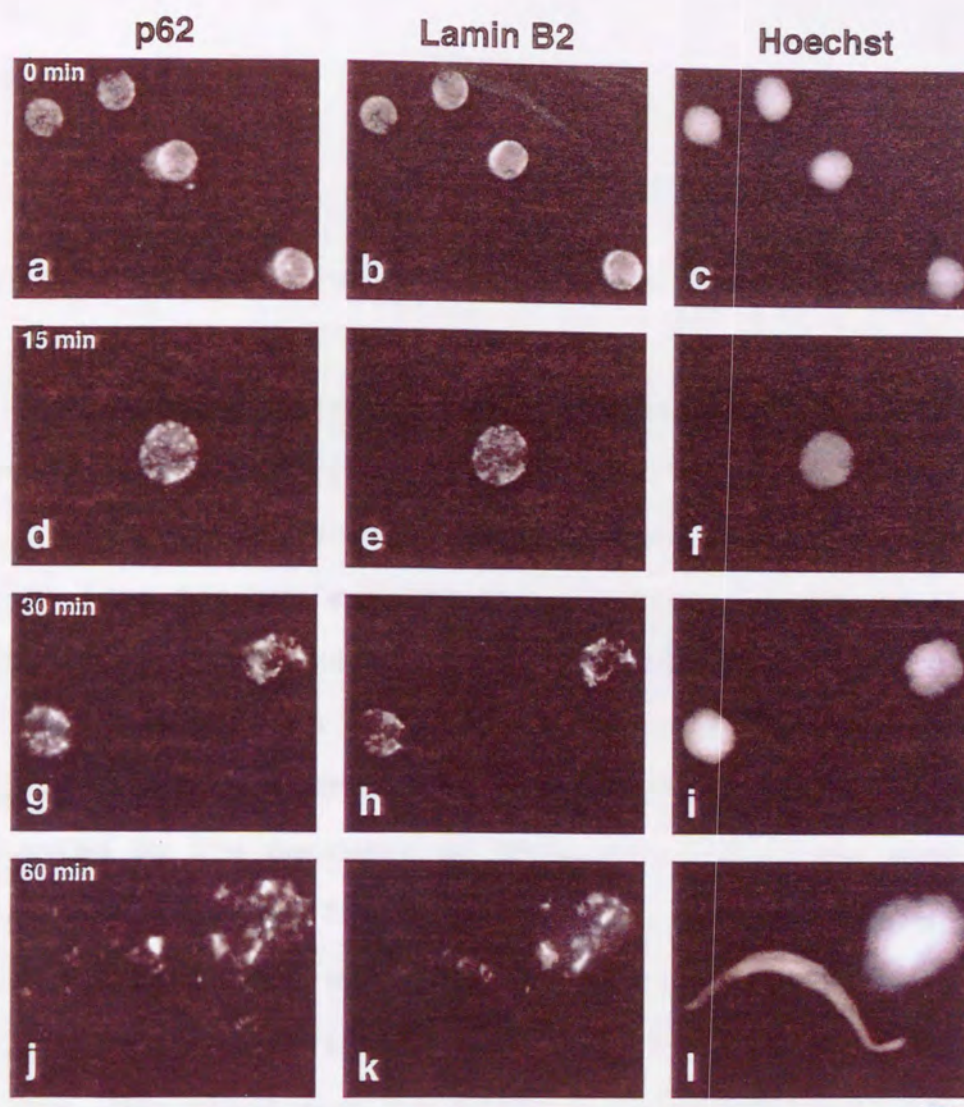**p62**   **Lamin B2**   **Hoechst**

Figure 6. Immunofluorescence staining of *Xenopus* erythrocyte
nuclei in M-phase egg extracts with anti-lamin B2 rod domain and
anti-p62 antibodies.   Isolated *Xenopus* erythrocyte nuclei were
incubated in *Xenopus* egg M-phase extracts for 0 min. (a to c), 15
min. (d to f), 30 min. (g to i) and 60 min. (j to l). After
fixation, each nuclei were double-labeled with a mixture of mouse
anti-lamin B2 rod domain (a, d, g and j) and rabbit anti-p62 (b,
e, h and k) antibodies. DNAs were visualized with Hoechst dye
33258 (c, f, i and l). Bar, 10 μm.

DISCUSSION

Nuclear envelope breakdown in Xenopus M-phase egg extracts

We investigated nuclear envelope breakdown of isolated *Xenopus* nucleus using *Xenopus* egg M-phase extracts. The results obtained in this report are summarized schematically in Fig. 7. Isolated nuclei from *Xenopus* erythrocyte and liver were incubated in M-phase egg extracts at 25 °C in the presence of ATP (a). After 15 minutes, nuclei were enlarged and swelled (b). After 30 minutes, nuclear envelope started to break down (c). This process was monitored by the decrease of DHCC signal (nuclear membrane) and dispersion of lamin B (nuclear lamina) and p62 (NPC). After 60 minutes, chromosome was condensed and elongated (d).

Lohka and Maller (1985) reported that preincubation with *Xenopus* interphase egg low speed supernatant (LSS) is necessary to induce nuclear envelope break down (NEBD) on *Xenopus* brain or liver nucleus. They explained that cytoplasmic component involved in NEBD and chromosome condensation is incorporated into nucleus and proceed the following events. Miake-lye and kirschner (1985) performed the similar experiments that the isolated nucleus derived from rat cells were preincubated with *Xenopus* interphase egg LSS and following addition of M-phase high speed supernatant (HSS). Newport and Spann (1987) reported that the time required for complete NEBD varied with the type of exogenous nuclei added. They explained that the time required for NEBD is depend on the
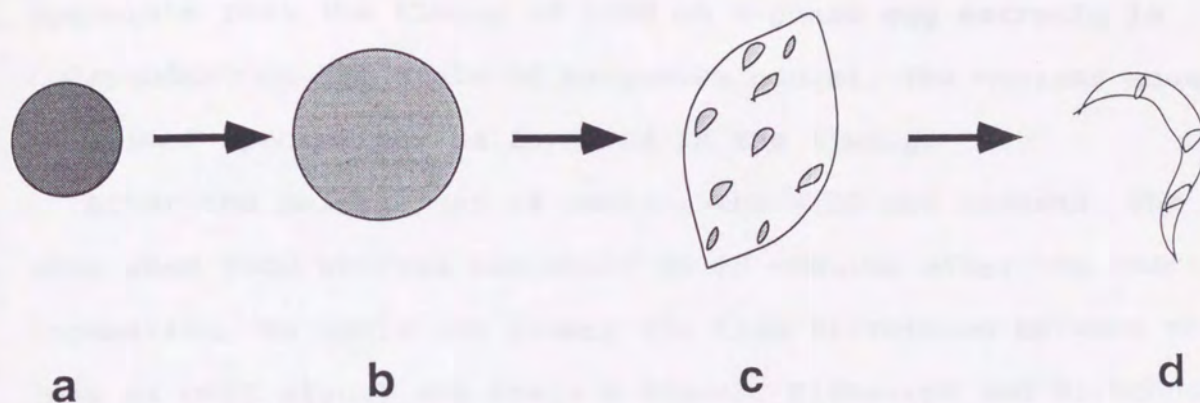
a   b   c   d

Figure 7. Schematic illustration for dynamics of nucleus in M-phase egg cell-free extracts. The mitotic events of nucleus in *Xenopus* M-phase egg extracts are shown (a to d). First, the incubated nucleus begins to enlarge and B-type lamin and p62 signals become weak (b). Second, the nucleus starts to elongate and the nuclear envelope breaks down (c). The B-type lamin and p62 signals become dot-like pattern. Finally, the nuclear envelope is almost dispersed and the chromosome is condensed and elongate like "worm shape" (d).

state of exogenous nuclei. However, in our experiments using *Xenopus* erythrocyte and liver nucleus, which are in G0-phase, NEBD are induced by incubation for 30-60 minutes. So, we speculate that the timing of NEBD on M-phase egg extracts is independent on the state of exogenous nuclei. The nuclear souse of animal species may be involved in the timing.

After the enlargement of nuclei, the NEBD are induced. The time when NEBD started was about 30-60 minutes after the start of incubation. We could not detect the time difference between the loss of DHCC signal and lamin B signal. Miake-Lye and Kirschner (1985) reported the difference of timing between the phosphorylation and dispersion of lamin and the NEBD. They showed that phosphorylation and dispersion of lamin occurred as a first step, and the nuclear structure became weak. The NEBD were induced 10 minutes later. So, the time lag may exist between the two process. Newport and Spann (1987) reported that at high concentration of nuclei in egg extracts, the NEBD was blocked but the lamin depolymerization was induced normally. These results suggest these reactions are regulated separately .

After NEBD, the chromosome condensation occurred. In our experiment, nucleus was condensed and elongated like a "worm shape", and individual chromosome was not appeared. The individual chromosome were probably fused before each chromosome became condensed completely. In addition, the chromosome condensation occurred after NEBD. The order of these two events was different from normal cell cycle (discussed later). This result agrees with previous reports using *Xenopus* cell-free

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system (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985, 1987; Newport and Spann, 1987).

Regulation of nuclear mitotic events

On normal cell division, chromosome condensation and NEBD occur sequentially during prophase and metaphase (Georgatos et al., 1997). At prophase, the first sign of mitotic events is the chromosome condensation. As the next event, nuclear envelope invaginations are formed, and nuclear lamina structure becomes loose (Georgatos et al., 1997; Takamori and Inoue, prepared for submit). The A-type lamins are solubilized faster than NEBD, but B-type lamins are not (Georgatos et al., 1997). Then, NEBD is induced and the components of nuclei and nuclear envelope are dispersed into cytoplasm.

The nuclear mitotic events are regulated firmly, probably by several mitotic inducers and checkpoint system. The key factor that induce mitotic events have been called MPF (M-phase promoting factor), and was previously characterized as cdc2/cyclin B kinase (Masui and Markert, 1971; for reviews, see Maller, 1991; Coleman and Dunphy, 1994; Stern and Nurse; 1996). When cdc2/cyclin B kinase is once activated, the most of nuclear mitotic events are induced. So, cdc2/cyclin B kinase is thought to be as a master regulator for mitosis. In addition to nuclear mitotic inducers, the targets of mitotic phosphorylation that exists on NE have been characterized. The well known target

proteins are lamin, p58/LBR, LAP1, LAP2, and nucleoporin, such as p62, and gp210 (Foisner and Gerace, 1993; Macaulay et al., 1995; Favreau et al, 1996; Nikolakaki et al, 1996, 1997). The phosphorylation of these proteins may be important for NEBD and dispersion of nuclear structures, such as nuclear lamina and NPC.

The mechanism of regulation of nuclear membrane structure in M-phase egg extracts is probably different from that in normal cell division. The cell cycle of *Xenopus* egg at early developmental stage is 60-90 minutes, and is much faster than that of normal cell cycle. In addition, the sequence of cell cycle in normal cell are regulated by checkpoint system. For example, M-phase activity is not increased before DNA replication is complete, and induced only at G2-phase. Such a system is not clear in *Xenopus* egg extract. The isolated nucleus derived from G0 or G1-phase cells also induced mitotic events.

Finally, recent reports demonstrated that at the time when NEBD occurs at prometaphase, nuclear membrane is fused to ER membrane and incorporated into mitotic ER/NE membrane network (Ellenberg et al., 1997; Yang et al., 1997; Wiese et al., 1997). This ER-NE interaction is probably lost on isolated nucleus. The involvement of ER membrane on NEBD regulation is not clear.

The system reported in this paper is very useful system to investigate the mechanism and regulation of NEBD. By studying the different and common mechanism of the cell-free system and normal cell cycle, the process of NEBD may be understood well in the future.

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