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Evaluation of mammalian cell-free systems of nuclear assembly and disassembly

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Table of contents

Page

List of tables iv
List of figures v
Abbreviations vii
Abstract ix
Résumé x
Acknowledgments xi

I. Introduction 1
1. Objectives 1
2. The nucleus, a functional compartment 1
3. The nuclear lamina 4
3.1. Lamins 4
3.2. Non-lamin components 7
3.3. Do plants and fungi have nuclear lamins? 8
4. Proteins of the inner nuclear membrane 9
4.1. Lamin B receptor 9
4.2. Lamina-associated polypeptides 9
4.3. Emerin 10
4.4. MAN 11
4.5. Other inner nuclear membrane proteins 12
4.6. Pore membrane proteins 13
5. Network of nuclear envelope protein interactions 14
6. Retention at the nuclear envelope 14
7. Nuclear disassembly and reassembly 15
8. Cell-free systems 17
9. New objectives 19

II. Materials and Methods 20
1. Materials 20
2. Cell culture 20
3. Cell synchronization and harvesting 20
4. Disassembly systems 21
4.1. Mitotic cell extracts for disassembly assays 21
4.2. Preparation of nuclei for disassembly assays 22
4.3. Loading of nuclei with antibodies 23
4.4. Nuclear disassembly 24
5. Assembly systems 24
5.1. Mitotic homogenate for nuclear assembly 24
5.2. Digitonin system of nuclear assembly 25
6. Cell fractionation for biochemical analysis 25
7. Gel electrophoresis and immunoblotting 26
8. Indirect immunofluorescence staining 26
9. Antibodies 28

III. Results 30
1. Localization of nuclear antigens during the cell cycle 30
1.1. Interphase localization and co-localization of nuclear antigens 30
1.2. Mitotic fate of nuclear antigens 43
1.3. Accessibility to the nucleus during mitosis 48
2. Nuclear disassembly 51
3. Nuclear assembly 63
3.1. Crude mitotic homogenate 68
3.2. Digitonin assembly system 74
3.3. Yield of nuclear assembly in intact nocodazole synchronized cells . . . 86
4. Adherence of nocodazole released cells 86

IV. Discussion 96
1. Nuclear antigens in micronuclei 96
2. Nuclear access during mitosis 98
3. Cell synchrony 98
4. Validity of cell-free systems of nuclear disassembly and assembly 101
5. Future research 103

V. References 106

List of tables

page

TABLE 1. Nuclear assembly upon addition of an exogenous supplement of mitotic soluble (membranous and cytosolic) extract. 73

TABLE 2. Nuclear assembly of LAP2 in synchronized HeLa cells. 91

TABLE 3. Adherence of nocodazole synchronized HeLa cells. 95

List of figures

page

FIGURE 1. Structure of the nuclear envelope and proteins of the inner nuclear membrane. 3

FIGURE 2. Spatial relationship of the LEM proteins to lamins A/C in HeLa cells during the cell cycle. 32

FIGURE 3. Spatial relationship of the LEM proteins to lamin B in HeLa cells during the cell cycle. 34

FIGURE 4. Spatial relationship of the LEM proteins to LAP2α in HeLa cells during the cell cycle. 36

FIGURE 5. Localization of 2A7 proteins in HeLa cells during the cell cycle. 38

FIGURE 6. Localization of LAP2 proteins in HeLa cells during the cell cycle. 40

FIGURE 7. Presence of LAP2, 2A7 antigens and nuclear pore proteins in micronuclei and cytoplasmic inclusions. 42

FIGURE 8. Presence of LEM proteins, lamin B and lamins A/C in micronuclei and cytoplasmic inclusions. 45

FIGURE 9. Localization of nuclear proteins in Fanconi's anemia cells. 47

FIGURE 10. Accessibility to the nucleus during the cell cycle. 50

FIGURE 11. Cyclin B1 is present in mitotic soluble extracts. 53

FIGURE 12. Presence of intermediate filaments around isolated HeLa nuclei. 55

FIGURE 13. Isolated nuclei failed to disassemble after 2h in mitotic soluble extract. 57

FIGURE 14. Presence of vimentin surrounding isolated nuclei. 60

FIGURE 15. Isolated CHO nuclei failed to disassemble after 2h in HeLa mitotic soluble extract. 62

FIGURE 16. Isolated nuclei permeabilized with lysolecithin can take up antibodies. 65

FIGURE 17. Presence of cytoskeletal components in the cytoplasm of digitonin permeabilized cells. 67

FIGURE 18. Localization of nuclear antigens in a homogenate of mitotic HeLa cells. 70

FIGURE 19. Incomplete assembly of nuclei in a crude homogenate of mitotic HeLa cells. 72

FIGURE 20. Incomplete assembly of LAP2 in nuclei of digitonin permeabilized mitotic HeLa cells. 76

FIGURE 21. Incomplete assembly of lamin B in nuclei of digitonin permeabilized mitotic HeLa cells. 78

FIGURE 22. Incomplete assembly of LAP2 in nuclei of digitonin permeabilized mitotic CHO cells. 81

FIGURE 23. Incomplete assembly of lamin B in nuclei of digitonin permeabilized mitotic CHO cells. 82

FIGURE 24. Western blot analysis of intact and digitonin permeabilized mitotic cells. 85

FIGURE 25. Western blot analysis of reassembled intact, control digitonin permeabilized mitotic cells and permeabilized cells exposed to goat polyclonal antibodies against lamin B. 87

FIGURE 26. Incomplete assembly of LAP2 in nuclei of nocodazole synchronized HeLa cells. 89

FIGURE 27. Tubulin distribution in intact or digitonin permeabilized, nocodazole synchronized HeLa cells. 92

Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BAF	Barrier to autointegration factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CDK1	Cyclin B1-p34cdc2 kinase complex
CHO	Chinese hamster ovary
CMT	Charcot-Marie-Tooth
cPBS	Cell culture phosphate buffered saline
DEB	DNA-end binding
$DiOC_6$	3,3'-dihexyloxacarbocyanine iodide
D-MEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDMD	Emery-Dreifuss muscular dystrophy
EDTA	Ethylenediamine-tetra-acetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLIP	Fluorescence loss in photobleaching
FPLD	Familial partial lipodystrophy
FRAP	Fluorescence recovery after photobleaching
GCL	Germ-cell-less
GFP	Green fluorescent protein
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HP1	Heterochromatin protein 1
ICMT	Isoprenylcysteine carboxymethyltransferase
IF	Immunofluorescence
INM	Inner nuclear membrane
LAP	Lamina-associated polypeptide
LBR	Lamin B receptor
LEM	LAP2-emerin-MAN1 domain
MEM	Minimal essential medium
MPF	M-phase promoting factor
mRNA	Messenger RNA
NE	Nuclear envelope
NEBD	Nuclear envelope breakdown
NLS	Nuclear localization signal
NPC	Nuclear pore complex
ONM	Outer nuclear membrane
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)

PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluoride
PNB	Prenucleolar body
PPHD	p-phenylenediamine
RB	Retinoblastoma
rDNA	Ribosomal DNA
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA
TCA	Trichloroacetic acid
TGFβ	Transforming growth factor-β
TX-100	Triton X-100
WB	Western blot
YA	Young arrest

Abstract

Mammalian cell-free systems are very useful for the biochemical and structural study of nuclear disassembly and assembly. Through manipulation, the role of specific proteins in these processes can be investigated. I first intended to examine the involvement of integral and peripheral inner nuclear membrane proteins in nuclear disassembly and assembly. However, I was unable to achieve disassembly when isolated interphase HeLa nuclei were exposed to mitotic soluble extracts containing cyclin B1. Homogenates of synchronized mitotic HeLa cells left to reassemble nuclei resulted in incomplete nuclear envelope assembly on chromatin masses. Digitonin permeabilized mitotic cells also assembled incomplete nuclei, generating a lot of cytoplasmic inclusions of inner nuclear membrane proteins as an intermediate. These results were therefore used as a basis for the experimental evaluation of mammalian cell-free systems. Synchronization itself induced incomplete nuclear assembly. This may be caused by the prior aberrant nuclear disassembly, or by the abnormal number of mitotic spindles.

Résumé

Les systèmes acellulaires de mammifères sont très utiles pour l'étude biochimique et structurale du désassemblage et de l'assemblage nucléaire. Le rôle dans ces processus de certaines protéines peut être étudié en manipulant le système. J'ai tout d'abord voulu examiner l'implication des protéines intégrales et périphériques de la membrane interne du noyau dans le désassemblage et l'assemblage nucléaire. Toutefois, des noyaux provenant de cellules HeLa en interphase ne se sont pas désassemblés lorsqu'exposés à un extrait cytoplasmique de mitose contenant de la cycline B1. De plus, le système d'assemblage nucléaire basé sur des homogénats de cellules HeLa mitotiques a engendré des membranes nucléaires incomplètes. Des cellules mitotiques perméabilisées à la digitonine ont également assemblé des enveloppes nucléaires incomplètes, générant une grande quantité d'inclusions cytoplasmiques pendant l'assemblage. Ces résultats ont donc été utilisés dans une évaluation expérimentale des systèmes acellulaires de mammifères. La synchronisation des cellules elle-même a causé un assemblage nucléaire incomplet, peut-être à cause d'un désassemblage aberrant, ou d'un nombre anormal de fuseaux mitotiques.

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I. Introduction

1. Objectives

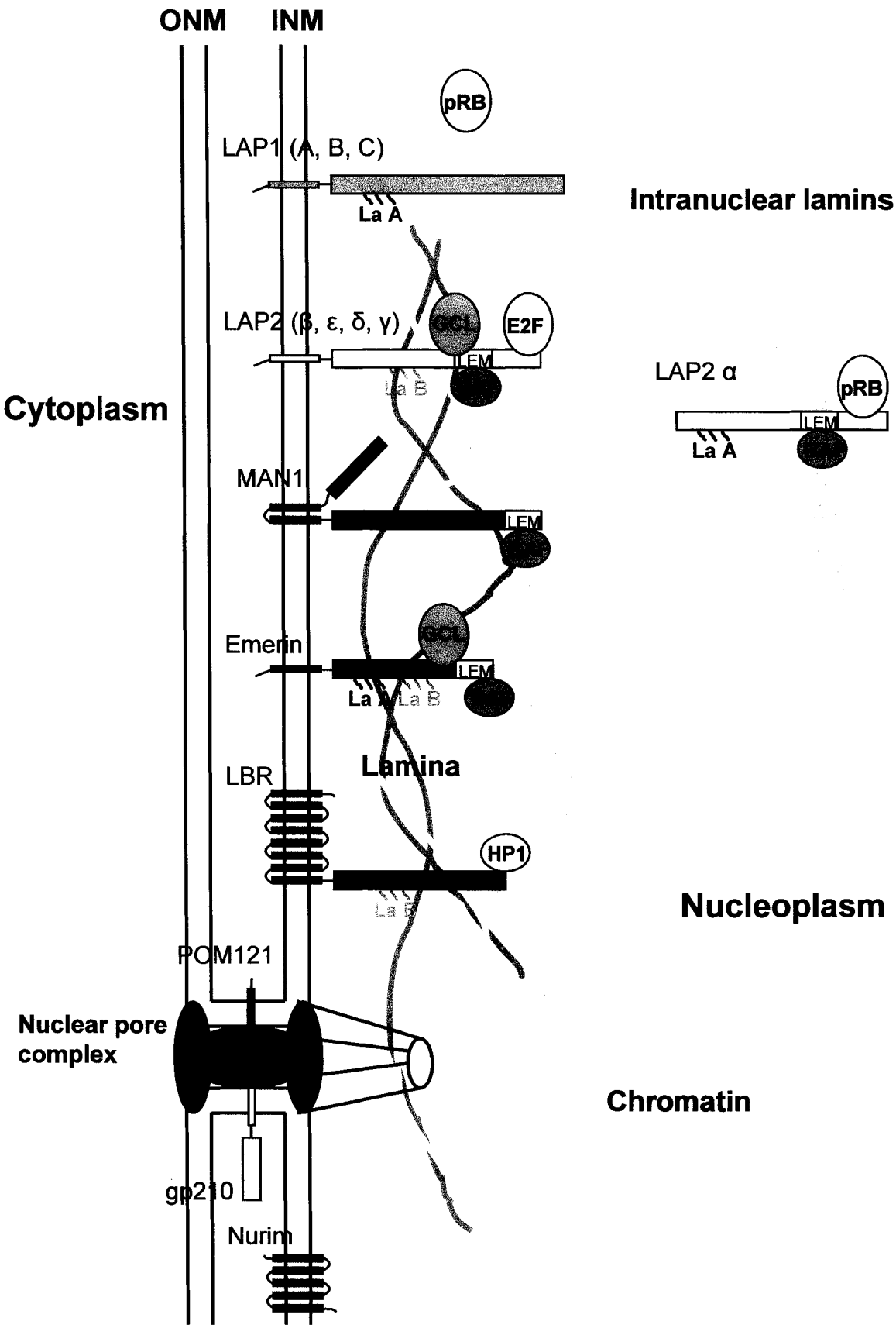
The primary objective of this project was to study the involvement of selected nuclear proteins in nuclear disassembly and assembly using human cell-free systems. Proteins to be studied were primarily nuclear envelope (NE) membrane proteins such as MAN1 and LAP2, as well as NE peripheral proteins like lamins. Clues as to the role of integral and peripheral nuclear membrane proteins in mitosis have been accumulating, but the picture is still incomplete. Furthermore, since the majority of previous cell-free experiments having been performed with amphibian meiotic systems, it was important to verify if the results applied to mammalian cells. Experiments in this study were therefore devised on a few promising mammalian cell-free systems previously described in the literature. Yet, results proved to be less reliable than could be expected from published evidence. Consequently, the relevance of these cell-free systems is questioned here.

2. The nucleus, a functional compartment

The nucleus of eukaryotic cells is a highly organized structure (reviewed by Cremer *et al.*, 2004). The NE separates the nucleus from the rest of the cell, segregating nuclear functions. The main physiological roles of the nucleus are to direct RNA synthesis and processing, as well as duplication of the genome at each cell cycle. In interphase cells, the nucleus is the site of major metabolic activities. The nucleoplasm contains mostly chromatin, which is attached to the NE and to specialized internal sites. Inside the nucleoplasm, there are many known subnuclear compartments: the nucleolus, coiled bodies, speckled compartments (interchromatin granules) and DNA replication foci (Cardoso *et al.*, 1999). It is at the site of the nucleoli that rDNA (ribosomal genes) is transcribed and where ribosome biogenesis happens.

The NE consists of two concentric lipid bilayers, which are separated into three distinct but interconnected domains (Fig. 1): the outer nuclear membrane (ONM), the inner nuclear membrane (INM), and the pore membranes. The ONM is continuous with the endoplasmic reticulum (ER), and similar in composition. The ONM can therefore be considered as a specialized region of the ER membrane. The lumen of the ER

Figure 1. Structure of the nuclear envelope and proteins of the inner nuclear membrane. BAF (barrier to autointegration factor), chromosomal factor; E2F, transcription factor; GCL (germ-cell less), transcription factor; HP1, heterochromatin protein 1; INM, inner nuclear membrane; La A, binding site for lamin A; La B, binding site for lamin B; LAP, lamina-associated protein; LBR, lamin B receptor; LEM, LEM domain; ONM, outer nuclear membrane pRB, retinoblastoma protein.


ONM
INM
pRB
LAP1 (A, B, C)
La A
Intranuclear lamins
LAP2 (β, ε, δ, γ)
E2F
LEM
La B
Cytoplasm
LAP2 α
pRB
LEM
La A
MAN1
LEM
Emerin
LEM
La A
La B
Lamina
LBR
HP1
La B
Nucleoplasm
POM121
Nuclear pore complex
Chromatin
gp210
Nurim

communicates with the perinuclear cisternae space, making the space between the membranes of the NE an "extracellular space". The pore membranes connect the inner and outer nuclear membranes and are associated with nuclear pore complexes (NPC) through specific integral proteins (reviewed by Worman and Courvalin, 2000). The INM possesses unique characteristics. It is associated with chromatin, and with the nuclear lamina which supports it. The interactions between lamina, chromatin and INM proteins are widely recognized as crucial to the structural maintenance of the NE, its disassembly and reformation during mitosis, and the higher organizational level of the genome in interphase (Ellenberg *et al.*, 1997).

3. The nuclear lamina

The NE is structurally supported by the fibrous nuclear lamina, which is also believed to participate in chromatin organization.

3.1. Lamins

Nuclear lamins were the first peripheral INM proteins to be characterized (Gerace, 1978). They are type V intermediate filament proteins that polymerize into 10 nm-diameter filaments (Aebi *et al.*, 1986). They form the nuclear lamina, an insoluble protein meshwork situated between the inner nuclear membrane and the chromatin, but are also present throughout the nucleus (Goldman *et al.*, 1992; Bridger *et al.*, 1993). The nuclear lamina provides support and strength for the NE (reviewed by Hutchison *et al.*, 2001) and acts as a framework for attachment and organization of chromatin. Lamins have been subdivided into types A and B based on their molecular sequences (reviewed by Nigg, 1992). Type A lamins are encoded by a common gene termed *LMNA*, and differences in splicing give rise, in vertebrates, to lamin isoforms A, AΔ10, C_1 and germ-cell specific C_2. Type B lamins B1 and B2 are encoded by different genes closely related in sequence, *LMNB1* and *LMNB2*, while lamin B3 is a product of alternatively spliced *LMNB2* present only in spermatocytes. Differential expression of the lamin types varies according to cell lines. For example, B-type lamins seem to be expressed in all cell lines, while A-type lamins are absent from early developing embryos as well as undifferentiated or rapidly proliferating cells (Lin and Worman, 1997; Worman and

Courvalin, 2000; Guilly *et al.*, 1987, 1990; Paulin-Levasseur *et al.*, 1996). During mitosis, the lamina is reversibly depolymerized by phosphorylation (Gerace and Blobel, 1980). Type A lamins become soluble in the cytoplasm while B-type lamins tend to remain associated with nuclear membranes (Gerace and Burke, 1988).

The structure of all intermediate filament proteins is organized around a central rod domain, also termed α-helical coiled-coil dimerization domain, with four coiled-coil sub-domains (1a, 1b, 2a and 2b) separated by non α-helical flexible linker regions, and flanked by globular head and tail domains (reviewed by Hutchison, 2002). Contrary to cytoplasmic intermediate filament proteins, nuclear lamins have a shortened head domain, an additional 42 residues (six heptad repeats) in the second coiled-coil domain (Fisher *et al.*, 1986; McKeon *et al.*, 1986), and a nuclear-localization signal (NLS) in the tail domain (Frangioni and Neel, 1993). The C-terminal end of all the B-type lamins and lamin A also harbors a CaaX (C, cysteine; a, any aliphatic amino acid; X, any amino acid) box which is a target for post-translational modifications. The first modification, which is necessary for association with the INM, is a farnesylation of the cysteine residue. Following membrane localization, the three terminal amino acids are removed by proteolysis, and the cysteine is carboxymethylated. Lamin A undergoes an additional step of endoproteolysis of its 15 C-terminal residues, including the modified cysteine, before yielding the mature lamin A form. Lamin C on the other hand does not undergo these modifications on its shorter C-terminus, and its integration in the nuclear lamina is dependent upon lamin A (reviewed in Moir *et al.*, 1995).

Lamins are thought to be involved in chromatin organization. In cell-free systems, lamins A and C associate with isolated chromosomes in the absence of lamin B and membranes (Burke, 1990). The C-terminal domain of lamin Dm_0 (the *Drosophila* B-type lamin) binds to histones H2A and H2B in *Drosophila* extracts (Goldberg *et al.*, 1999). Lamins are present in the nucleoplasm (Bridger *et al.*, 1993), supporting the theory that lamins are implicated in chromatin organization. During interphase of mammalian cells, lamin B co-localizes with sites of DNA replication labeled by bromodeoxyuridine incorporation and recognized by the proliferating cell nuclear antigen (PCNA; Moir *et al.*, 1994). Lamins A/C were not found to co-localize with these sites, but are nonetheless present in foci inside the nucleus (Goldman *et al.*, 1992).

Nuclear lamins offer structural support for the nucleus. As seen in live cells expressing GFP-lamin B1, the nucleus undergoes constant deformation, but the original shape is restored almost immediately (Daigle *et al.*, 2001). The idea that the mechanical resistance of the nucleus is due to the lamina is supported by studies conducted in *lmn-1* knock-down *Caenorhabditis elegans*, in which any deformation of the NE during a cell cycle is maintained until the next division (Liu *et al.*, 2000). Furthermore, lamins A/C also bind *in vivo* with the nuclear and cytoplasmic forms of actin in differentiating myoblasts (Lattanzi *et al.*, 2003).

The nuclear lamina is involved in the positioning of NPCs. Lamina filaments are seen to interact with the nuclear rings of the NPCs and to interconnect adjacent NPCs (Aebi *et al.*, 1986; Zhang *et al.*, 1996). When lamin expression is knocked down by RNA interference (RNAi) in *C. elegans*, the NPCs move around laterally in the NE before clustering together (Liu *et al.*, 2000). In normal interphase cells, NPCs are evenly spaced and only move slowly and synchronously as large arrays, with the lamina exhibiting identical movements (Daigle *et al.*, 2001).

A-type lamins, and their binding partners, have been associated with a number of genetic disorders called laminopathies. The best known of these diseases are the autosomal dominant form of Emery-Dreifuss muscular dystrophy (EDMD; Bonne *et al.*, 1999), familial partial lipodystrophy (FPLD) and Charcot-Marie-Tooth (CMT) disorders. They are characterized by abnormalities in particular tissues, for example muscle wasting in EDMD, while all other tissues seem to be unaffected by the mutant or missing protein. There are two hypotheses as to why certain mutations of NE proteins present in all cells give rise to tissue-specific diseases (reviewed by Moir and Spann, 2001). The 'structural hypothesis' proposes that the mutations weaken the lamina, resulting in a fragile NE that breaks in the muscles of affected individuals, leading to cell death and tissue degeneration. In unaffected people, the nucleoplasmic skeleton functions normally and helps protect muscle from mechanical stress. The 'gene-expression hypothesis' states that the tissue-specific changes in gene expression associated with some mutations promote disease, possibly by the lack of interaction with specific transcription factors required to maintain cellular integrity (Ostlund *et al.*, 1999), or by disrupting heterochromatin attachment to the NE in cells lacking lamins A/C (Wilson *et al.*, 2001).

3.2. Non-lamin components

Additional peripheral proteins associated with the INM have been discovered. Otefin has been identified as a 45 kDa *Drosophila* peripheral protein of the INM whose association with the NE is stronger than that of lamins, but it has yet to be found in mammals (Harel *et al.*, 1989; Padan *et al.*, 1990; Ashery-Padan *et al.*, 1997a). The hydrophilic NH_2-terminal domain of otefin interacts with the rod domain of lamins (Goldberg *et al.*, 1998) and reduction of lamin Dm_0 expression significantly alters the distribution of otefin (Wagner *et al.*, 2004). This protein is necessary for the attachment of membrane vesicles to chromatin during nuclear assembly *in vitro* (Ashery-Padan *et al.*, 1997b; Ulitzur *et al.*, 1997). However, down-regulation of otefin by RNAi has no influence on the viability of *Drosophila* Kc167 cells (Wagner *et al.*, 2004). Otefin is also a phospho-protein *in vivo*, possessing a major site for phosphorylation by cdc2 kinase and cyclin AMP-dependent protein kinase at serine 36 (Ashery-Padan *et al.*, 1997b).

Young arrest (YA), a developmentally regulated, cell-cycle dependent protein of the nuclear lamina, has been found in *Drosophila* embryos. The phenotype of null mutation for the *fs(1)Ya* gene (which codes for YA), arresting development shortly after fertilization, indicates that this protein is essential for the initiation of embryonic cleavage divisions (Lin and Wolfner, 1991). YA is localized to the NE, mirroring the distribution of lamins (Lopez *et al.*, 1994). The yeast two-hybrid system showed that both the rod and tail, plus part of the head domain of lamin Dm_0, are necessary for interaction with YA, as well as for its proper localization (Goldberg *et al.*, 1998). The hydrophilic C-terminal lamin-binding domain of YA, more precisely residues 556-696, is sufficient for both lamin binding and nuclear periphery targeting (Mani *et al.*, 2003).

Circumferin, also called the P1 antigen or peripherin, was detected with monoclonal antibodies produced by immunizing mice with nuclear matrices isolated from lymphocytes (Chaly *et al.*, 1984). This antigen is found at the nuclear rim of interphase cells, but coats the surface of chromosomes during mitosis (Chaly *et al.*, 1984; Schatten *et al.*, 1985). The P1 epitope is highly conserved, and the labeling pattern can be found in mammals, *Drosophila*, sea urchin, cells of the higher plant *Vicia faba*, and in organisms with a closed mitosis (where the NE does not disassemble during mitosis) such as the unicellular alga *Polymella* and the yeast *S. cerevisiae* (reviewed by Chaly and

Stochaj, 1999). Extraction experiments suggested that circumferin is associated with chromatin as well as NE components, and that the association depends partially on disulfide bonds (Chaly *et al.*, 1985).

Statin, a 57 kDa protein, was discovered as a nuclear protein present in aged cultures of human fibroblasts but absent from young, proliferating cultures (Wang, 1985a). Expression of statin can be induced in young cultures by proliferation arrest (Wang, 1985a) whereas reinitiation of proliferation causes its disappearance (Wang, 1985b; Wang and Lin 1986). The function of statin is still unknown. However, evidence that it coprecipitates with p45 serine/threonine kinase in a complex with the retinoblastoma (RB) protein, a cell cycle inhibitor in its unphosphorylated form, gives a hint. It has been proposed that statin maintains the nonproliferative state by interacting with p45 kinase, which prevents the phosphorylation of RB proteins (Lee *et al.*, 1992; Wang *et al.*, 1994).

3.3. Do plants and fungi have nuclear lamins?

Lamins are essential to the viability of animal cells (Steen and Collas, 2001), which would lead to the assumption that they are also necessary in plants and fungi. There have been some reports of lamin-like proteins in different plant species, but the results are inconsistent. A purification method for animal lamins adapted for pea nuclei yielded four proteins between 49 and 66 kDa, which is in the size range for lamins (McNulty and Saunders, 1992). These are recognized by antibodies directed against animal intermediate filament proteins, and by antibodies against lamin B. However, immunofluorescence shows their localization to be in the nucleoplasm, and not predominantly at the nuclear rim (McNulty and Saunders, 1992; Beven *et al.*, 1991). Similar results were obtained with intermediate filament antibodies (Minguez and Moreno Diaz de la Espina, 1993; Frederick *et al.*, 1992). Still, these promising results have not been supported by molecular evidence. Searches of publicly available plant and yeast genomes did not reveal any direct orthologues of lamins (Meier, 2000; Georgatos *et al.*, 1989; Mewes *et al.*, 1998). This would imply that plants do not require lamins, but it seems more likely that non-animal eukaryotes possess a distinct set of NE proteins that replace lamins functionally.

4. Proteins of the inner nuclear membrane

The number of known proteins specifically associated with the INM is growing, especially due to the interest in such proteins as causes of human genetic diseases. They all possess at least one transmembrane domain, and are associated with lamins and/or DNA. Because of these associations, they are believed to be crucial in chromatin organization and in the processes of nuclear assembly and disassembly.

4.1. Lamin B receptor

The first integral INM protein to be discovered was the so-called lamin B receptor (LBR), also termed p58, a protein of 637 amino acid residues in chicken, which binds to B-type lamins and chromatin proteins (Worman *et al.*, 1988; Worman *et al.*, 1990). LBR forms a complex with heterochromatin protein 1 (HP1) and two of the core histones, namely H3/H4 (Polioudaki *et al.*, 2001). The nucleoplasmic N-terminal extremity is organized into two globular domains separated by a hinge region. This section is followed by eight transmembrane segments and a hydrophilic C-terminal tail (Smith and Blobel, 1993). It seems to be an essential protein in mitosis, since antibodies against LBR block nuclear assembly in sea urchin egg extract (Collas *et al.*, 1996). The hydrophobic domain of LBR shares extensive structural similarities with sterol reductase enzymes. The protein even possesses sterol C14 reductase activity when expressed in *Saccharomyces cerevisiae* strains defective in this enzyme (Silve *et al.*, 1998).

4.2. Lamina-associated polypeptides

Lamina-associated polypeptides (LAPs) are proteins that, as their name implies, are associated with the nuclear lamina. The LAP1 group of three related type II integral membrane proteins, LAP1A, LAP1B and LAP1C, has been identified by a single monoclonal antibody against the INM (Senior and Gerace, 1988). LAP1C, the only isoform to have been sequenced, consists of 506 amino acids and possesses a single membrane spanning region (Martin *et al.*, 1995). *In vitro* experiments show that LAP1A and LAP1B bind to lamins A/C and lamin B1, whereas LAP1C does not have a lamin-binding affinity *in vitro* (Foisner and Gerace, 1993). However, it has been reported that LAP1C is mobile in undifferentiated cells that do not express A-type lamins, but becomes

stably anchored to the NE when lamins A/C are expressed, indicating some sort of interaction between LAP1C and lamins A/C (Powell and Burke, 1990). LAP1 proteins also form *in vivo* complexes with B-type lamins (Maison *et al.*, 1997).

LAP2s, also known as thymopoietins (Harris *et al.*, 1995), were identified by another monoclonal antibody (Foisner and Gerace, 1993; Furukawa *et al.*, 1995) and comprise many isoforms encoded by the LAP2 gene, which are unrelated to the LAP1 protein family. The best known are α, β and γ. Additional isoforms are found in mouse but not in human cells: β', ε, δ and ζ (Berger *et al.*, 1996). Except for LAP2α and LAP2ζ, all mammalian LAP2 isoforms are type II integral proteins of the INM, and possess a closely related N-terminal nucleoplasmic domain of variable length, a single membrane spanning region and a short lumenal domain at the C-terminus (reviewed by Dechat *et al.*, 2000a). LAP2β has the longest nucleoplasmic domain of the isoforms with 408 residues. LAP2α is unique among the isoforms by its structure and function. It shares only the 187 residue N-terminal domain common to all LAPs, and contains a unique 506 residue C-terminal domain with no transmembrane region (reviewed by Vlcek *et al.*, 2001). All LAP2 isoforms bind to chromatin by themselves (Cai *et al.*, 2001) or through chromosomal protein barrier to autointegration factor (BAF; Worman and Courvalin, 2000). LAP2β and probably LAP2γ bind to lamin B1 (Foisner and Gerace, 1993), while LAP2α, the nucleoplasmic isoform, interacts with intranuclear A-type lamins (Dechat *et al.*, 2000b). The DNA interactions of LAP2 suggest that these proteins are involved in gene expression. LAP2β is capable of reducing the transcriptional activity of the E2F-DP complex, a transcription factor, either by itself or through its binding partner germ-cell-less (GCL), which is localized at the NE (Nili *et al.*, 2001).

4.3. Emerin

Emerin, first identified as a mutated protein in X-linked EDMD (Bione *et al.*, 1994), is located at the inner nuclear membrane. Emerin binds lamin A, via its central region, as well as BAF via its amino terminus (Lee *et al.*, 2001; Shimi *et al.*, 2003). The interaction of emerin with BAF is necessary for the incorporation of the protein into the reforming NE (Haraguchi *et al.*, 2001). Moreover, A-type lamins are necessary to the

proper localization of emerin (Sullivan *et al.*, 1999). The transcriptional repressor GCL also competes for binding of emerin with BAF (Holaska *et al.*, 2003). Hence emerin forms stable complexes with either lamin A plus GCL or lamin A plus BAF. Emerin can also bind to actin, both nuclear and cytoplasmic forms, during the late stages of myotube differentiation and in mature muscle (Lattanzi *et al.*, 2003). Emerin is deficient in the nuclear membrane of patients with X-linked EDMD, suggesting that it is an essential protein in humans (Nagano *et al.*, 1996). Some mutations related to diseases have been mapped in the lamin-binding domain, disrupting lamin A binding *in vitro*, while no mutation has been found in the BAF-binding domain (Lee *et al.*, 2001). Cells transfected with disease specific emerin mutants show mislocation of emerin and lamins A/C. The amount of emerin mislocation is associated with alterations in the NE morphology, as well as with longer cell-cycle times (Fairley *et al.*, 2002).

4.4. MAN

The "MAN antigens" are three polypeptides specifically recognized by autoantibodies found in a patient with an ill-defined collagen vascular disease. These polypeptides are located exclusively at the NE and co-partition biochemically with nuclear lamins during *in situ* isolation of nuclear matrices (Paulin-Levasseur *et al.*, 1996). One of the antigens has been identified as LAP2β (Lang *et al.*, 1999). Another has been characterized as an 82.3 kDa integral protein of the inner nuclear membrane termed MAN1 (Lin *et al.*, 2000). Homologues of MAN1 have been found in *C. elegans* (Lee *et al.*, 2000) and in *Xenopus* (XMAN1; Osada *et al.*, 2003). The remaining 40 kDa "MAN antigen" has yet to be characterized. Loss of function mutations in the MAN1 gene *LEMD3* have recently been found to cause osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis, disorders characterized by increased bone density (Hellemans *et al.*, 2004). There is also recent evidence that MAN1 plays a role as a regulator of R-Smad proteins, which are critical intracellular mediators of transforming growth factor-β (TGFβ), bone morphogenic proteins (BMPs) and activin signaling. MAN1 interacts with Smads through its RNA recognition motif in the C-terminus, whose function is still unclear (Pan *et al.*, 2005).

MAN1 shares a common feature with LBR, LAP1, LAP2 and emerin: they all contain nucleoplasmic, amino-terminal domains that bind to lamins or chromatin (Worman and Courvalin, 2000). MAN1 also shares with LAP2 and emerin a region of about 40 residues called the LEM (LAP2-emerin-MAN1) motif, which defines them as a family (Lin *et al.*, 2000). Other family members include otefin (Wagner *et al.*, 2004), Lem-3 (Lee and Wilson, 2004; Lee *et al.*, 2000), and two protein isoforms recently discovered in *Drosophila* from the CG9424 gene termed Bocksbeutel-α (which possesses a transmembrane domain) and Bocksbeutel-β (localized predominantly in the nucleoplasm; Wagner *et al.*, 2004). Mounting evidence suggests that the MAN antiserum is specific to the LEM domain, hence recognizing an entire family of proteins (Lin *et al.*, 2000). Nuclear magnetic resonance and molecular modeling shows that the three-dimensional structure of the LEM motif is of two large parallel α helices (Laguri *et al.*, 2001).

The LEM domain of LAP2β, MAN1 and emerin binds BAF (Cai *et al.*, 2001), a small protein with a dimer mass of 20 kDa highly conserved among metazoans (Cai *et al.*, 1998). BAF recruits nuclear proteins to chromatin during nuclear assembly of mammalian cells, and this recruitment is essential to the localization of emerin, LAP2β and A-type lamins at the reforming NE (Haraguchi *et al.*, 2001). BAF is dynamic and mobile during interphase, contrary to integral INM proteins, as shown by fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analyses in living human cells (Shimi *et al.*, 2003). The constant N-terminal region of LAP2 also contains an additional LEM-like motif that binds DNA directly (Cai *et al.*, 2001).

4.5. Other inner nuclear membrane proteins

Nurim (nuclear rim), discovered by visual screening of a GFP-fusion library, is a 29 kDa inner nuclear membrane protein very tightly associated with the NE. Analogous to nuclear matrix constituents like lamins, it resists extraction with 1% Triton X-100 (a detergent) and high salts (Rolls *et al.*, 1999). It is only released in significant quantities with 4 M urea extraction, conditions under which most INM proteins and even lamins A/C are completely extracted (Hofemeister and O'Hare, 2005). Unlike other INM

proteins, nurim is devoid of a large hydrophilic N-terminal domain, containing just four or five residues before its first transmembrane segment. The N-terminus of all other INM proteins analyzed to date contains domains necessary to their nuclear retention and targeting. However, many regions of nurim seem to be necessary for its nuclear rim localization, which may indicate an unusual targeting mechanism for this protein (Rolls *et al.*, 1999). Containing six transmembrane domains with short intervening loops, it has both ends on the nucleoplasmic side of the INM. Nurim has striking sequence similarities with a class of enzymes, isoprenylcysteine carboxymethyltransferases (ICMTs). These enzymes are involved in the processing of proteins containing a CaaX motif at their C-terminus, like nuclear lamins, which would indicate an enzymatic activity at the INM for nurim (Hofemeister and O'Hare, 2005).

Nesprins (NE spectrin repeats) are a family of type II integral membrane proteins localized at the nuclear membrane and expressed upon early muscle differentiation. Two genes, coding for nesprins 1 and 2, give rise to a number of differentially spliced isoforms. They are characterized by multiple, clustered spectrin repeats, bipartite nuclear localization sequences and a conserved C-terminal single transmembrane domain (Zhang *et al.*, 2001). Nesprin-1, also known as myne-1 (myocyte nuclear envelope; Mislow *et al.*, 2002a; Mislow *et al.*, 2002b), colocalizes with LAP1, emerin and lamins at the NE and with heterochromatin inside the nucleus (Zhang *et al.*, 2001). The smaller of the eight major isoforms of nesprin-2 identified to date co-localizes and binds with lamin A and emerin, while the larger isoforms co-localize with heterochromatin (Zhang *et al.*, 2004).

4.6. Pore membrane proteins

Pore membranes are distinct domains from the INM and ONM. The only known integral membrane proteins of pore domains are gp210 (Gerace *et al.*, 1982; Wozniak *et al.*, 1989) and POM121 (Hallberg *et al.*, 1993). These proteins link the nuclear membrane to the nuclear pore complexes, which actively control the passage of macromolecules to and from the nucleus (reviewed in Vlcek *et al.*, 2001).

5. Network of nuclear envelope protein interactions

A unifying concept is beginning to emerge suggesting that NE proteins interact with multiple partners, forming a network of attachments between the NE and chromatin (Fig. 1). Lamins can interact with LAP1 (Foisner and Gerace, 1993), LAP2 (Foisner and Gerace, 1993; Dechat *et al.*, 2000b), LBR (Worman *et al.*, 1990), emerin (Lee *et al.*, 2001), MAN1 (Worman and Courvalin, 2000), otefin (Goldberg *et al.*, 1998), and YA (Goldberg *et al.*, 1998). Chromatin can interact with LAP2 (Worman and Courvalin, 2000), emerin (Haraguchi *et al.*, 2001) and MAN1 (Foisner and Gerace, 1993) through BAF, with LBR through HP1 (Polioudaki *et al.*, 2001), and with lamins through histones. Emerin and lamins also bind to nuclear actin (Lattanzi *et al.*, 2003). NE proteins are even found to interact with transcriptional factors: emerin (Holaska *et al.*, 2003) and LAP2β (Nili *et al.*, 2001) with GCL, and LAP2β with E2F (Nili *et al.*, 2001). They are also involved with cell cycle regulators: LAP2α, lamin A and statin all interact with the RB protein, a key regulator of the cell cycle and differentiation (Vlcek *et al.*, 2002; Markiewicz *et al.*, 2002; Lee *et al.*, 1992). The first INM protein shown to be part of a larger protein complex is LBR; this complex contains several components, including an LBR-kinase (RS-kinase), lamin B and a small integral membrane protein termed p18 (Simos *et al.*, 1996).

6. Retention at the nuclear envelope

The favored view on localization of integral membrane proteins to the INM is by a 'diffusion-retention' mechanism (Soullam and Worman, 1995). After synthesis and insertion into ER membranes, they diffuse laterally from the membrane of the ER to the ONM, then through the nuclear pore membrane, and finally to the inner nuclear membrane. The integral membrane proteins are therein retained, apparently through selective interactions with other resident nuclear proteins. Supporting this model are FRAP experiments with green fluorescent protein (GFP) tagged LBR proteins. These show that LBR-GFP diffuses rapidly and freely within ER membranes, but that it is essentially immobilized when it reaches the inner nuclear membrane (Ellenberg *et al.*, 1997). Moreover, a segment of the MAN1 protein containing the N-terminus (responsible for DNA and lamin binding) and the first transmembrane domain localizes

to the nuclear rim, whereas a segment with both transmembrane domains and the C-terminus fails to be targeted to the INM (Wu *et al.*, 2002).

7. Nuclear disassembly and reassembly

Mitosis in higher eukaryotes involves complete disassembly and reassembly of the nucleus, referred to as "open mitosis". Disassembly occurs in three main independent steps (Newport and Spann, 1987): chromosome condensation, nuclear lamina depolymerization and NE breakdown (NEBD), this last step defining the end of prophase and the beginning of prometaphase. Disassembly of the nuclear lamina correlates with its hyperphosphorylation, elicited by the cyclin B1-$p34^{cdc2}$ kinase complex (CDK1), previously known as the M-phase promoting factor (MPF), and by protein kinase C (PKC) (Peter *et al.*, 1990; reviewed in Buendia *et al.*, 2001). Phosphorylation is the only detectable charge-altering modification of lamins specifically happening during mitosis (Ottaviano and Gerace, 1985). A number of integral and peripheral NE proteins, including LBR, LAP2β, LAP2α (Dechat *et al.*, 1998) and emerin, are also believed to be phosphorylated at prophase by kinase $p34^{cdc2}$. Phosphorylation is thought to abolish the structural protein-protein integrity of the NE, promoting membrane release from chromatin. The discovery that phosphorylation by $p34^{cdc2}$ leads to disassembly of the nuclear lamina suggested that NEBD is mostly a 'cataclysmic' process (reviewed by Nigg, 1992). It was at the time thought that disassembly of the lamina suddenly left the NE without support and its integrity simply collapsed. However, the more recent evidence that lamins A and B dissociate at different times from the NE (Georgatos *et al.*, 1997), which is incompatible with a 'cataclysmic' event, points to an orchestrated chain of events leading to complete NEBD. Disassembly of NPCs also seems to be an important step in nuclear disassembly, leading to a fenestration of the NE prior to NEBD (Collas, 1998; Terasaki *et al.*, 2001).

Microtubules affect NE structure. At the onset of prophase, the microtubules cause two symmetrical indentations on antidiametric sites of the NE where the centrosomes are located (Pawaletz and Lang, 1988). Microtubule bundles are also found deep within NE invaginations (Georgatos *et al.*, 1997). They have been shown to facilitate NEBD by literally tearing the NE open (reviewed by Burke and Ellenberg,

2002). Amazingly, NEBD and fragmentation of the nuclear lamina can still occur in the absence of microtubules, as in nocodazole-treated cells, or cell-free systems. However, the mode of nuclear disassembly of nocodazole-treated cells differs significantly from normal ones. The fragmentation pattern of lamins B is more rapid, and no nuclear indentations are present (Georgatos *et al.*, 1997). It also seems that soluble tubulin regulates binding of HP1, a gene regulator, to the NE. Addition of excess soluble tubulin to an *in vitro* reassembly system abolishes recruitment of LAP2β and lamin B to the surface of chromosomes (Kourmouli *et al.*, 2001).

As mitosis progresses, reassembly of the nucleus is characterized by dephosphorylation by phosphatases (reviewed by Earnshaw and Pluta, 1994). Assembly of the NE is also a stepwise process which starts in anaphase and is completed in early G1. Proteins start being targeted to chromosomes in anaphase A and B. LAP2α associates with chromatin very early in nuclear reassembly, before most of the lamins (Dechat *et al.*, 1998) and membrane-bound LAP2β (Vlcek *et al.*, 1999). Amongst the first markers to be targeted to chromosomes, LBR and LAP2β are associated with the lateral margins of chromosomes that emerge from the mitotic apparatus. Emerin, LAP2α and BAF are targeted to specific chromosome surfaces related to the mitotic apparatus, while the nucleoporin Nup153 attaches to all surfaces of chromosomes (reviewed by Buendia *et al.*, 2001). As nuclear vesicles attach to chromosomes, they must fuse to enclose them. Nuclear pore complexes also resume their location sequentially, and the nuclear lamina continues to assemble, resulting in a functional nucleus. Lamin A associates only with the nucleus after pore complexes are assembled. Starting as a soluble form in the nucleus, it gradually becomes incorporated in the nuclear lamina in early G1 and throughout the first few hours of this cell cycle stage (Moir *et al.*, 2000). After the nucleus is enclosed, it enlarges as the NE and lamina grow to let the chromatin decondense and permit formation of intranuclear structures, such as the nucleolus (Gant and Wilson, 1997).

Many nuclear proteins have been shown to be essential in nuclear assembly. Experiments in *Xenopus* cell-free systems with truncated LAP2β proteins indicate that lamina assembly and membrane-chromatin attachment may be mediated by LAP2

proteins (Gant *et al.*, 1999). More specifically, LAP2α fragments block assembly of nuclear membranes and lamins A/C *in vitro* (Vlcek *et al.*, 2002).

There are two apparently mutually exclusive models of NE breakdown and assembly. The first model suggests that the NE is fragmented into vesicles during mitosis, giving rise to NE-derived vesicles distinct from ER (Vigers and Lohka, 1991; Chaudary and Courvalin, 1993; Buendia and Courvalin, 1997). This is based mostly on the isolation of vesicles by immunoprecipitation. In *Xenopus* extracts, vesicles containing lamin B3 (the major B-type lamin found in *Xenopus* egg; Lourim *et al.*, 1996) bind to chromatin and fuse together. Inhibition of lamin B3 blocks this association, subsequently blocking nuclear assembly. However, another vesicle population lacks lamin B3, and binds to chromatin but does not fuse (Lopez-Soler *et al.*, 2001). The second model, based on live cell experiments (Ellenberg *et al.*, 1997; Yang *et al.*, 1997), suggests that there is no vesiculation of the NE, and that proteins of the nuclear membranes re-localize by diffusion to the continuous ER. Segregated vesicles are thought to be an artifact of the homogenization of mitotic cells. It has yet to be concluded if either or a combination of both these models (Cotter *et al.*, 1998) is the right scenario.

8. Cell-free systems

In vitro systems of nuclear disassembly and assembly are especially attractive for structural and biochemical studies of mitosis. Many teams have conducted experiments using a cell-free system to study nuclear assembly. A definitive model is starting to emerge from all this evidence, gathered from a few key species. In contrast, only a limited number of cell-free disassembly studies have been done. This may be because it is more difficult to study the function of proteins in a system that is breaking down, than in a structure that is forming. Cell-free extracts for nuclear disassembly studies have been isolated from *Spisula* (clam) oocytes (Dessev *et al.*, 1989), sea urchin eggs (Collas, 1998), *Drosophila* (Maus *et al.*, 1995), *Xenopus laevis* eggs (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Newport and Spann, 1987), chicken hepatoma cells (Nakagawa *et al.*, 1989), and even mammalian cells like Chinese hamster ovary (CHO; Suprynowicz and Gerace, 1986) and HeLa (Collas *et al.*, 1999; Martins *et al.*, 2000).

The most common cell-free system takes advantage of cellular mitotic extracts of *Xenopus laevis* (Lohka and Masui, 1983; Newport and Spann, 1987). Although strictly speaking it is a meiotic system, its widespread use can be explained by its many advantages over mammalian somatic cell systems (Burke, 1998). The most obvious are the low cost, and short period of time for harvesting. Furthermore, *Xenopus* extracts efficiently package naked DNA, and NE precursors are not limiting. However, because this system is amphibian and embryonic, there are major differences from what happens in somatic mammalian cells. Somatic cells have limited NE precursors, and some proteins involved in nuclear disassembly and assembly have isoforms that are differentially expressed during development. It is therefore important to use systems of nuclear assembly/disassembly prepared from mammalian cells.

There have been a limited number of studies done with mammalian cell-free systems. Suprynowicz and Gerace (1986) disassembled nuclei isolated from CHO cells using a postribosomal supernatant from synchronized metaphase CHO. They observed prophase/prometaphase-like changes in the isolated nuclei, including chromosome condensation, NEBD and lamina disassembly. More recently, cell-free nuclear disassembly experiments have been executed with human cells (Collas *et al.*, 1999; Martins *et al.*, 2000). Using a HeLa cell-free system, the function of some nuclear proteins were blocked by introducing antibodies in the permeabilized isolated nuclei affecting disassembly (Collas *et al.*, 1999). Cell-free assembly systems were developed as total homogenates of metaphase CHO cells incubated in an appropriate physiological buffer (Burke, 1998; Burke and Gerace, 1986). As time passes during these assays, MPF is inactivated and nuclear envelopes reform around chromosome clusters. The function of NE proteins in assembly can be blocked by adding specific antibodies to the homogenate (Burke and Gerace, 1986). A novel *in vitro* assembly assay was developed by Kourmouli and colleagues (2001) where nocodazole-synchronized HeLa cells are pre-incubated in low concentrations of digitonin to permeabilize the plasma membrane, allowing the addition of exogenous elements to the system.

9. New objectives

I first intended to study the involvement of integral and peripheral INM proteins in nuclear disassembly and assembly using mammalian cell-free systems. These systems seemed quite promising, but after trying out existing methods as well as modifying them, they proved to be unreliable. Many difficulties were encountered in working out efficient assays. For disassembly, I was unable to obtain a functioning system using published protocols (Collas *et al.*, 1999). Reactions following the original protocol were attempted many times and varied in many ways, but to no avail. As for assembly, mitotic cell homogenates and digitonin permeabilized mitotic cells turned out to give questionable results and generally incomplete nuclear membrane assembly. Hence, this thesis essentially conveys a critical assessment of mammalian cell-free systems of nuclear disassembly and assembly, based on experimental evidence.

II. Materials and Methods

1. Materials

General biochemical and chemical supplies were purchased from BDH (Ville St-Laurent, Québec, Canada) and Sigma (St Louis, MO).

2. Cell culture

HeLa cells (CCL 2) and Fanconi's anemia cells (CRL 1196) were obtained from the American Type Culture Collection (ATCC; Manassas, VA), while CHO cells were kindly provided by Dr. Marc Ekker (Department of Biology, University of Ottawa, Ottawa, Ontario, Canada). Fanconi's anemia and HeLa cells were cultured in Eagle's minimum essential medium (MEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Gibco BRL) while CHO cells were grown in Dulbecco's modified Eagle medium (D-MEM; Gibco BRL) supplemented with FBS and antibiotics. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

3. Cell synchronization and harvesting

Cells were grown up to 90% confluence (plated at a density of 1.5 x 10^4/cm^2 and grown for 48 h, or at 7.5 x 10^3/cm^2 and grown for 72 h), and either left untreated and asynchronous, or treated for 18 h with nocodazole (1 μM from a 3.3 mM stock in dimethyl sulfoxide; DMSO; Sigma) to arrest cells in mitosis. Untreated cells were washed in cell culture phosphate buffered saline (cPBS; 137 mM NaCl, pH 7.0, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl) and detached in trypsin / ethylenediamine-tetra-acetic acid (EDTA; 0.025% trypsin, pH 7.4, 1 mM EDTA, in cPBS), while cells synchronized with nocodazole were collected by the shake-off technique (Tobey *et al.*, 1967). Subsequently, cells were recovered by centrifugation at 500 g for 6 min.

To obtain cells synchronized in G2, a double thymidine block was applied. The method was adapted for HeLa cells from Stein *et al.* (1994). Cells were seeded at a density of 1.5 x 10^4/cm^2 and grown for 24 h. The growth medium was then replaced with fresh medium containing 2 mM thymidine (from a 100 mM stock in serum-free medium).

After 12 hours, the thymidine-containing medium was removed. Cells were washed twice with an equal volume of serum-free medium and covered with complete medium containing 24 μM deoxycytidine for 9 hours to release them from the first block. Thymidine was then added to a final concentration of 2 mM. After 12 hours, the cells were washed twice with an equal volume of serum-free medium, and covered with complete medium containing 24 μM deoxycytidine for another 12 hours to release them from the second block. Cells were subsequently detached with trypsin / EDTA and harvested by centrifugation.

Serum deprivation (Sayed *et al.*, 2001) was also used to obtain G2-synchronized HeLa cells. HeLa cells were grown for 24h at a density of 5 x $10^4/cm^2$ in complete medium. Cells were washed twice with cPBS and cultured for 30h in medium containing 0.5% FBS to synchronize them in G0 phase. The medium was replaced with fresh medium supplemented with 10% FBS to induce the cells to re-enter the cell cycle. After 19 hours, released cells were collected by detaching them with trypsin / EDTA and centrifugating them.

4. Disassembly systems

4.1 Mitotic cell extracts for disassembly assays

Cell extracts were obtained as previously described (Collas *et al.*, 1999). Mitotic HeLa cells were washed in ice-cold phosphate buffered saline (PBS; 130 mM NaCl, pH 7.0, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and centrifuged at 500 g. The pellet was washed in 20 volumes of ice-cold lysis buffer (20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 8.2, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol (DTT), 20 μg/ml cytochalasin B, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) and centrifuged at 800 g for 10 min. In some preparations, EDTA was replaced with ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). The pellet was resuspended in 1 volume of lysis buffer and incubated for 30 min on ice. Homogenization was achieved by sonicating (Braun-Sonic 2000 Sonicator) twice for 2 min on ice. The homogenate was centrifuged at 10,000 g for 15 min at 4°C, and then the supernatant was centrifuged at 200,000 g (45,000 rpm) for 3 h at 4°C in a Beckman SW55 Ti rotor with adaptors for 0.8

ml Ultra-Clear tubes. Clear supernatant, later referred to as the soluble mitotic extract, was separated into aliquots and snap-frozen in liquid nitrogen to be stored at –80°C. Both low-speed and high-speed pellets were kept in sample buffer (2% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM Tris-Base pH 6.8, 25 mM mercaptoethanol, 0.005% bromophenol blue) for analysis.

An alternative method was devised from a protocol by Burke (1998). After incubation in lysis buffer, cells were homogenized on ice by 25 strokes of a tight fitting pestle in a dounce homogenizer. The resulting homogenate was centrifuged at 100,000 g (32,500 rpm) in a SW55 Ti Beckman rotor with 0.8 ml tube adaptors at 4°C for 30 min. The supernatant, which is the soluble mitotic extract, was frozen as in the previous method, and the pellet was kept in sample buffer for analysis.

4.2 Preparation of nuclei for disassembly assays

Interphase nuclei were isolated as described by Collas *et al.* (1999). Unsynchronized confluent HeLa cells were harvested, washed in PBS and centrifuged at 400 g. The pellet was resuspended in 20 volumes of ice cold nuclear isolation buffer (buffer N; 10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose, 25 mM KCl, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) containing 10 μg/ml cytochalasin B, and incubated for 30 min on ice. Cells were then homogenized on ice with 150 strokes of a tight fitting glass pestle in a homogenizer. The homogenate was centrifuged at 400 g for 10 min at 4°C. The pellet was recovered, washed twice in buffer N and centrifuged for 10 min at 400 g and 4°C. The recovered nuclei were used fresh in buffer N or frozen at -80°C in buffer N containing 70% glycerol.

An alternative method for isolating interphase nuclei was adapted from Suprynowicz and Gerace (1986). Harvested cells were washed in 40 volumes of isolation buffer (10 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 0.5 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin), centrifuged for 6 min at 1,500 g then resuspended in 3 volumes of isolation buffer and incubated on ice for 30 min. Swollen cells were then homogenized with 50 strokes of a dounce homogenizer. The homogenate was layered over a cushion of 30%

sucrose (w/v) in a buffer (10 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$ and 1 mM DTT) and centrifuged for 2 min at 500 g. Nuclei recovered in the pellet were treated as in the original method.

Digitonin permeabilized cells were also utilized instead of a nuclear suspension. In a protocol adapted from nuclear import assays (Moore and Blobel, 1992), unsynchronized HeLa cells grown on cover-slips were permeabilized by a 5 min exposure on ice to 40 μg/ml digitonin in buffer A* (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin), then washed in buffer A*. Alternatively, in a protocol for CHO by Wu and colleagues (1997), suspension cells recovered by exposure to trypsin/EDTA (as described above in the cell culture section) were used in place of isolated nuclei. Cells were recovered by centrifugation at 500 g for 5 min, washed in ice cold transport buffer (20 mM HEPES, pH 7.3, 110mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA) at a concentration of 5 X 10^6 cells/ml, centrifuged again, and resuspended in transport buffer at a concentration of 1 X 10^7 cells/ml. An equal volume of transport buffer containing 80 μg/ml digitonin was added and the cells were incubated 5 min on ice. An equal volume of 3% bovine serum albumin (BSA) in transport buffer was then added to the mix to quench the remaining digitonin. The digitonin ghosts were recovered by centrifugation and resuspended in buffer N. These were treated as isolated nuclei from this point on.

4.3 Loading of nuclei with antibodies

According to the method of Collas *et al.* (1999), purified nuclei (2000/μl) were permeabilized in 500 μl of buffer N containing 0.75 μg/ml lysolecithin (Sigma) for 15 min at room temperature. Excess lysolecithin was inactivated by adding 1 ml of 3% BSA in buffer N for 5 min on ice. Nuclei were sedimented at 400 g for 10 min, washed once in buffer N and sedimented again. Nuclei were resuspended in 100μl of buffer N containing antibodies and incubated on ice for 1 h with gentle agitation. Nuclei were sedimented at 500 g through 1 M sucrose for 20 min and held in buffer N on ice until use.

4.4 Nuclear disassembly

The following method was taken from Collas *et al.* (1999). A reaction consisted of 20 µl soluble mitotic extract, 1 µl nuclear suspension (~2 x 10^4 nuclei) and 0.6 µl ATP-generating system (1 mM ATP, 10 mM creatine phosphate, and 25 µg/ml creatine kinase), which initiated the reaction. In the case of digitonin permeabilized cells grown on cover-slips, these were inversed on a drop of the incubation mixture (consisting of 20 µl soluble mitotic extract and 0.6 µl of the ATP-generating system described above) deposited on parafilm in a humid chamber. The reaction proceeded at 30°C for up to 2 h. Chromatin condensation was monitored by staining with 0.1 µg/ml Hoechst 33258.

5. Assembly systems

5.1 Mitotic homogenate for nuclear assembly

The procedure was adapted from Burke (1998). Mitotic cells were incubated in 100 volumes of complete medium containing 1 µM nocodazole and 20 µM cytochalasin B for 30 min at 37°C. Cells were harvested by centrifugation at 1500 g for 6 min and washed twice in 50 volumes of ice-cold PBS. Cells were then washed in 1.5 ml of ice-cold KHM (78 mM KCl, 50 mM HEPES-KOH, pH 7, 4 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT, 20 µM cytochalasin B) and centrifuged at 1000 g for 5 min at 4°C. Mitotic cells were resuspended in 1 volume of KHM and homogenized on ice with 10-25 strokes of a tight fitting pestle in a 1 ml dounce homogenizer (aiming for 95% cell breakage).

Crude homogenate was used fresh by being diluted twofold with KHM and incubated for up to 2h at 37°C to study nuclear reassembly. Alternatively, to obtain a post-chromosomal supernatant containing mitotic membranes and cytoplasm, the homogenate was centrifuged at 1000 g for 5 min at 4°C to pellet chromatin, nuclei and unbroken cells. This supernatant was used fresh or kept at -80°C. In some experiments, 1 volume of post-chromosomal supernatant was added to the crude homogenate prior to reassembly.

5.2 Digitonin system of nuclear assembly

The procedure was adapted from Kourmouli and colleagues (2000). Mitotic cells were quickly washed three times in ice-cold piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM PMSF), centrifuged for 30 sec at 1,500 g and resuspended at a concentration of 10^6 cells/ml PIPES* buffer (PIPES buffer plus 1 mM DTT, 2 µg/ml each leupeptin, pepstatin A, aprotinin and antipain). Cell membranes were permeabilized with 50 µg/ml digitonin (from a 1 mg/ml stock in DMSO) for 5 min on ice. Antibodies were sometimes added to 200 µl aliquots of the digitonin ghosts suspension, and the final volume adjusted to 300 µl with PIPES* buffer. Suspensions were then incubated at 33°C for up to 2h.

6. Cell fractionation for biochemical analysis

The following fractionation procedure was adapted from Chaudhary and Courvalin (1993). Cells were centrifuged at 500 g for 5 min at 4°C. The first supernatant (S1) was kept at –80°C until use. Cells were resuspended in hypotonic buffer A (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 20 µM cytochalasin B, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 2.5 mM sodium pyrophosphate, 0.1 mM orthovanadate, 2 mM sodium fluoride, 5 µM aluminium ammonium sulphate) and left on ice for 15 min. Swelled cells were ruptured by 20 strokes of a tight fitting pestle in a Dounce homogenizer. To separate nuclei or chromatin (P) from the membranes and cytoplasm (S2), the homogenate was layered over a 3 ml cushion of 30% sucrose (w/v) in hypotonic buffer A and centrifuged at 2000 g for 10 min at 4°C in a swinging-bucket rotor (Sorvall HB-4 at 3,700 rpm). Proteins from both supernatants (S1 and S2) were precipitated with 10% trichloroacetic acid (TCA) for 15 min at 40°C, and then recovered by centrifugation at 15,000 g for 10 min in a fixed-angle rotor. These protein samples were washed in 1 ml of 5% TCA, and centrifuged for 10 min at 3,000 g. They were subsequently washed in acetone, and centrifuged again. Finally, the protein pellets were left to dry for 15 min to evaporate acetone residues. Sample buffer was added to obtain equal volumes of each fraction, namely S1, P and S2.

7. Gel electrophoresis and immunoblotting

For total cell homogenates, cells were scraped from culture dishes in Tris-acetate buffer (10 mM Tris-acetate, pH 7.5, 150 mM NaCl, 1 mM EGTA) with a rubber policeman and transferred to conical centrifuge tubes. Cells were harvested by centrifugation at 1,500 g for 6 min and washed twice in the same buffer. For gel electrophoresis, cell pellets were resuspended in SDS-sample buffer to solubilize proteins, and sonicated. Samples were boiled for 5 min before loading. Proteins were then separated by electrophoresis in 5% stacking and 12% resolving SDS-polyacrylamide gels according to the method of Laemmli (1970).

Protein profiles were visualized by Coomassie blue (0.1% (w/v) Coomassie blue R-250, 10% acetic acid, 25% methanol) staining of gels. Otherwise, the separated polypeptides were electrophoretically transferred from gels to nitrocellulose membranes and processed for immunoblotting (Western blot, WB) as recommended by Amersham Canada (Oakville, Ontario, Canada). Briefly, membranes were blocked for 1 hour in milk (5% low-fat powdered milk in PBS with 0.05% Tween) and then incubated for 1 hour in primary antibodies. Detection was performed with the appropriate biotinylated secondary antibody followed by streptavidin-horseradish peroxidase (1:4000), and developed with a chemiluminescence kit (Amersham Canada). Reactivity was visualized on Hyperfilm ECL (Amersham Canada).

8. Indirect immunofluorescence staining

Intact interphase cells were fixed, permeabilized and stained as previously described (Chaly *et al.*, 1984). Briefly, cells attached to coverslips were washed in PBS twice for 30 seconds, and then fixed for 5 min with 3% paraformaldehyde in PBS. They were washed again in PBS, reduced with 0.1% sodium borohydride ($NaBH_4$) in PBS three times for 4 min, and then permeabilized for 20 min in PBS containing 0.2% Triton X-100.

Intact interphase cells were alternatively permeabilized with digitonin. Cells attached to coverslips were washed in buffer A* (20 mM HEPES, pH 7.3; 110 mM potassium acetate; 5 mM sodium acetate; 2 mM magnesium acetate; 1 mM EGTA; 2 mM DTT; 1 μg/ml each leupeptin, aprotinin and pepstatin) twice for 30 seconds, and then

permeabilized for 5 min with 35 μg/ml digitonin (from 10 mg/ml stock in DMSO) in buffer A*. They were washed again in buffer A*, fixed for 15 min with 3% paraformaldehyde in PBS. The cells were washed in PBS, and then reduced with 0.1% sodium borohydride in PBS three times for 4 min.

Cells were sometimes simultaneously fixed and permeabilized in ethanol. Mitotic cells in suspension were centrifuged 1 min at 700 g to remove supernatant, then resuspended in cold 95% ethanol and incubated at -20°C for 15 min. Cells were centrifuged again to remove ethanol, resuspended in PBS and kept on ice until all samples could be centrifuged on coverslips at 2,000 g for 10 min at 4°C (plate rotor of a Hermle Z360K at 3,500 rpm).

Mitotic cells in suspension, nuclei or mitotic chromosomes were also centrifuged for 5 min at 1,500 g, then resuspended in 3% paraformaldehyde in PBS for 5 minutes, centrifuged again, resuspended in PBS and kept on ice until all samples could be centrifuged on coverslips at 2,000 g for 10 min at 4°C (plate rotor of a Hermle Z360K at 3,500 rpm). The material that adhered to coverslips was reduced with 0.1% sodium borohydride in PBS three times for 4 min, then permeabilized for 20 min in 0.2% Triton X-100 in PBS.

Fixed and permeabilized material on coverslips was washed 3 times for 5 min in PBS, incubated for an hour with the primary antibody, washed again 3 times for 5 min in PBS, then incubated for an hour with the secondary antibody. This procedure was repeated if double immunolabelling was done. For some of the experiments, after the secondary antibody incubation, membranes were labeled with 2.5 μg/ml (from a 10 mg/ml stock in ethanol) of the lipid dye 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma-Aldrich) in PBS for 5 min. The cells were then washed in PBS and stained with Hoechst 33258 (1 μg/ml in PBS), and coverslips were put cells side down on a drop of mounting medium (0.1% p-phenylenediamine (PPHD) and 50% glycerol in PBS). Conventional epifluorescence microscopy was carried out on a Zeiss Axiophot and observations were either recorded on Ilford XP2-400 film and the negatives scanned with a CanoScan FS 4000 US (Canon) using Adobe Photoshop v 5.0 LE at 1000dpi, or the images were captured with a Hamamatsu C5985 cooled CCD camera using Metamorph v 4.01 (Universal Imaging).

9. Antibodies

The primary antibodies used were: the MAN antiserum at a dilution of 1:5000 previously described in Paulin-Levasseur *et al.* (1996); a mouse monoclonal antibody against human lamin B (MatriTect, Cambridge, MA) at a dilution of 1:30; a mouse monoclonal antibody against lamin B1 (Oncogene, San Diego, CA) at a dilution of 1:50 for immunofluorescence (IF) and 1:100 for WB; a mouse monoclonal antibody against lamin B2 termed X223 (provided by Dr. G. Krohne, Germany) at a dilution of 1:100, as previously described (Lourim *et al.*, 1996); a goat polyclonal antibody against lamin B1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:100 for IF; a mouse monoclonal antibody specific for lamins A/C termed R27 (provided by Dr. G. Krohne, Germany) at a dilution of 1:100 for IF and 1:1000 for WB, as previously described (Hoger *et al.*, 1990); a rabbit antibody against lamins A/C (provided by Dr. H. J. Worman, New York, NY) at a dilution of 1:400, as previously described (Barton and Worman, 1999); a goat polyclonal antibody against the N-terminus of lamins A/C (Santa Cruz) at a dilution of 1:100 for IF; a mouse monoclonal antibody against LAP2 termed 13d4 (provided by Dr. R. Benavente, Germany) at a dilution of 1:20 for IF and 1:50 for WB, as previously described (Alsheimer *et al.*, 1998); a mouse monoclonal antibody against LAP2α (provided by Dr. R. Foisner, Vienna, Austria) at a dilution of 1:5, as previously described (Harris *et al.*, 1994); a mouse monoclonal antibody against the N-terminus of human emerin (Novocastra Laboratories Ltd, Newcastle, UK) at a dilution of 1:40 for IF; a mouse monoclonal antibody against cyclin B1 (BD Biosciences Pharmingen, Franklin Lakes, NJ) at 1 μg/ml for WB; a mouse monoclonal antibody against α-tubulin termed DM1A (Sigma, Saint Louis, MO) at a dilution of 1:500 for IF and 1:5000 for WB; a mouse monoclonal antibody against actin (Cedarlane, Hornby, Ontario, Canada) at 10 μg/ml for WB; a goat polyclonal antibody against vimentin termed ZAK (provided by Dr. P. Traub, Germany) at a dilution of 1:1500 for IF and 1:3000 for WB; a mouse monoclonal antibody against calnexin (BD Transduction Laboratories, BD Biosciences) at a dilution of 1:1000 for WB; a mouse monoclonal antibody termed 2A7 (hybridoma supernatant) at a dilution of 1:20 for IF, as previously described (Paulin-Levasseur and Julien, 1999); a mouse monoclonal antibody against

nuclear pore complex proteins termed MAb414 (Babco Berkeley Antibody Company, Richmond, CA) at a dilution of 1:1500 for IF.

The following secondary antibodies were used for IF: a donkey anti-rabbit IgG conjugated with CY3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at a dilution of 1:400; a rabbit anti-human IgG, IgA and IgM conjugated with fluorescein isothiocyanate (FITC; ICN ImmunoBiologicals) at a dilution of 1:400; a goat anti-human IgG conjugated with CY3 (Jackson ImmunoResearch) at a dilution of 1:400; a rabbit anti-goat conjugated with FITC (MP Biomedicals Cappel, Irvine, CA) at a dilution of 1:250; and a donkey anti-mouse IgG conjugated with CY3 (Jackson ImmunoResearch) at a dilution of 1:400. Appropriate biotinylated secondary antibodies were used for WB: an anti-human Ig from sheep (Amersham) at a dilution of 1:4000 and an anti-mouse Ig from sheep (Amersham) at a dilution of 1:4000.

III. Results

1. Localization of nuclear antigens during the cell cycle

A first step in this study was to examine the *in vivo* cell cycle fate of nuclear antigens that would be used as markers of nuclear assembly and disassembly in human HeLa cells. It was important to validate the tools as well as the system by verifying the efficiency of antibody labeling in control cells. This was done by observing if the patterns of staining corresponded to published evidence, thus demonstrating that the cell line and antibodies behaved as expected in control conditions. An untreated population was used for these preliminary experiments because it was crucial to avoid the artifacts usually encountered in synchronized cultures, even though asynchronous cultures contain only a small fraction of mitotic cells (3 to 5%).

1.1. Interphase distribution and co-localization of nuclear antigens

To gain more insight into the distribution of nuclear proteins, double immunolabelling experiments were performed on HeLa cells. During interphase, LEM-bearing proteins, lamins A/C and lamin B displayed perinuclear localization under conventional immunofluorescence microscopy. This can be observed as a bright ring at the periphery of the nucleus (Fig. 2 A, A'; Fig. 3 A, A'; Fig. 4 A). In some instances, lamins A/C could also be detected in intranuclear foci (data not shown; see Goldman *et al.*, 1992). In contrast to the LEM proteins and lamins, LAP2α was seen within the nucleoplasm and did not present a characteristic NE perinuclear distribution (Fig 4 A') as previously reported by Dechat and collaborators (2000b).

Single immunolabelling experiments were also carried out for other antigens. The nucleocytoplasmic shuttling protein 2A7 was located throughout the nucleoplasm during interphase (Fig. 5 A) in a diffuse manner, and was also present to a lesser extent in the nucleoli of some cells (data not shown), as previously described (Paulin-Levasseur and Julien, 1999). LAP2 proteins were found at the periphery of the nucleus (Fig. 6 A), as was expected from integral INM proteins. Nuclear pore proteins were also located at the nuclear periphery (Fig. 7 C) in a dotted pattern.

Figure 2. Spatial relationship of the LEM proteins to lamins A/C in HeLa cells during the cell cycle. Cells were double-labeled for immunofluorescence with the MAN antiserum (A-E) and anti-lamins A/C from rabbit (A'-E'). Preparations were counter-stained with Hoechst 33258 (A''-E'') to visualize DNA and observed by phase-contrast microscopy (A'''-E'''). Pictures show the progression of cells through the stages of mitosis: interphase (A-A'''), prophase (B-B'''), prometaphase (C-C'''), metaphase (D-D''') and late telophase (E-E'''). The arrow in A'' points to a micronucleus containing both LEM bearing proteins (A) and lamins A/C (A'). Bar = 10 μm.

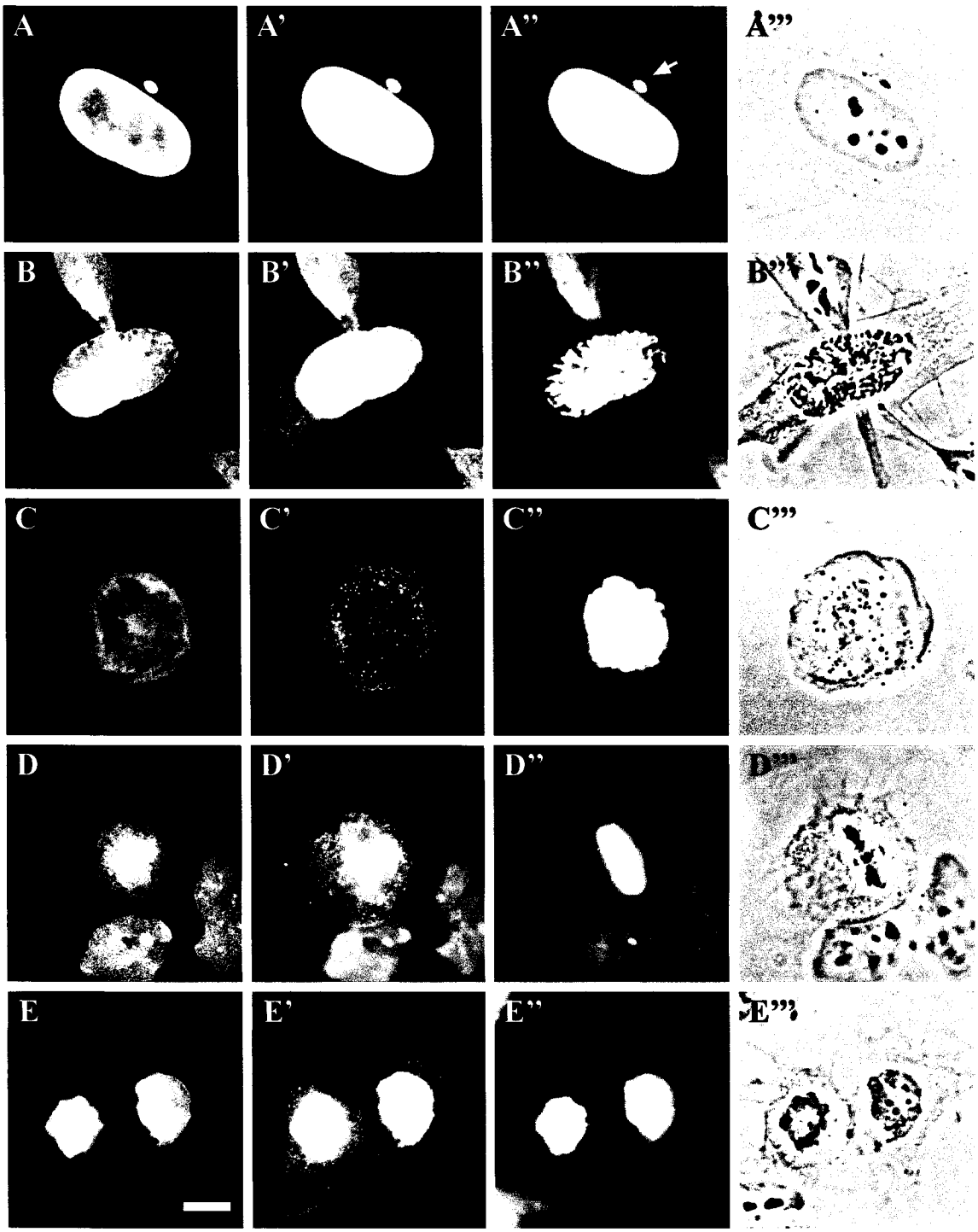

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Figure 3. Spatial relationship of the LEM proteins to lamin B in HeLa cells during the cell cycle. Cells were double-labeled for immunofluorescence with the MAN antiserum (A-F) and anti-lamin B (A'-F'). Preparations were counter-stained with Hoechst 33258 (A''-F'') to visualize DNA and observed by phase-contrast microscopy (A'''-F'''). Pictures show the progression of cells through the stages of mitosis: interphase (A-A'''), prophase (B-B'''), prometaphase (C-C'''), metaphase (D-D'''), anaphase (E-E''') and late telophase / early G1 (F-F'''). The arrowhead in A'' points to a micronucleus that contains both MAN (A) and lamin B (A'). Bar = 10μm.

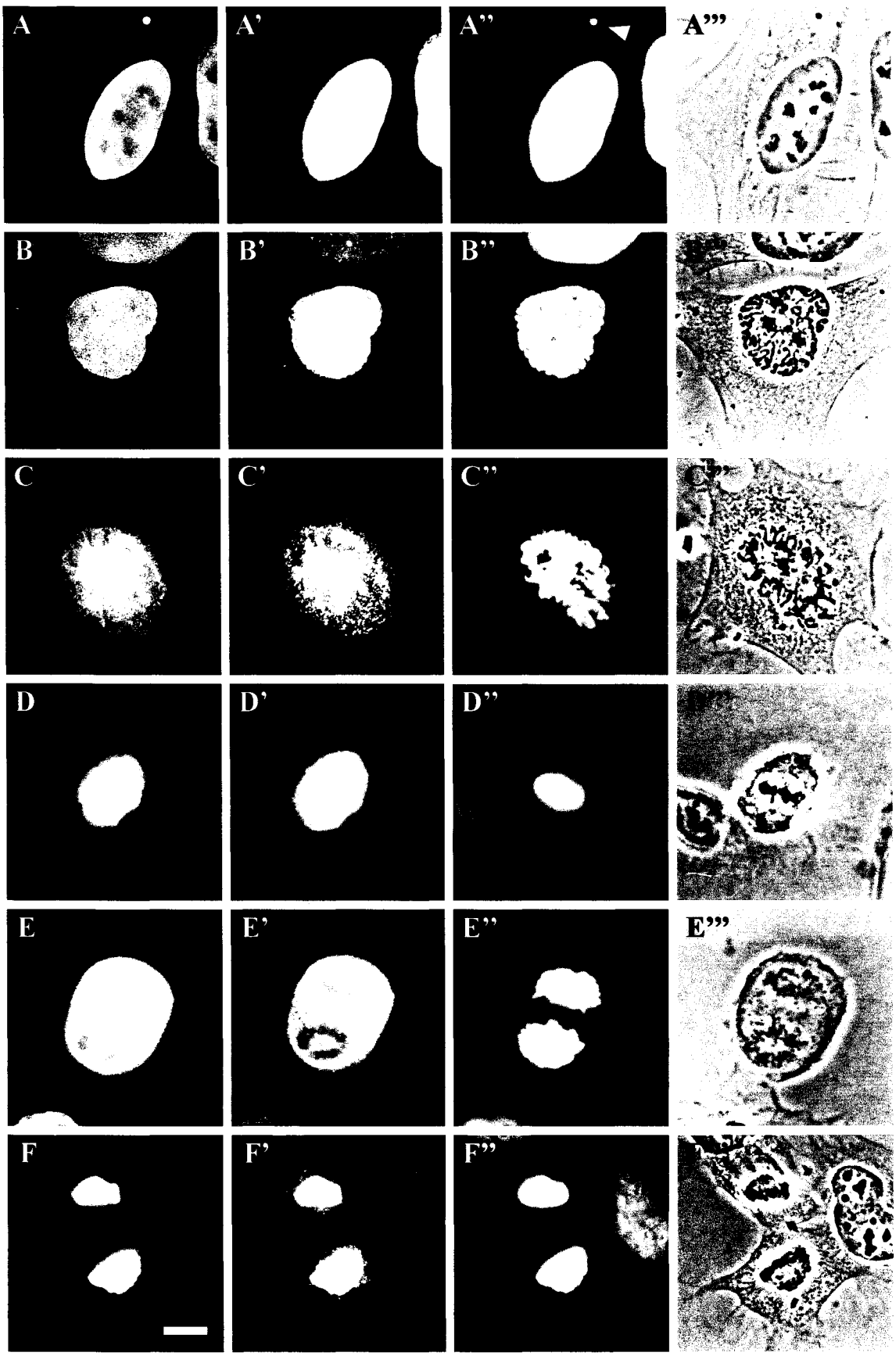
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Figure 4. Spatial relationship of the LEM proteins to LAP2α in HeLa cells during the cell cycle. Cells were double-labeled for immunofluorescence with the MAN antiserum (A-F) and anti-LAP2α (A'-F'). Preparations were counter-stained with Hoechst 33258 (A''-F'') to visualize DNA and observed by phase-contrast microscopy (A'''-F'''). Pictures show the progression of cells through the stages of mitosis: interphase (A-A'''), prophase (B-B'''), prometaphase (C-C'''), metaphase (D-D'''), anaphase (E-E''') and late telophase (F-F'''). The arrow in A'' points to a micronucleus; LEM proteins (A) and LAP2α (A') are present inside. The arrowhead in F points to a MAN positive cytoplasmic inclusion which does not have LAP2α (F'). Bar = 10μm.

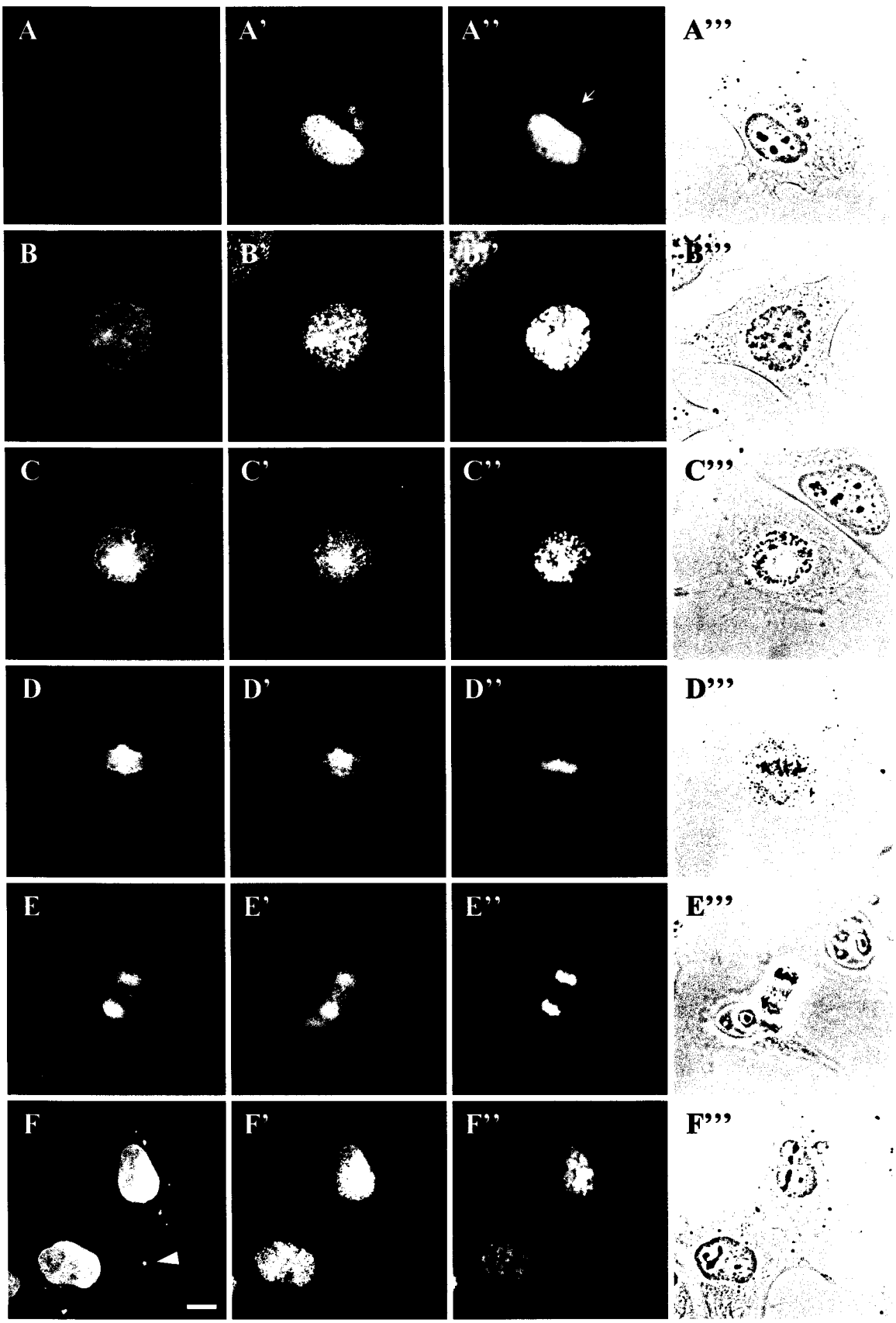
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Figure 5. Localization of 2A7 proteins in HeLa cells during the cell cycle. Cells were labeled for immunofluorescence with the 2A7 antibody (A-F). Preparations were counter-stained with Hoechst 33258 (A'-F') to visualize DNA and observed by phase-contrast microscopy (A''-F''). Pictures show the progression of cells through the stages of mitosis: interphase (A-A''), prophase (B-B''), late prometaphase (C-C''), anaphase (D-D''), and late telophase / early G1 (E-E''). Bar = 10 μm.

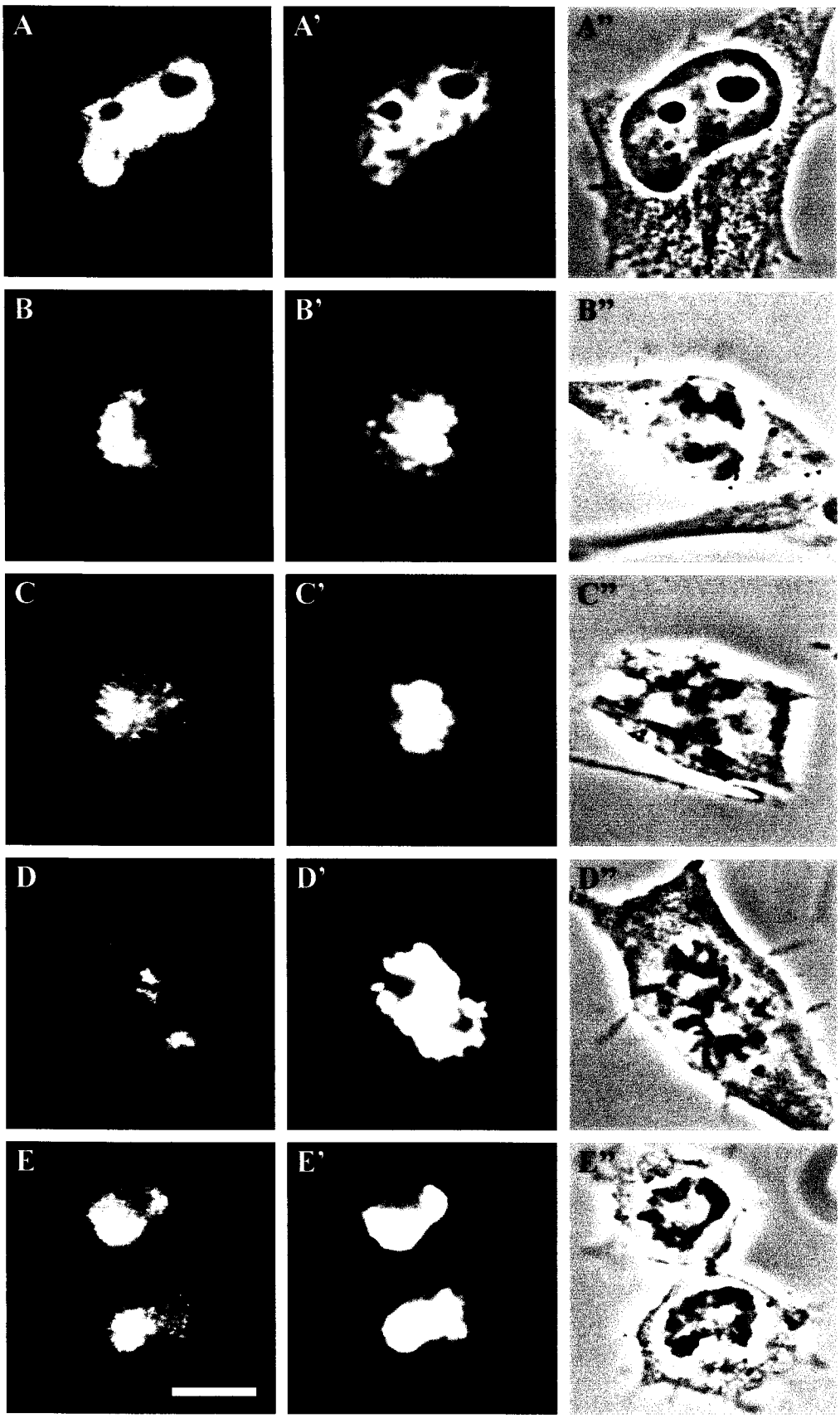
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Figure 6. Localization of LAP2 proteins in HeLa cells during the cell cycle. Cells were labeled for immunofluorescence with the 13d4 antibody directed against LAP2 proteins (A-F). Preparations were counter-stained with Hoechst 33258 (A'-F') to visualize DNA and observed by phase-contrast microscopy (A''-F''). Pictures show the progression of cells through the stages of mitosis: interphase (A-A''), prophase (B-B''), late prometaphase (C-C'') and late telophase / early G1 (D-D''). Bar = 10 μm.

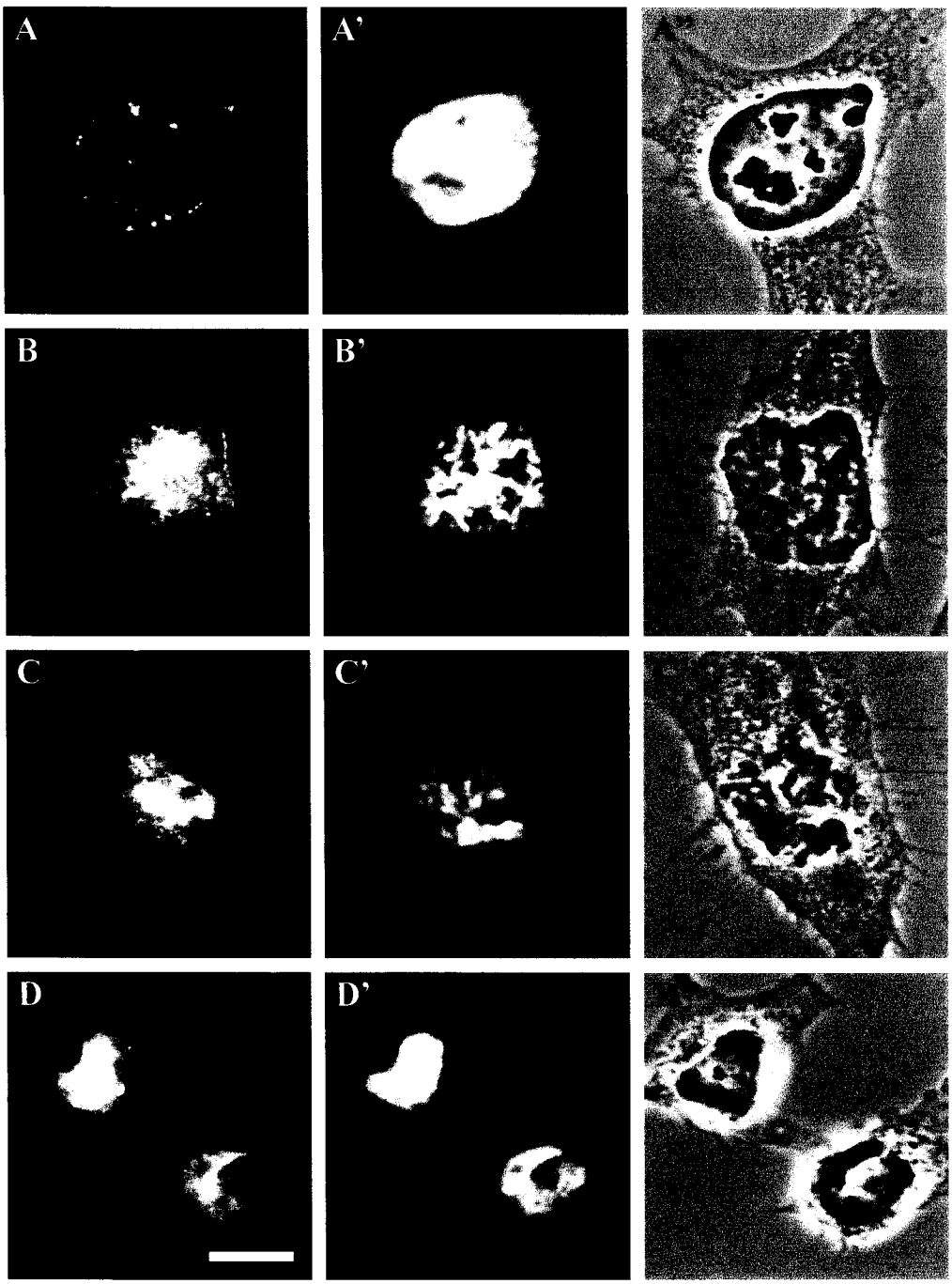
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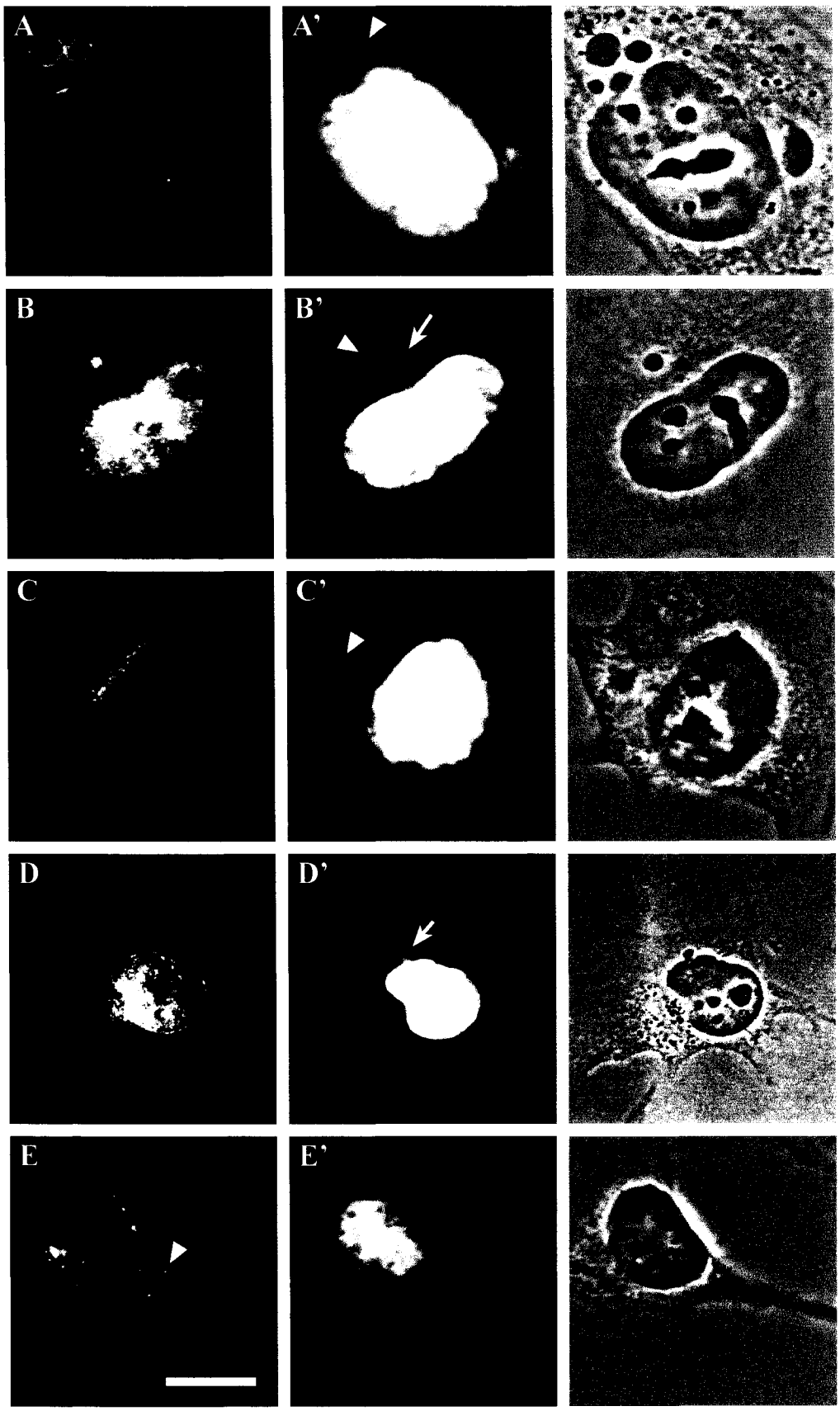
Figure 7. Presence of LAP2, 2A7 antigens and nuclear pore proteins in micronuclei and cytoplasmic inclusions. Cells were labeled for immunofluorescence with the anti-LAP2 termed 13d4 (A), 2A7 antibody (B) and anti-nuclear pore proteins (C-E). Preparations were counter-stained with Hoechst 33258 (A'-E') to visualize DNA and observed by phase-contrast microscopy (A''-E''). The arrowhead in A' points to a micronuclei containing LAP2 (A'). The arrowhead in B' points to a micronuclei with 2A7, while the arrow to one lacking 2A7. The arrowhead in C' points to a micronuclei surrounded by nuclear pores, while the arrow in D' points to one without nuclear pores. The arrowhead in E' points to a cytoplasmic inclusion of nuclear pores. Bar = 10 μm.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Interphase HeLa cells sometimes contain micronuclei, which are whole or fragments of chromosomes that lag behind in anaphase and are excluded from the nucleus in telophase. Micronucleation can be induced by mutagens, for example by prolonged exposure of cultured cells to colchicine (Bell and Scheer, 1996), but also occurs naturally in some cell lines. In interphase HeLa cells with micronuclei, the LEM proteins were always found to occupy these structures (Fig 4 A), as was LAP2α (Fig. 4 A') or other LAP2 proteins (Fig. 7 A, arrowhead in A'), and lamins A/C (Fig. 8 C', arrowhead in C''). Lamin B, the 2A7 antigen and nuclear pore proteins were found in some micronuclei (Fig. 3 A', arrowhead in A''; Fig. 7 B, arrowhead in B'; C, arrowhead in C') but absent in others (Fig. 7 B, arrow in B'; D, arrow in D'; Fig. 8 B', arrowhead in B'').

To support the observations made on the HeLa cell line, human Fanconi's anemia cells were also investigated by immunofluorescence. Interphase cells showed the expected peripheral nuclear staining for the LEM proteins (Fig. 9 A), lamin B (Fig. 9 B) and lamins A/C (Fig. 9 C).

1.2. Mitotic fate of nuclear antigens

To investigate further the behavior of, and relationships between these nuclear proteins during cell division, unsynchronized mitotic HeLa cells were observed in double immunolabelling experiments. Lamins A/C disassembled as the cells progress into late prophase/early prometaphase (Fig. 2 B'), whereas the LEM proteins, lamin B and LAP2α were released into the cytoplasm during late prometaphase (Fig. 3 C, C'; Fig. 4 C). During metaphase, the LEM proteins, lamins A/C, lamin B and LAP2α were diffusely distributed in the cytoplasm (Fig. 2 C, C'; 3 D, D'; 4 D, D'). LAP2α had been reported previously to associate with chromosomes extremely early in postmitotic nuclear assembly (Dechat *et al.*, 1998). At late anaphase, there was a slight association of the LEM proteins and lamin B with chromosomes (Fig. 4 E; Fig. 3 E, E'). At the end of cytokinesis, we can see that the LEM proteins, lamin B and LAP2α have all returned to the reformed nuclei (Fig. 2 F; 3 F, F'; 4 F, F'). However, lamins A/C stayed in a soluble form for an extended period before they were integrated in the nuclear lamina, as can be

Figure 8. Presence of LEM proteins, lamin B and lamins A/C in micronuclei and cytoplasmic inclusions. Cells were double-labeled for immunofluorescence with the MAN antiserum (A-C), and with anti-lamin B (A', B') or anti-lamins A/C from rabbit (C'). Preparations were counter-stained with Hoechst 33258 (A''-C'') to visualize DNA. The arrowhead in A points to a MAN positive cytoplasmic inclusion that does not contain lamin B (A'), while the one in c points to one containing lamins A/C. The arrowhead in B'' points to a micronucleus positive for LEM proteins but lacking lamin B. Bars = 10 μm.

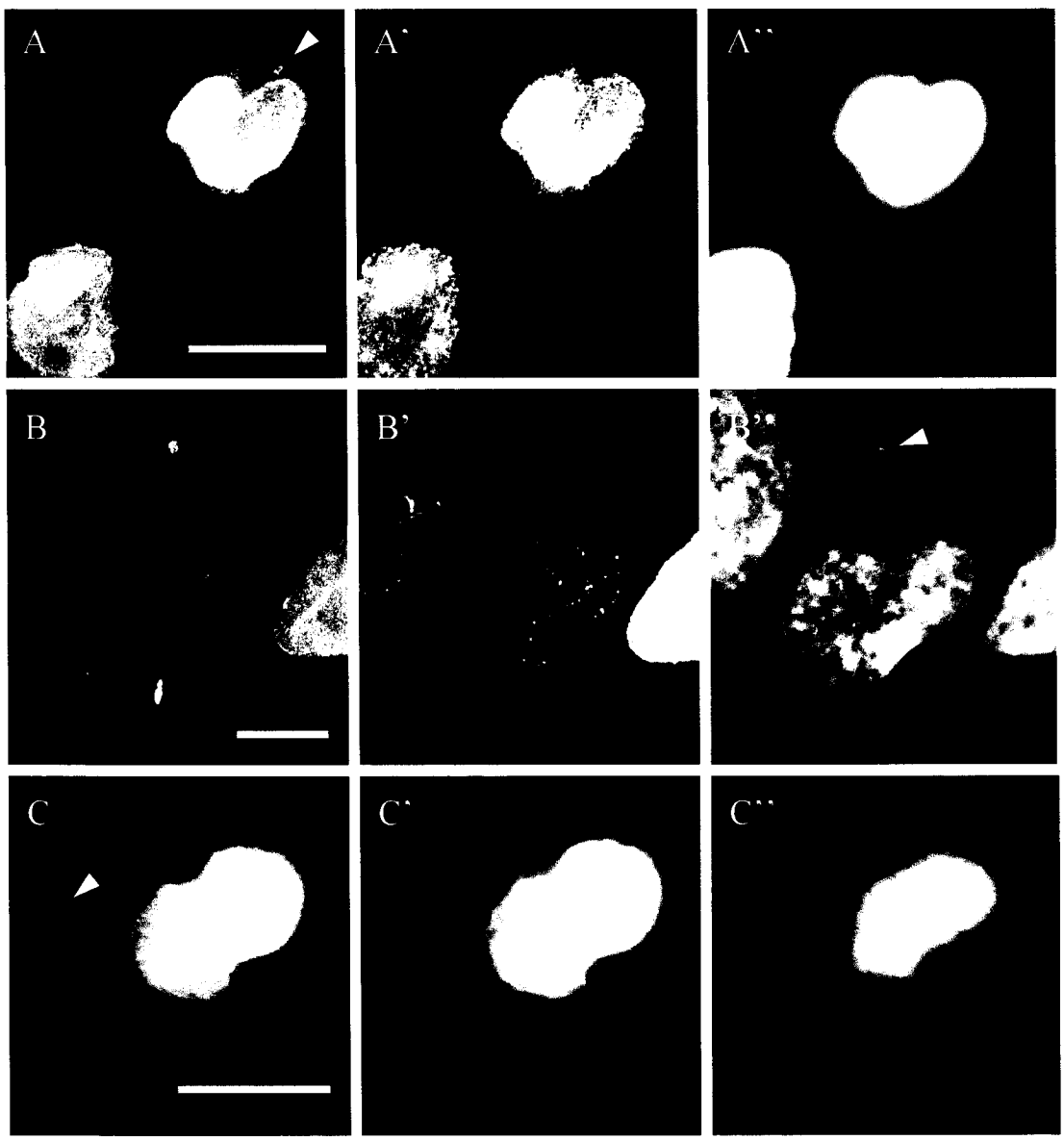
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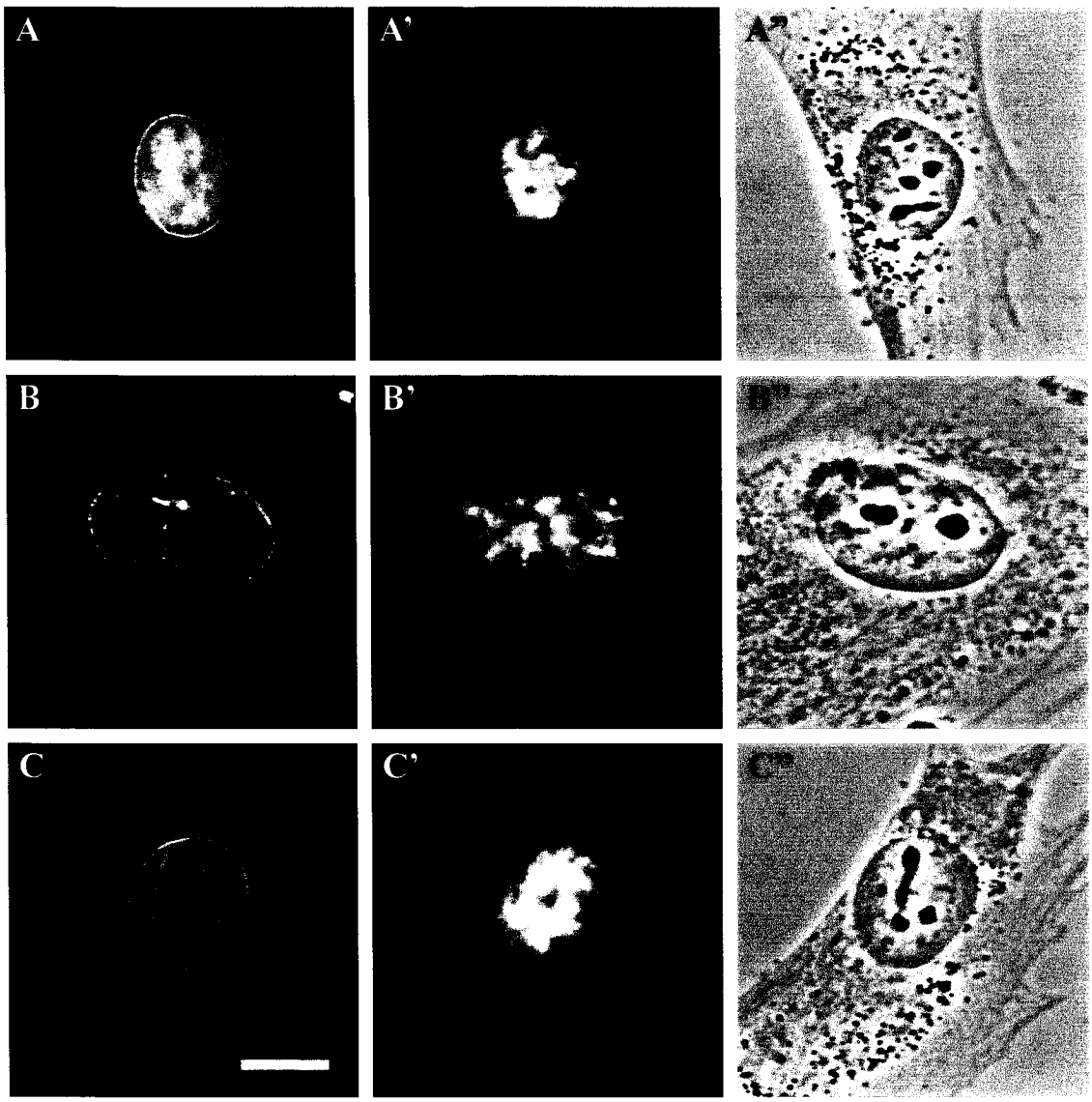
Figure 9. Localization of nuclear proteins in Fanconi's anemia cells. Cells were labeled for immunofluorescence with the MAN antiserum (A), anti-lamin B termed X223 (B), anti-lamins A/C from goat (C). Preparations were counter-stained with Hoechst 33258 (A'-C') to visualize DNA and observed by phase-contrast microscopy (A''-C''). Bar = 10 μm.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seen by the lack of peripheral staining and continued presence of staining in the cytoplasm in telophase (Fig. 2 F').

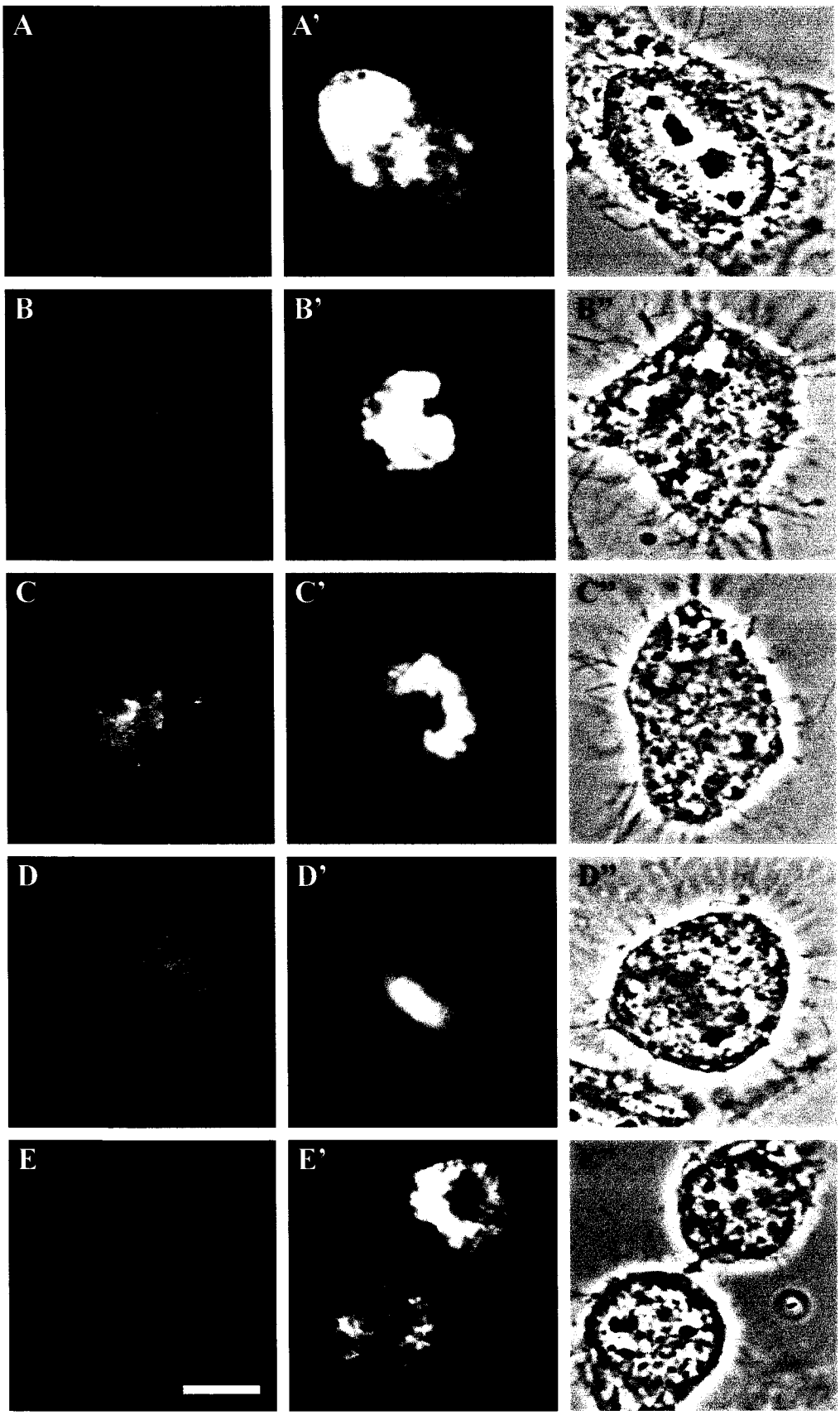
Single immunolabelling experiments were also carried out for other antigens to study their behavior in unsynchronized mitotic HeLa. During prophase, as previously described (Paulin-Levasseur and Julien, 1999), the 2A7 antigen was mostly confined to the nucleoplasmic space in between condensing chromosomes (Fig. 5 B). As the NE disassembled in prometaphase, staining was still concentrated on the chromosome mass, but was also found throughout the cytoplasm (Fig. 5 C). During anaphase, 2A7 continued to be associated with chromatin (Fig. 5 D). The 2A7 antigen reintegrated the nucleoplasm of late telophase/early G1 cells (Fig. 5 E). LAP2 proteins were still found at the nuclear periphery during prophase (Fig. 6 B), but started to leave the nucleus and scatter in the cytoplasm in prometaphase (Fig. 6 C). In late telophase/early G1 cells, LAP2 proteins had fully reintegrated the nucleus (Fig. 6 D).

Late telophase / early G1 cells sometimes contain cytoplasmic inclusions of nuclear antigens that have not yet been integrated in the newly reformed nuclei. Contrary to micronuclei, they do not contain any DNA. Cytoplasmic inclusions containing the LEM proteins lacked LAP2α (Fig. 4 F, F'), while they appear always to include lamins A/C (Fig. 3 F, F'). Lamin B was present in some but absent in other LEM proteins positive cytoplasmic inclusions (Fig. 8 A, A'). Nuclear pore proteins were also found in cytoplasmic inclusions (Fig. 7 E).

1.3. Accessibility to the nucleus during mitosis

To determine the time point at which the nucleus becomes freely permeable during mitosis, access to the LEM proteins from the cytoplasm was examined. This was assessed by permeabilizing HeLa cells with digitonin before fixing them. Digitonin affects only the cholesterol-rich plasma membrane but not the nuclear membranes nor other internal membranes, permitting access of antibodies in interphase cells to the cytoplasm but not to the nucleus. During interphase, no staining was observed with the MAN antiserum (Fig. 10 A), indicating a strictly nuclear localization of the LEM proteins, as well as a lack of access to the nucleus. At early prometaphase, the nuclear periphery was stained (Fig. 10 B) as diffusion of antibodies to the nucleus was permitted.

Figure 10. Accessibility to the nucleus during the cell cycle. HeLa cells were exposed to digitonin to permeabilize the plasma membrane, fixed and labeled for immunofluorescence with the MAN antiserum (A-E). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-E') and observed by phase-contrast microscopy (A''-E''). Pictures show the progression of cells through the stages of mitosis: interphase (A-A''), early prometaphase (B-B''), late prometaphase (C-C''), metaphase (D-D'') and telophase (E-E''). Bar = 10 μm.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This may be caused by the start of NEBD, but also by NPC disassembly, which has been shown to leave the nucleus leaky in starfish oocytes while the NE is still intact (Terasaki *et al.*, 2001). At this point, the nucleus presented a distorted shape (Fig. 10 A), believed to be caused by microtubules from the mitotic spindle which facilitate NEBD by tearing the NE open (Beaudouin *et al.*, 2002). As mitosis progressed into later prometaphase and metaphase, the LEM proteins were released and dispersed throughout the cellular space (Fig. 10 C-D). Following nuclear reassembly in telophase (Fig. 10 E), the LEM proteins were no longer accessible from the cytoplasm as staining was not visible anymore.

2. Nuclear disassembly

The cell-free system used at first for nuclear disassembly was based on previous experiments performed with HeLa cells (Collas *et al.*, 1999). As demonstrated in a series of articles by Collas's team (Collas *et al.*, 1999; Steen *et al.*, 2000; Martins *et al.*, 2000), interphase nuclei disassemble in the cyclin containing soluble fraction of mitotic cytoplasm (a soluble mitotic extract isolated from nocodazole synchronized cells) when supplemented with an ATP-generating system. The system is manipulated by loading antibodies into the nuclei by lysolecithin permeabilization prior to exposure to the mitotic extract to block the action of specific proteins during disassembly.

Soluble mitotic extracts were obtained from HeLa cells by two different methods (see Materials and Methods for more details; extracts were isolated on three separate occasions for each method). Upon analysis by Western blot, both extracts were shown to contain cyclin B1 (Fig. 11 A-B; lane 1) in the same proportion as whole mitotic cells by visual approximation (Fig. 11 A-B; lane 2). Extracts were sometimes prepared by replacing EDTA with EGTA in the preparation buffer (see Materials and Methods).

Nuclei were isolated also from the HeLa cells by the method suggested by Collas and colleagues (1999) as described in Materials and Methods, but were entangled in cytoplasmic material (Fig. 12 A''). These nuclei did not disassemble when exposed to the mitotic extracts (Fig. 13 A') and seemed to form clumps as incubation time elapsed (Fig. 13 B'). This was attempted 10 times with different batches of isolated nuclei. Further analysis of the nuclei by immunofluorescence staining with antibodies specific to vimentin (Fig. 12 A) indicated that the material surrounding them was at least partially

Figure 11. Cyclin B1 is present in mitotic soluble extracts. Proteins were separated by SDS-PAGE and immunoblotted with antibodies directed against cyclin B1. Mitotic HeLa cells were fractionated to obtain a mitotic soluble extract either by the method of Collas (A; lane 1), or by the method of Burke (B; lane 1) (see materials and methods for more details). These are compared to whole nocodazole synchronized mitotic HeLa cells homogenate (A and B; lane 2), and to whole unsynchronized HeLa cells homogenate (A and B; lane 3). The size of the band is 62 kDa. Samples were loaded to have extracts from approximately the same number of cells in all wells.

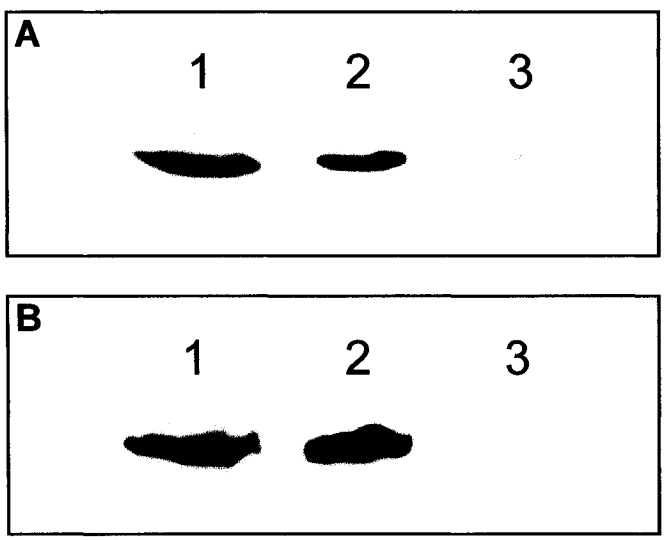
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Figure 12. Presence of intermediate filaments around isolated HeLa nuclei. Nuclei were labeled for immunofluorescence with anti-vimentin (A), counter-stained with Hoechst 33258 (A') to visualize DNA and observed by phase-contrast microscopy (A''). Bar = 10 μm.

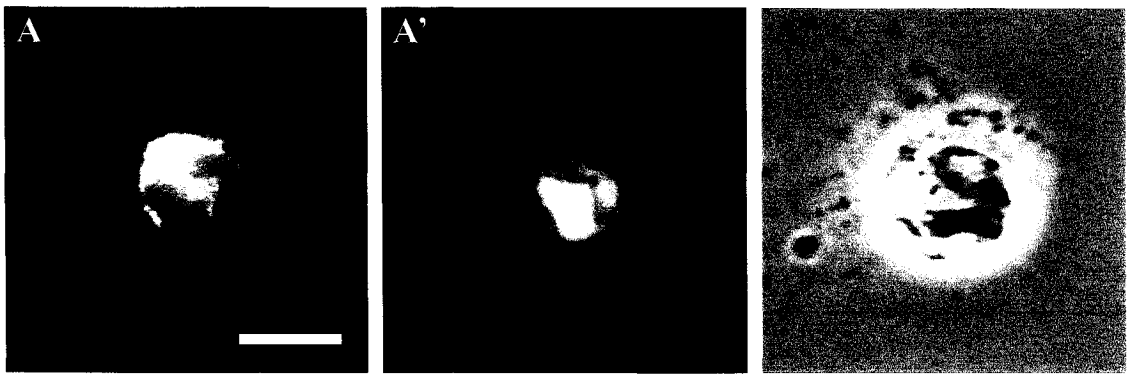
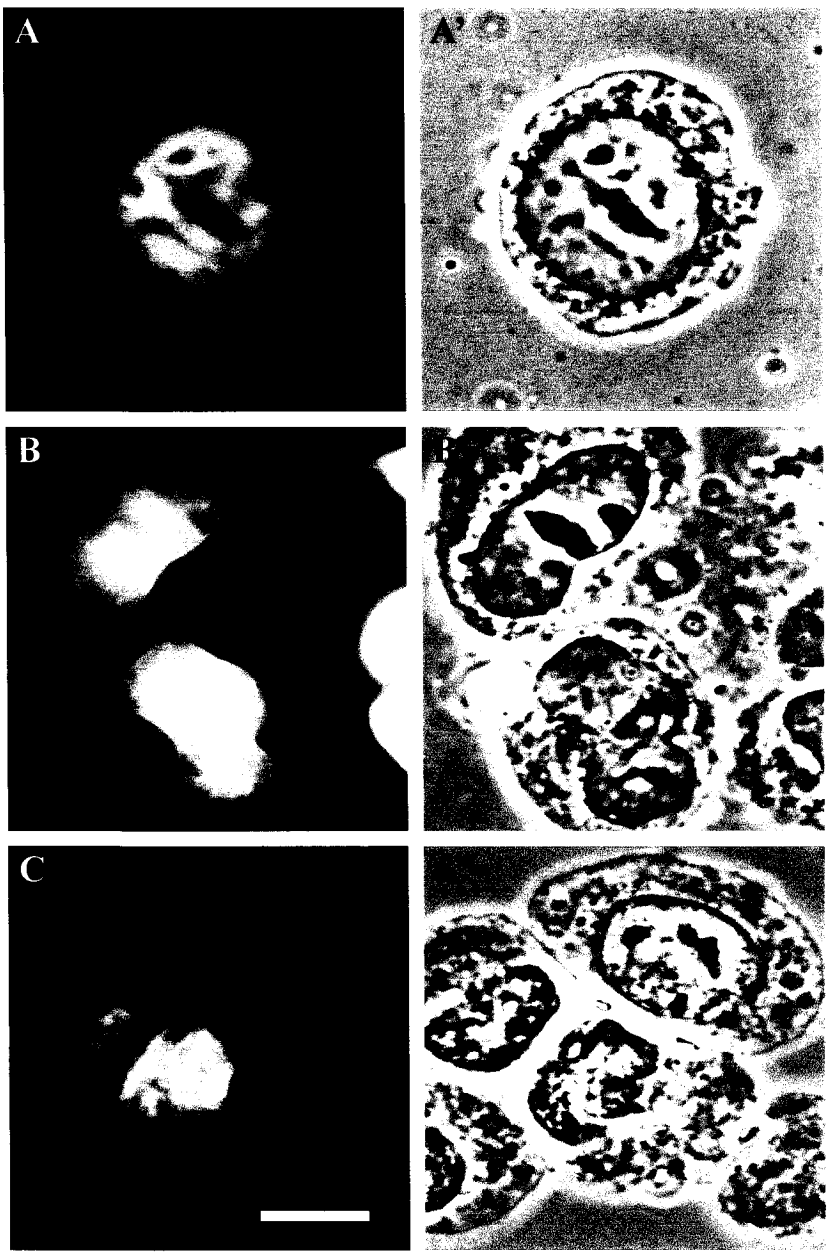

A
A'

Figure 13. Isolated nuclei failed to disassemble after 2h in mitotic soluble extract. Nuclei were stained with Hoechst 33258 to visualize DNA (A-C) and observed by phase-contrast microscopy (A'-C'). Pictures show nuclei after a 2h incubation either non disassembled and single (A-A'), or nuclei in clumps (B-B'). Nuclei were also permeabilized with lysolecithin prior to exposure to the mitotic extract, but they did not disassemble (C-C'). Bar = 10 μm.


A
A'
B
C

composed of intermediate filaments. The protocol for isolation of nuclei was modified in an attempt to get rid of them. To try depolymerizing intermediate filaments, the KCl concentration was reduced from 25 mM to 10 mM to have more hypotonic conditions, which had been previously shown to be effective (Traub and Nelson, 1982). The number of pestle strokes with the homogenizer was also varied (25 strokes, 75 and 150), but a reduction from the original 150 strokes left more vimentin with the nuclear isolated fraction (Fig. 14 lanes 2, 4). An alternative isolation method based on a protocol by Burke (1998; Burke and Gerace, 1986) was also carried out (see Materials and Methods for more details), but the results were the same: nuclei were surrounded by a lot of cytoplasmic material and no disassembly was observed.

Some disassembly experiments were also attempted with nuclei attached to coverslips beforehand (either by coating the coverslips with poly-L-lysine or by centrifugating the nuclei on them) to reproduce *in vivo* conditions better since HeLa cells grow attached to a substrate. However, the nuclei did not disassemble with the two different soluble mitotic extracts.

Furthermore, interphase nuclei isolated from CHO cells were used with the HeLa mitotic extract. This was in the hope that if the problem was with the HeLa nuclei alone, the cyclin-containing extracts would permit disassembly. Nevertheless, the CHO nuclei did not disassemble (Fig. 15 B-B').

Many disassembly reactions were performed, with different batches of mitotic extracts as well as with nuclei isolated on different occasions, used fresh as well as thawed, but without success. Since the G1 phase nuclei are more resistant to disassembly against mitotic extracts than cells in G2 and S phase (Hogner *et al.*, 1988), it was thought that the high incidence of G1 cells in an unsynchronized population might be affecting the experimental outcome. Attempts were therefore undertaken to synchronize cells in G2 before isolating nuclei so they would disassemble more readily. Two cell synchronization methods were tested: double thymidine block and serum starvation. However, these two methods did not yield higher percentages of mitotic figures at the expected time after release, or at any other time during the following 12h, indicating that synchrony was not successful (synchrony was attempted five times with serum starvation and twice with double thymidine block). Nuclei were nonetheless isolated from these

Figure 14. Presence of vimentin surrounding isolated nuclei. Proteins were separated by SDS-PAGE and immunoblotted with antibodies directed against vimentin. HeLa cells were homogenized with either 75 strokes (lanes 1-2) or 150 strokes (lanes 3-4) of a tight fitting pestle, and nuclei (lanes 2, 4) were isolated from cytoplasmic material (lanes 1, 3) by centrifugation. The size of the band is 57 kDa.

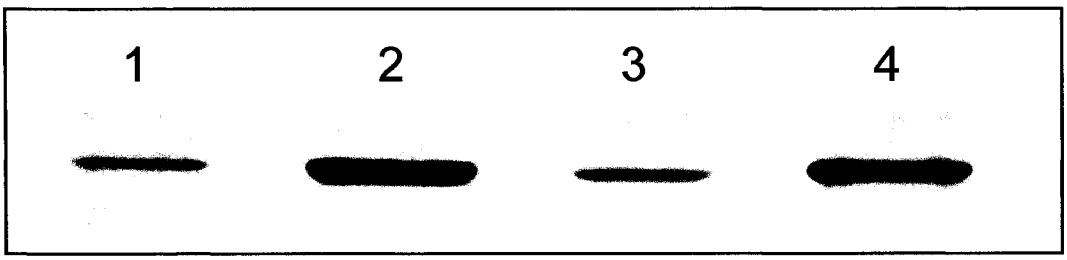
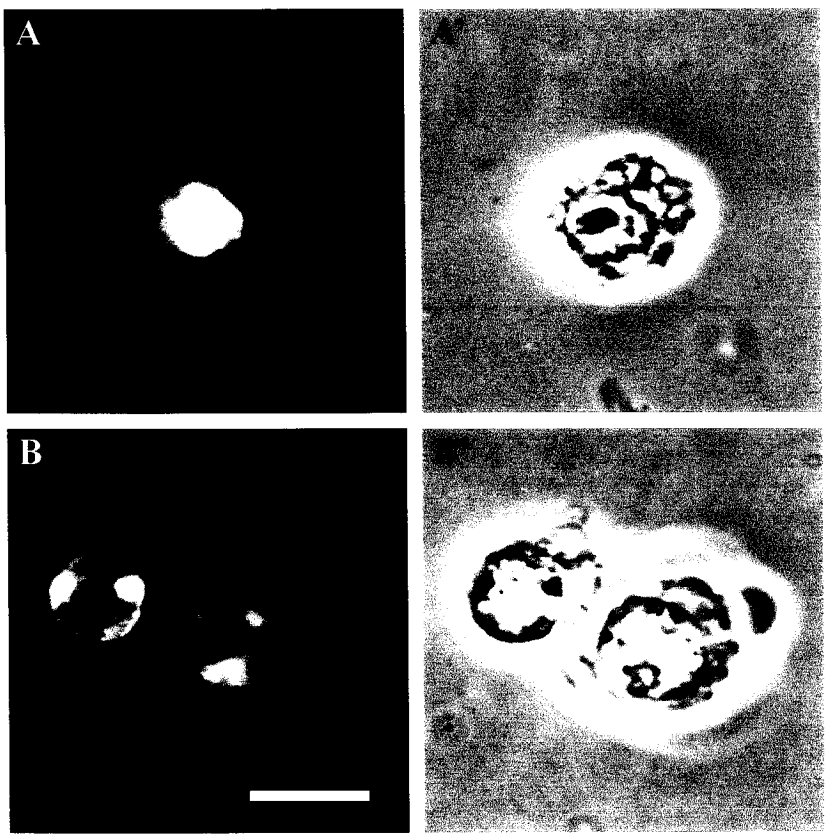

1
2
3
4

Figure 15. Isolated CHO nuclei failed to disassemble after 2h in HeLa mitotic soluble extract. Nuclei were stained with Hoechst 33258 to visualize DNA (A-B) and observed by phase-contrast microscopy (A'-B'). Pictures show nuclei when first exposed to the mitotic extract (A-A') and after a 2h incubation (B-B'). Bar = 10 μm.


A
B

cells (after release for the interval of time suggested by the protocols) and exposed to mitotic soluble extracts, but none disassembled on both attempts.

Since, in the experiments described in published articles (Collas, 1999), the nuclei were pre-permeabilized with lysolecithin to permit entry of antibodies prior to disassembly, the same treatment was also applied to isolated nuclei before exposing them to mitotic extracts. Nuclei were indeed shown to be permeabilized by their incorporation of 2H12 antibodies after exposure to lysolecithin (Fig. 16 A). However, the nuclei did not disassemble (Fig. 13 C') on either of the two attempts.

Because of the major difficulties encountered in obtaining a working nuclear disassembly system, other options were considered. Digitonin affects only the plasma membrane of cells and leaves the cytoplasm empty of its soluble components. This system is often used to study nuclear transport (Adam *et al.*, 1990; Merle *et al.*, 1999). It was hypothesized that the digitonin ghosts could be adapted to devise a novel nuclear disassembly assay. With such an assay, the nucleus could even disassemble in slightly more "normal" conditions, since microtubules and other cytoskeletal components would be left intact in the cell (Fig. 17). Thus, an asynchronous population of HeLa cells grown on coverslips was exposed on ice to digitonin. Then the coverslips were inverted on mitotic extracts containing an ATP-generating system and incubated at 30°C for 2 hours in a humid chamber. The nuclei did not disassemble on all three attempts. It was then thought that the attachment of the cells to a substrate might be preventing disassembly, since HeLa mitotic cells are rounded and only loosely connected to the plate when they are in culture. A suspension of interphase cells were therefore permeabilized by digitonin, rinsed, and then exposed to mitotic soluble extracts. Again, the nuclei did not disassemble on both attempts.

3. Nuclear assembly

Since the nuclear disassembly cell-free assays did not behave as expected in spite of sustained efforts, the aim of the research project described here was shifted towards studying cell-free nuclear assembly. This was done mainly because it is a related process, and the skills and tools acquired while working on the nuclear disassembly systems could be applied to assembly. Two protocols were tested for their effectiveness

Figure 16. Isolated nuclei permeabilized with lysolecithin can take up antibodies. Permeabilized nuclei were labeled for immunofluorescence with 2H12 (A) and counter-stained with Hoechst 33258 (A') to visualize DNA. Bar = 5 μm.

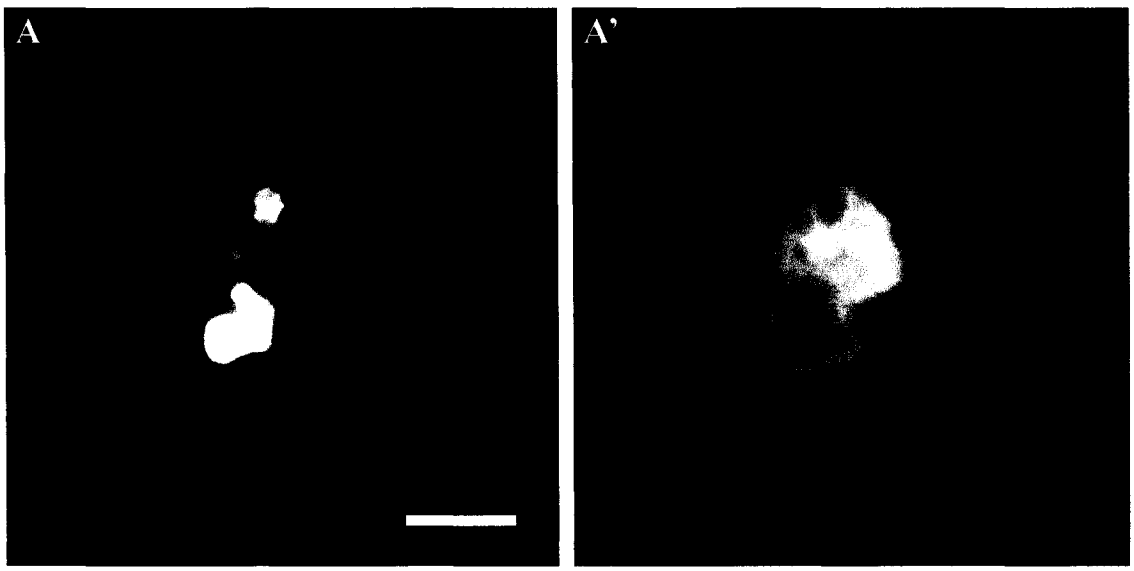
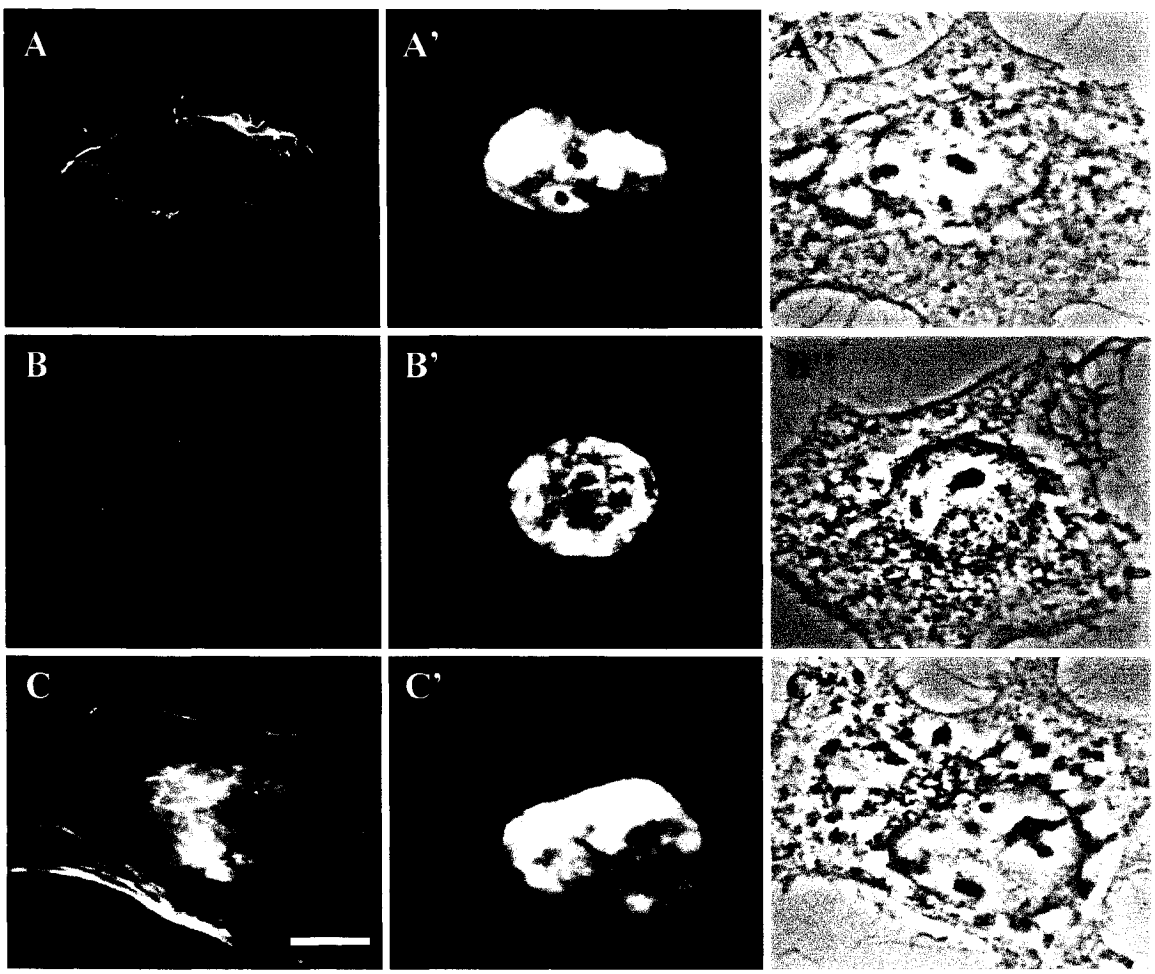

A
A'

Figure 17. Presence of cytoskeletal components in the cytoplasm of digitonin permeabilized cells. HeLa cells were exposed to digitonin to permeabilize the plasma membrane, fixed and labeled for immunofluorescence with anti-tubulin (A), anti-vimentin (B), and anti-actin (C). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-C') and observed by phase-contrast microscopy (A''-C''). Bar = 10 μm.


A
A'
A''
B
B'
B''
C
C'
C''

in the reassembly of the nuclear envelope and its associated components, which will be referred to as *crude mitotic homogenate* and *digitonin assembly system.*

3.1. Crude mitotic homogenate

The simplest method of cell-free nuclear assembly for mammalian cells is a crude homogenate of mitotic cells left to assemble nuclei in a buffer at 33°C for up to 2 hours. A method developed by Burke and Gerace (1986; Burke, 1998) in CHO cells was adapted as the main protocol for HeLa cells. At time 0 (immediately following homogenization and before any incubation time), the condensed chromatin was devoid of marker nuclear antigens (Fig. 18), as was expected. After 2 hours of reassembly in the crude mitotic homogenate, the chromatin was found to be surrounded by incomplete nuclear membranes as seen by staining for different nuclear antigens (Fig. 19). Some chromatin masses presented relatively regular peripheral nuclear staining (labeled with the serum to LEM proteins and LAP2 antibodies), but this happened only when the nuclei were surrounded by cytoplasmic material as visualized by phase contrast (Fig. 19 A''). Some nuclei had a relatively regular peripheral staining without cytoplasmic material (Fig. 19 D-D''), but these were rather rare. Addition of a surplus of a post-chromosomal supernatant containing mitotic membranes and cytoplasm to the mix (as suggested by Burke and Gerace, 1986; described in Materials and Methods) improved the numbers of regularly stained nuclei only slightly (Table 1). Different numbers of pestle strokes were tried to get maximum homogenization without breaking up chromatin masses, which could have resulted in increased percentages of small nuclei and therefore, in an insufficient membrane vesicles to chromatin surface ratio. The application of 15 strokes seemed to give the best results (results not shown). In total, nine separate assembly reactions were attempted.

With the system seemingly optimized, a preliminary experiment was performed where antibodies were introduced to block the function of specific proteins. The addition of MAN antiserum to LEM proteins in different concentrations (diluted 1:5000, 1:1000 and 1:200) yielded no appreciable difference in the reassembly of nuclear pore complexes or LAP2 proteins as compared to the control by immunofluorescence, on both attempts (results not shown). The same lack of difference from control conditions was seen with

Figure 18. Localization of nuclear antigens in a homogenate of mitotic HeLa cells. Material was labeled for immunofluorescence with antibodies against proteins of the nuclear pore complexes termed mAb414 (A), antibodies against LAP2 proteins termed 13d4 (B), the MAN antiserum (C), and anti-lamins A/C from goat (D). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-D') and observed by phase-contrast microscopy (A''-D''). Bar = 10 μm.

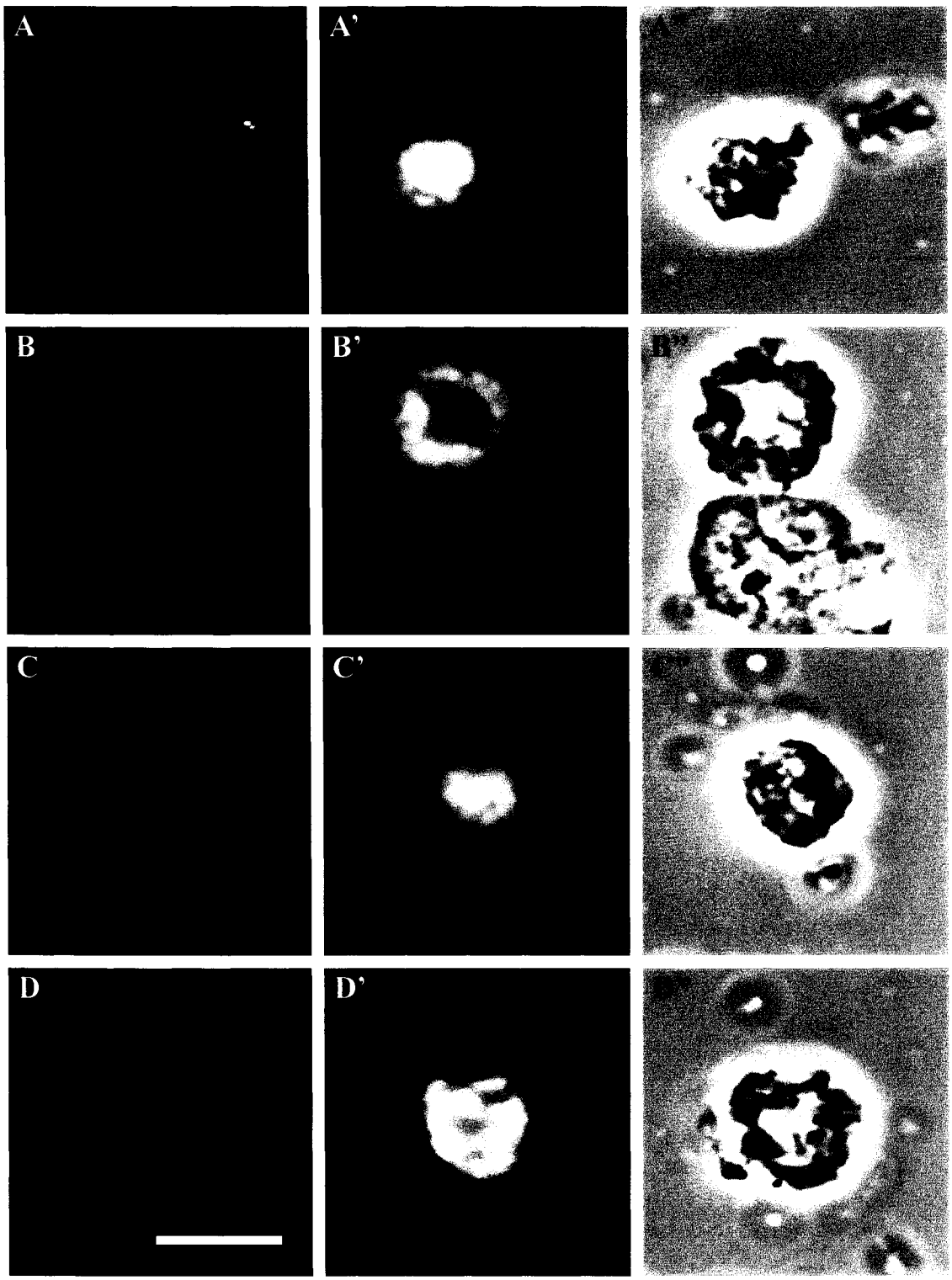

A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"

Figure 19. Incomplete assembly of nuclei in a crude homogenate of mitotic HeLa cells. Material was labeled for immunofluorescence with antibodies against proteins of the nuclear pore complexes termed mAb414 (A), antibodies against LAP2 proteins termed 13d4 (B), the MAN antiserum (C), and anti-lamins A/C from goat (D). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-D') and observed by phase-contrast microscopy (A''-D''). Bar = 10 μm.

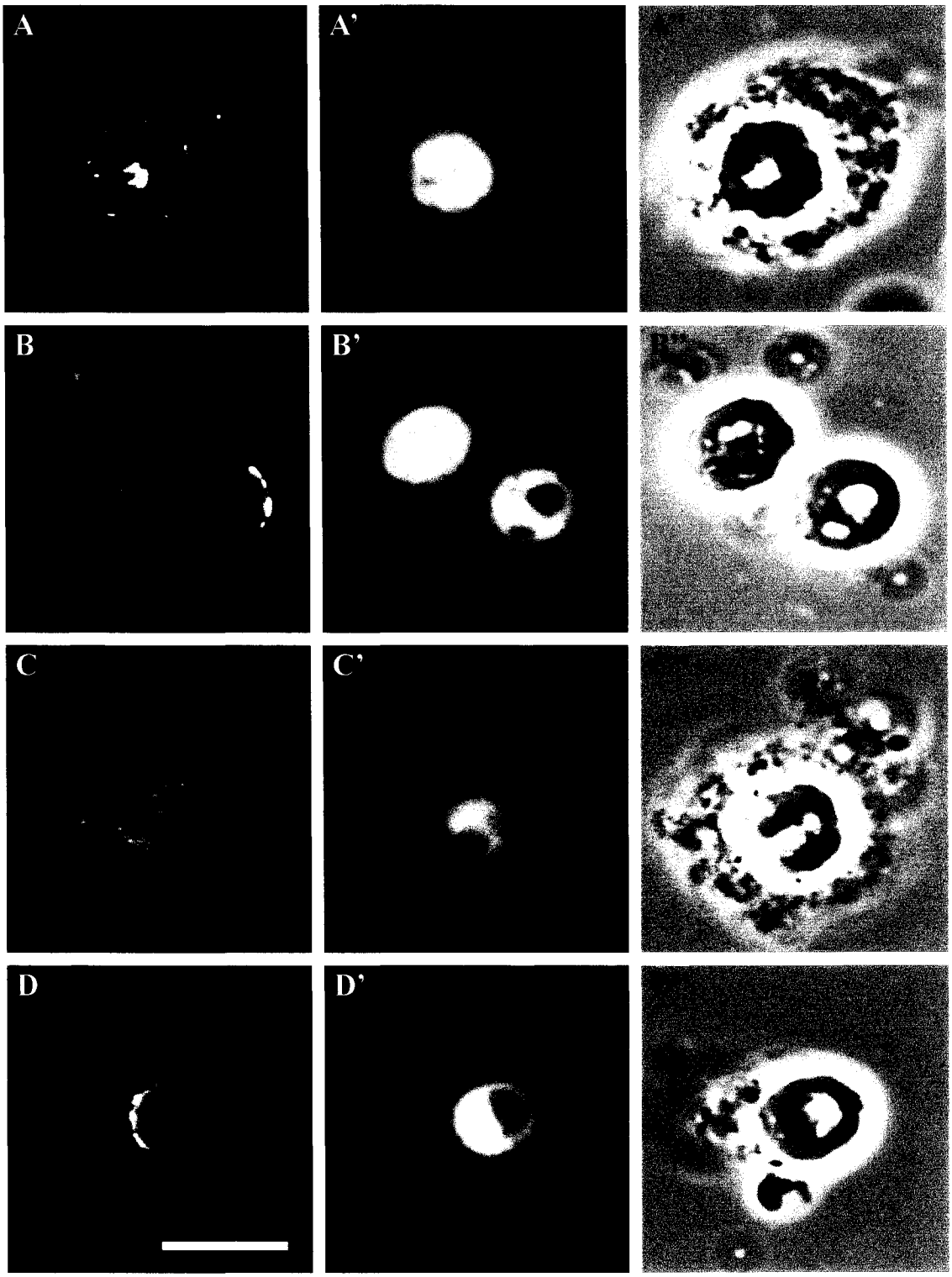

A
A'
B
B'
C
C'
D
D'

Table 1. Nuclear assembly upon addition of an exogenous supplement of mitotic soluble (membranous and cytosolic) extract. Synchronized mitotic HeLa cells were homogenized and a membranous and cytosolic fraction was either added (M) or not (NM); and the homogenate was left to reassemble nuclei at 33°C for 2 (t=2h) to 4 hours (t=4h). Assembly of nuclear membranes was assessed by perinuclear staining with the MAN antiserum (nuclei with MAN), while the total number of chromatin bundles, which would correspond to a nucleus, was assessed by staining with Hoechst DNA dye (total # of nuclei).

	T=2h		t=4h
	NM	M	NM
total # of nuclei	313	798	332
nuclei with MAN	135	384	142
% of assembly	43%	48%	43%

specific antibodies directed against emerin (diluted 1:50, 1:30 and 1:10) when the cells were stained for lamins A/C and LEM proteins (results not shown). This absence of effect was somewhat surprising since the antiserum to LEM proteins recognizes several components of the NE, and these proteins are deemed important as a group for their lamin and chromatin interactions. It is not as unexpected with emerin, since with EDMD, the effect of the mutant emerin protein is only felt in some types of cells and not others (Tsuchiya *et al.*, 1999). These results might be due to a failure by the antibodies to block the action of the proteins. They may also be caused by limitations in the detection of differences in immunofluorescence, particularly when assembly in control conditions is not complete, resulting in a false negative.

In regard to the above mentioned considerations, it appeared appropriate to investigate another method.

3.2. Digitonin assembly system

Digitonin was previously utilized in an unsuccessful attempt at nuclear disassembly. However, a digitonin system had been used by Kourmouli and colleagues (2000) to test the effects of recombinant forms of HP1 on nuclear envelope assembly. In the protocol elaborated by this group, synchronized mitotic cells are exposed to digitonin to permeabilize the plasma membrane and permit the entry of peptides or exogenous proteins, and then left at 33°C for 2h for the nuclei to reassemble (see Materials and Methods for details). Assembly reactions were attempted on ten separate occasions. Microscopic analysis of mitotic permeabilized cells indicated a diffuse cellular localization of marker nuclear antigens (Fig. 20 A; Fig. 21 A). After 30 minutes of incubation, lamin B and LAP2 formed cytoplasmic inclusions (Fig. 20 B; Fig. 21 B). After two hours, the assembly of nuclear envelopes was still incomplete in most cases as assessed by staining for LAP2 (Fig. 20 C) and lamin B (Fig. 21 C). Cytoplasmic aggregates of antigens were again present. Also, the chromatin remained condensed and the nuclei did not grow in size (Fig. 20 C'; Fig. 21 C'), even after 4 hours of incubation (results not shown).

To verify if the poor assembly of the HeLa cells was due to the cell line, assembly reactions were also performed with digitonin permeabilized mitotic CHO cells, which

Figure 20. Incomplete assembly of LAP2 in nuclei of digitonin permeabilized mitotic HeLa cells. Cells were labeled for immunofluorescence with anti-LAP2 proteins (A-C). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-C') and observed by phase-contrast microscopy (A''-C''). Pictures show cells after permeabilization (A-A''), after a 30 minute incubation (B-B''), and after 2h (C-C''). Bar = 10 μm.

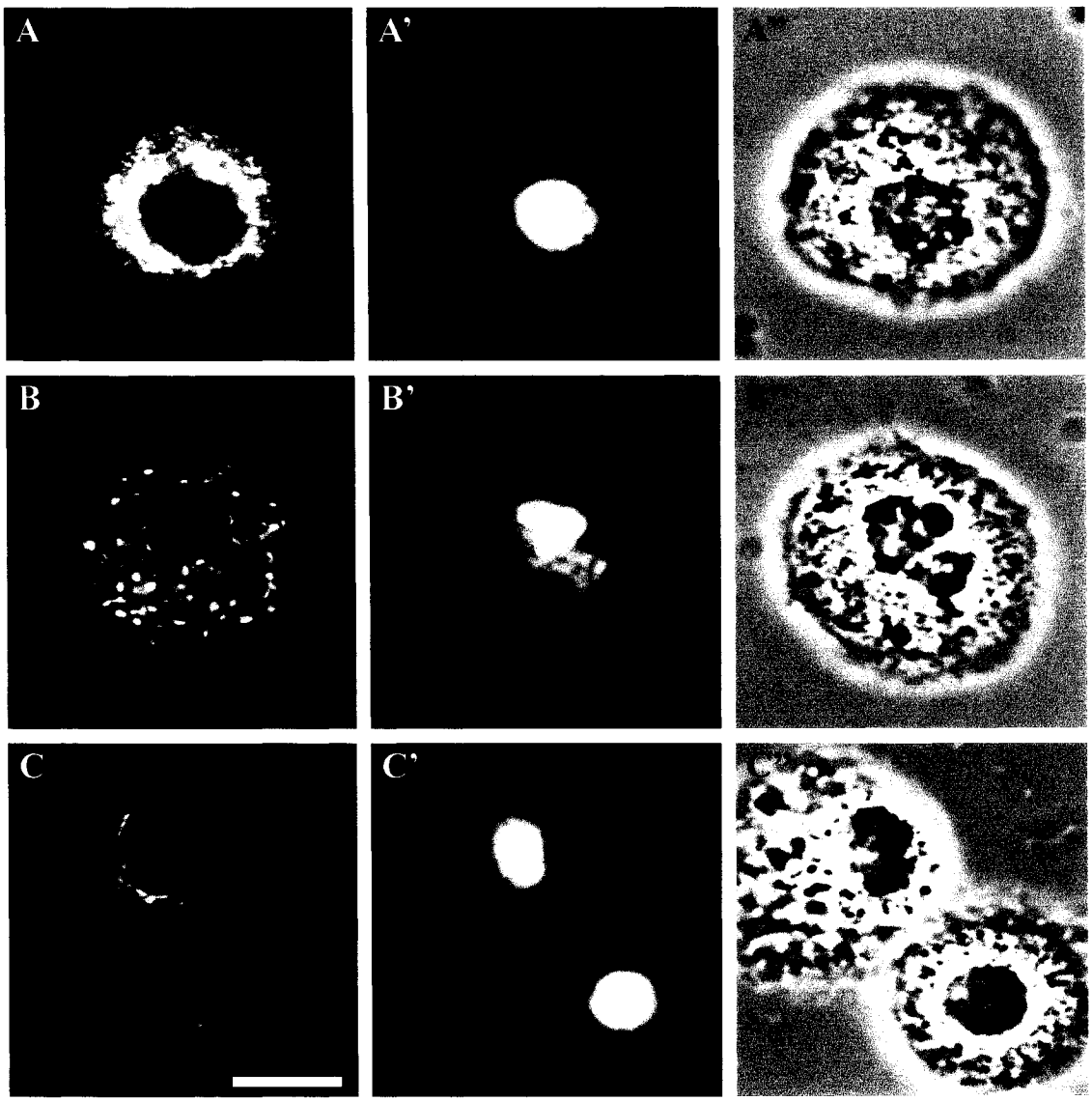
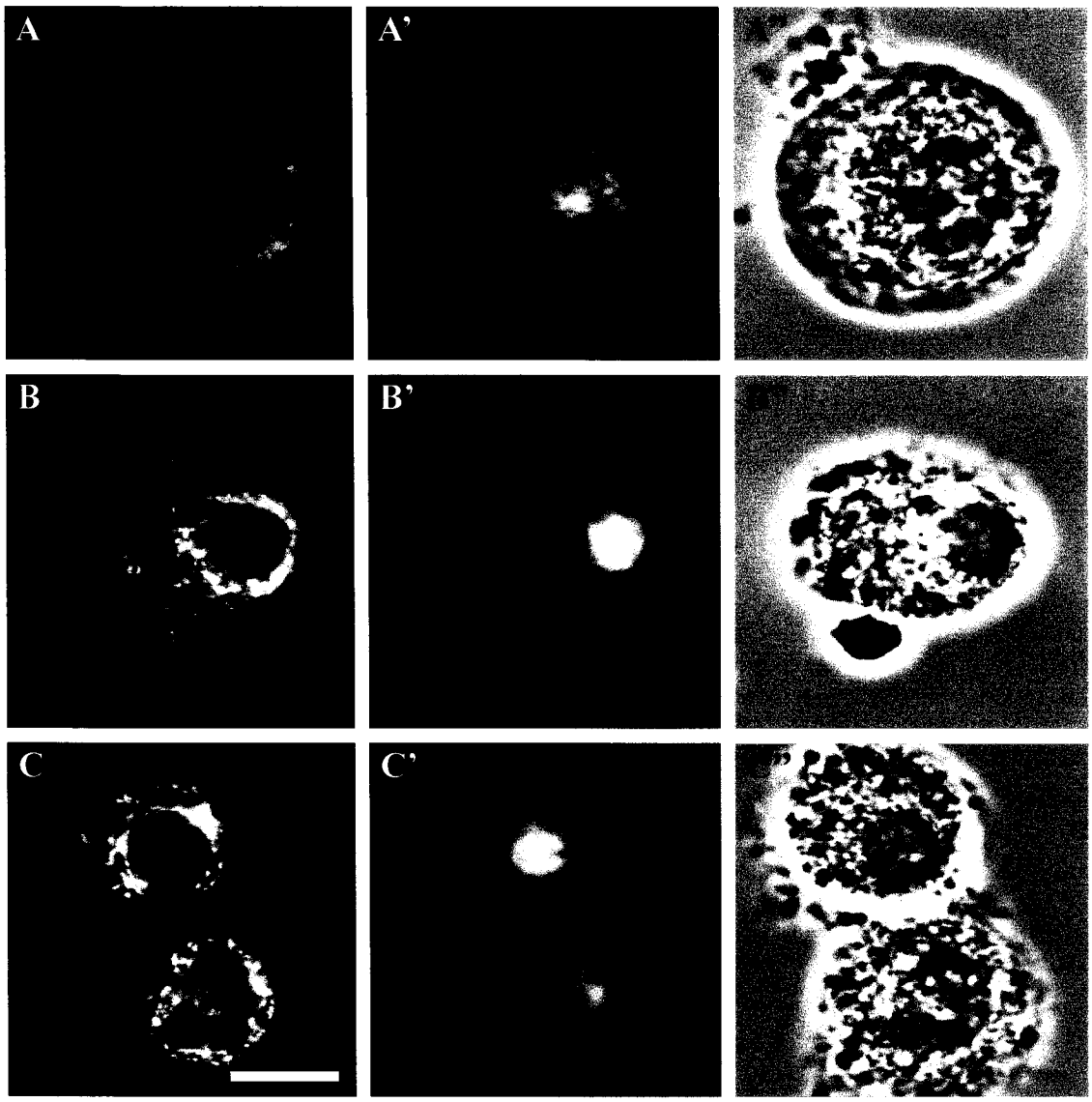

A
A'
A"
B
B'
C
C'

Figure 21. Incomplete assembly of lamin B in nuclei of digitonin permeabilized mitotic HeLa cells. Cells were labeled for immunofluorescence with an anti-lamin B1 (A-C). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-C') and observed by phase-contrast microscopy (A''-C''). Pictures show cells after permeabilization (A-A''), after a 30 minute incubation (B-B''), and after 2h (C-C''). Bar = 10 μm.


A
A'
B
B'
C
C'

were the cells used in the original method (Kourmouli *et al.*, 2000). However, for all three attempts, the yield of nuclear assembly of markers was even worse than with HeLa cells. LAP2 as well as lamin B had a diffuse cellular localization in synchronized mitotic CHO cells (Fig. 22 A; Fig. 23 A), comparable to what has been observed in HeLa cells. Similarly to HeLa cells, LAP2 and lamin B formed cytoplasmic inclusions as assembly proceeded and began to assemble around chromatin after a 45 minutes incubation (Fig. 22 B; Fig. 23 B). However, chromosomes were still visibly condensed and remained separate after 2 hours (Fig. 22 D'; Fig. 23 D'), augmenting the surface of chromatin to be covered and resulting in a poor yield of assembly (Fig. 22 D; Fig. 23 D). In an attempt to decondense chromatin further, an ATP-generating system (same as in disassembly systems; see Material and Methods) was added after 30 minutes of incubation. The results were the same in reactions with or without ATP at 15 minutes after its addition (Fig. 22 C; Fig. 23 C). However, after 2 hours of incubation, assembly was even poorer in reactions with ATP (Fig. 22 E; Fig. 23 E), indicating an inhibitory effect of the ATP on assembly.

Nuclear assembly had previously been shown to be prevented by depletion of lamins in mitotic extracts of CHO (Burke and Gerace, 1986) and by blocking lamin B in *Xenopus* cell-free extracts (Lopez-Soler *et al.*, 2001). Blocking lamin B function in HeLa cells with antibodies was therefore performed to appraise the assay. A preliminary blocking experiment was performed by addition of antibodies directed against lamin B1 after permeabilizing mitotic HeLa cells with digitonin. At the start of the assay, Western blot analysis showed that the digitonin permeabilized cells had lost some of their soluble proteins into the buffer, namely lamins A/C (Fig. 24, C; lane 4), tubulin (Fig. 24, E; lane 4) and part of LAP2 proteins (Fig. 24, D; lane 4). The presence of protein bands in the intact cell wash buffer (Fig. 24 A-E; lane 1) was probably due to some cell breakage during centrifugation. After 2 hours of assembly, the blocked and control preparations were observed by immunostaining with marker antibodies. No difference from controls was noticed by microscopy on both attempts (results not shown). However, western blot analysis of fractions revealed differences with controls. For instance, blocked cells seemed to have lost more material than controls from their cytoplasm. Less proteins were detected in the fraction containing the membrane and soluble components of the

Figure 22. Incomplete assembly of LAP2 in nuclei of digitonin permeabilized mitotic CHO cells. Cells were labeled for immunofluorescence with a monoclonal antibody against LAP2 proteins termed 13d4 (A-E). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-E') and observed by phase-contrast microscopy (A''-E''). Pictures show cells following permeabilization (A-A''), after 45 minutes of incubation (B-B''), after 45 minutes but with an ATP-generating system added after 30 minutes (C-C''), and after 2h without (D-D'') or with the addition of ATP (E-E''). Bar = 10 μm.

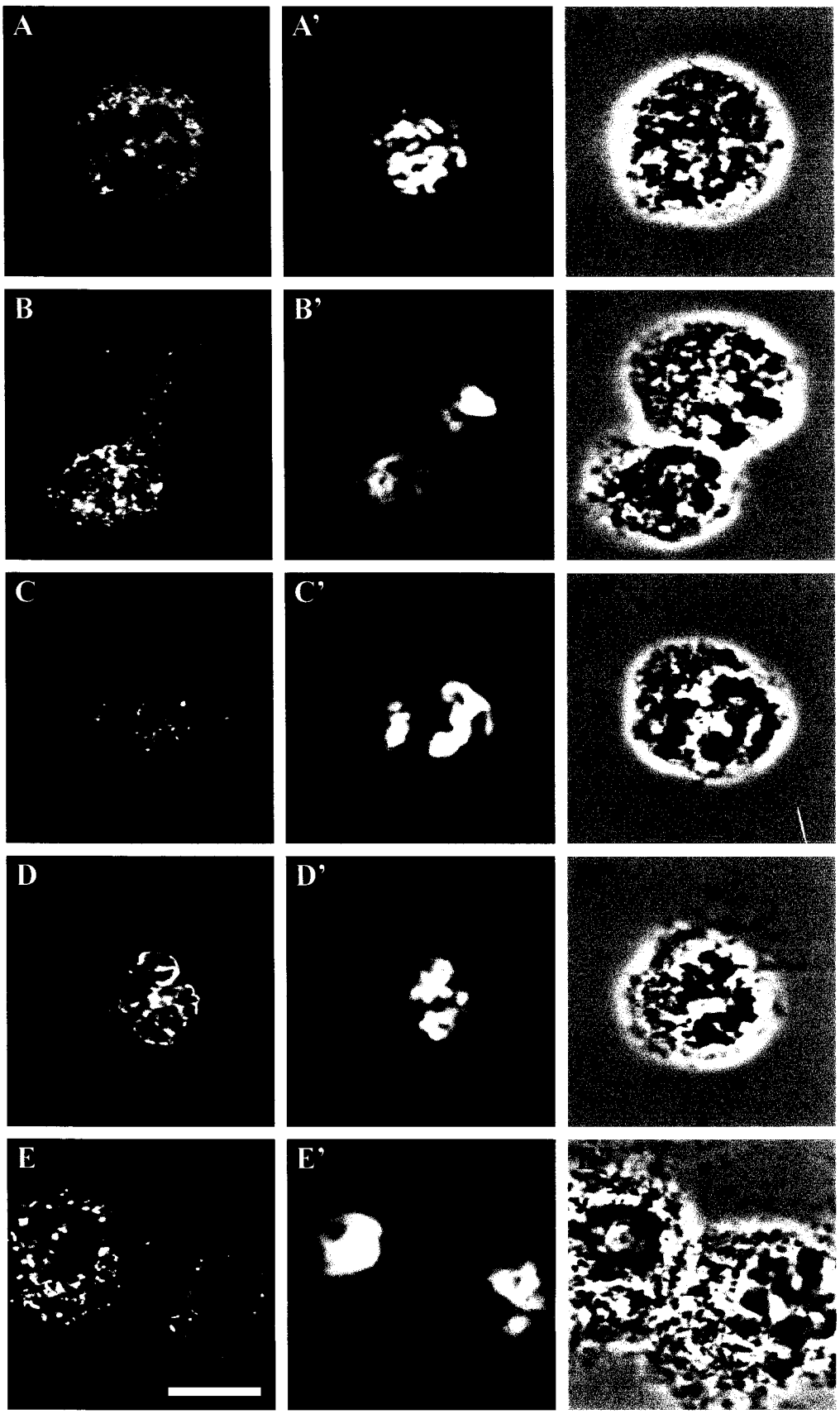
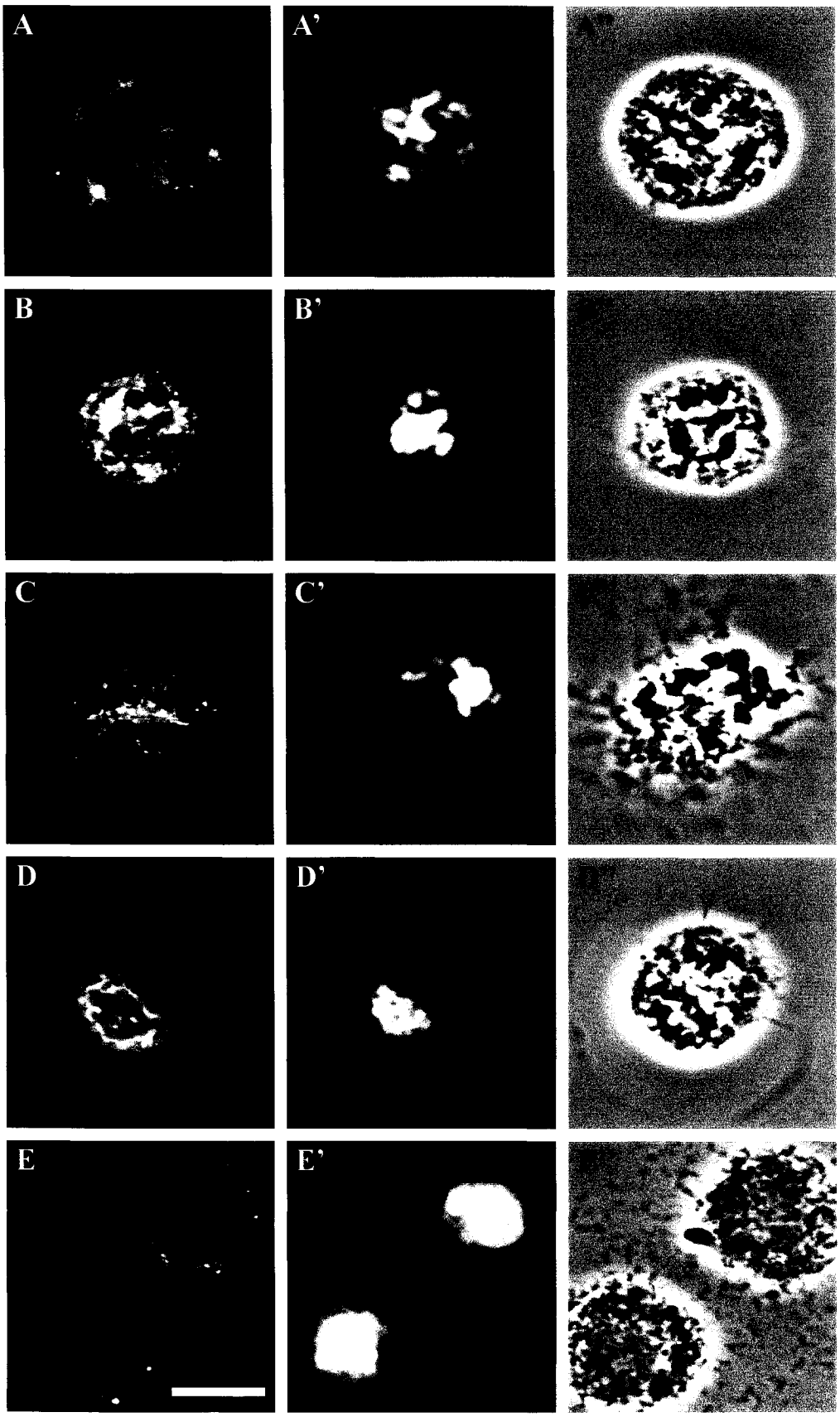

A
A'
B
B'
C
C'
D
D'
E
E'

Figure 23. Incomplete assembly of lamin B in nuclei of digitonin permeabilized mitotic CHO cells. Cells were labeled for immunofluorescence with anti-lamin B1 (A-E). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-E') and observed by phase-contrast microscopy (A''-E''). Pictures show cells following permeabilization (A-A''), after 45 minutes incubation (B-B''), after 45 minutes of but with an ATP-generating system added after 30 minute (C-C''), and after 2h without (D-D'') or with the addition of ATP (E-E''). Bar = 10 μm.


A
A'
B
B'
C
C'
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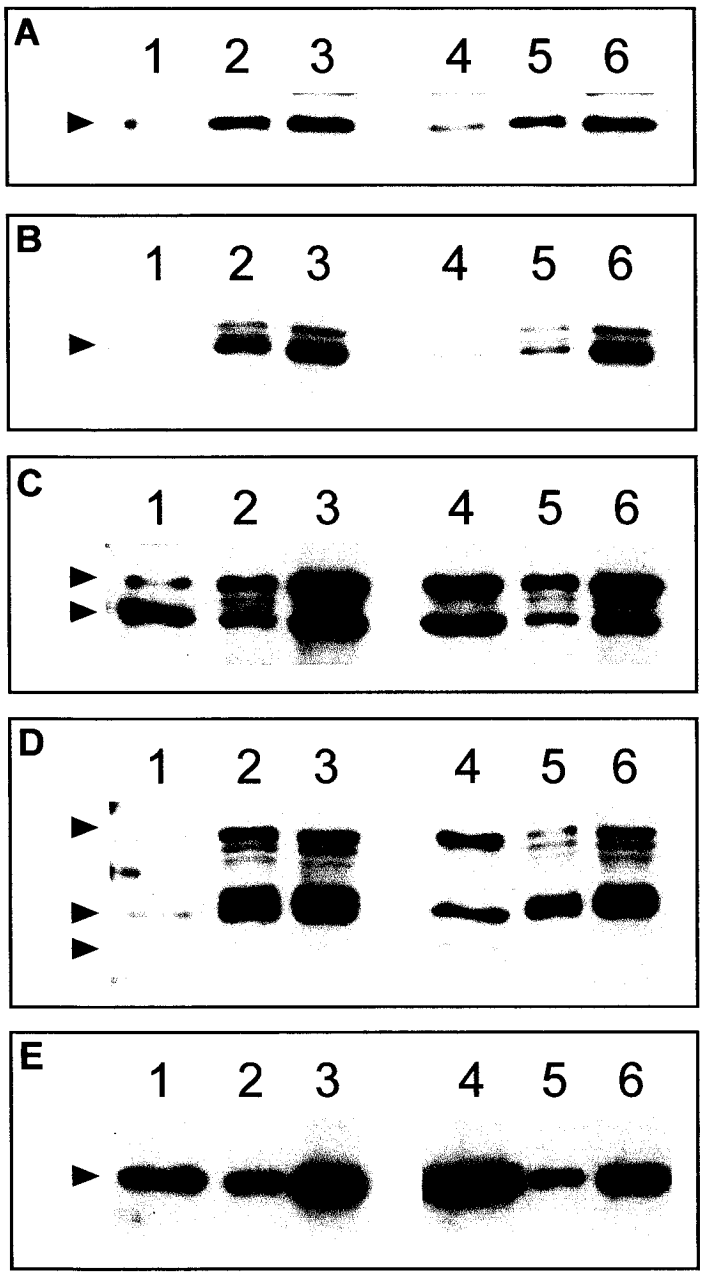
D'
E
E'

Figure 24. Western blot analysis of intact and digitonin permeabilized mitotic cells. Proteins were separated by SDS-PAGE and immunoblotted with antibodies directed against calnexin (A), lamin B (B), lamins A/C (C), LAP2 proteins (D) and tubulin (E). Mitotic cells either left intact (A-E; lanes 1-3) or permeabilized with digitonin (A-E; lanes 4-6) were isolated by centrifugation, the first supernatant corresponding to the buffer (A-E; lane 1, 4). The cells were then homogenized and fractionated into a pellet (A-E; lane 2, 5), and membranes and soluble fraction (A-E; lane 3, 6). Samples were loaded to have approximately the same number of cells in all wells. Arrows indicate relevant bands.


A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6
D
1 2 3 4 5 6
E
1 2 3 4 5 6

blocked permeabilized cells (Fig. 25 A-D; lane 9) than in the permeabilized controls (Fig. 25 A-D; lane 6). The missing proteins of the blocked cells seemed to be in the buffer (Fig. 25 A-D; lane 4 and 7). Otherwise, the protein content of nuclei and chromatin fractions did not appear to differ between preparations with added antibodies (Fig. 25 A-D; lane 8) and controls (Fig. 25 A-D; lanes 2, 5).

3.3. Yield of nuclear assembly in intact nocodazole synchronized cells

After the poor results obtained with both methods, the assembly of nuclei in intact nocodazole synchronized cells was assessed. HeLa cells were released from an 18h nocodazole block and incubated at 33°C for up to 2h, either in the buffer used for the digitonin permeabilized cells or in complete culture medium. Immediately following nocodazole block release, LAP2 antigens were in soluble or membrane bound form in the cytoplasm (Fig. 26 A). A relatively high number of interphase cells could be seen at the start of the experiment, representing a mean of 18% of the cell populations (Table 2). At 10 minutes after the release, LAP2 proteins started to associate with chromatin (Fig. 26 B). After 20 minutes, these proteins were found in cytoplasmic inclusions and started to assemble around the chromatin of some cells (Fig. 26 C). The yield of complete assembly was surprisingly poor after 2 hours of assembly (Fig. 26 D-E; Table 2), and the chromatin did not seem to decondense (Fig. 26 D'-E').

Microtubule organization was used to evaluate assembly in intact versus digitonin permeabilized nocodazole synchronized cells. At the start of the release, the tubulin was in soluble form in the cytoplasm of both intact and permeabilized cells (Fig. 27 A-B). After 45 minutes of incubation, the majority of intact nocodazole synchronized cells had assembled an aberrant number of mitotic spindle poles, e.g. three or more (Fig. 27 C, cell on the right), compared to the normal bipolar spindle (Fig. 27 C, cell on the left) of unsynchronized cells. Comparatively, permeabilized cells had lost most of their tubulin and displayed no staining (Fig. 27 D).

4. Adherence of nocodazole released cells

In light of the generally poor nuclear assembly of synchronized cells, the substrate adherence of cells released from a nocodazole block (1μM nocodazole for 18h) was

Figure 25. Western blot analysis of reassembled intact, control digitonin permeabilized mitotic cells and permeabilized cells exposed to goat polyclonal antibodies against lamin B. Proteins were separated by SDS-PAGE and immunoblotted with mouse monoclonal antibodies directed against calnexin (A), lamin B1 (B), lamins A/C (C) and LAP2 proteins (D). Cells were incubated for 2 hours at 33°C. These cells were either left intact (A-D; lanes 1-3), permeabilized with digitonin and nothing was added (A-D; lanes 4-6) or were permeabilized and lamin B1 antibodies from goat were added (at dilution 1:50) at the start of the reaction (A-D; lanes 7-9). All cells were isolated by centrifugation, the first supernatant corresponding to the buffer (A-D; lane 1, 4, 7). The cells were then homogenized and fractionated into a pellet (A-D; lane 2, 5, 8) and membranes and soluble fraction (A-D; lane 3, 6, 9). Samples were loaded to have approximately the same number of cells in all wells. Arrows indicate relevant bands.

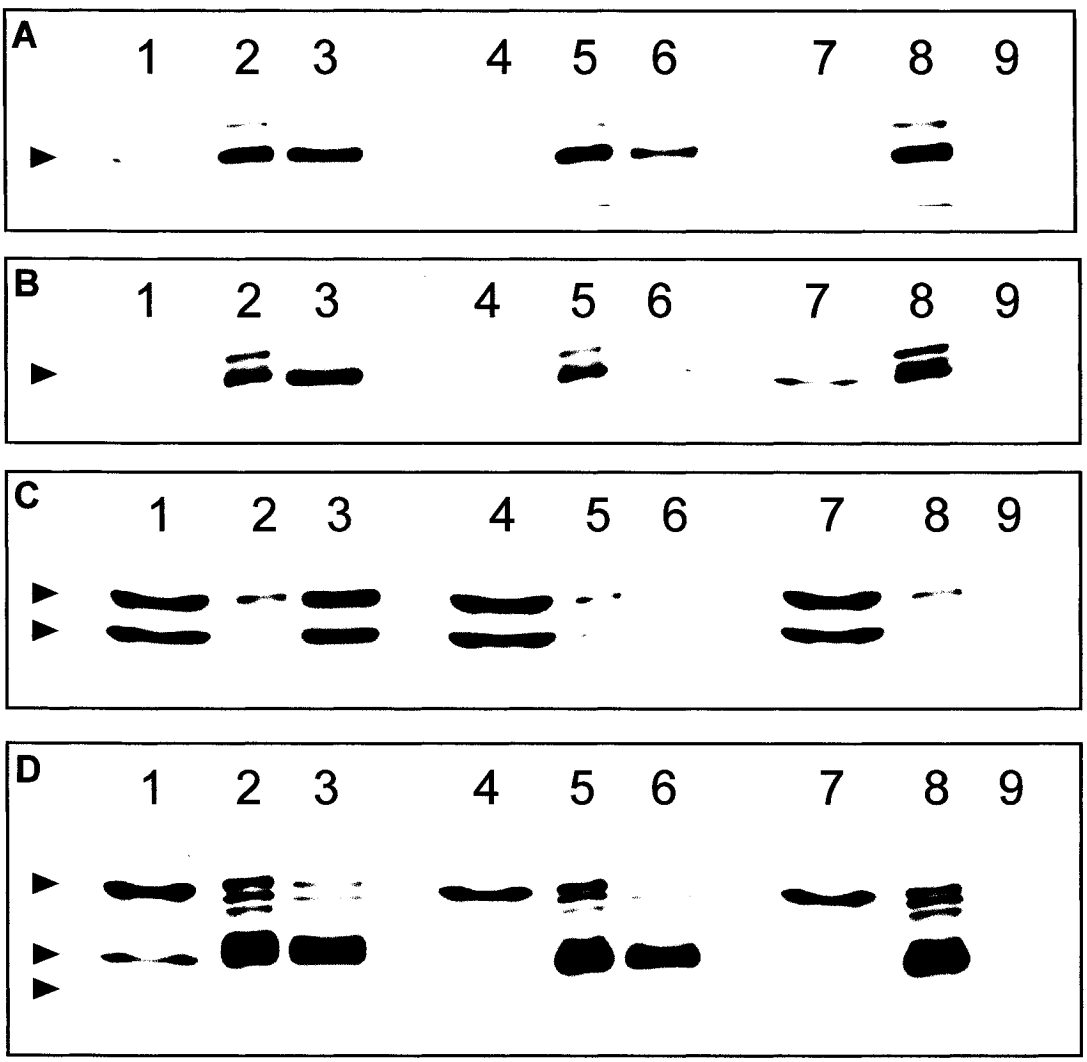
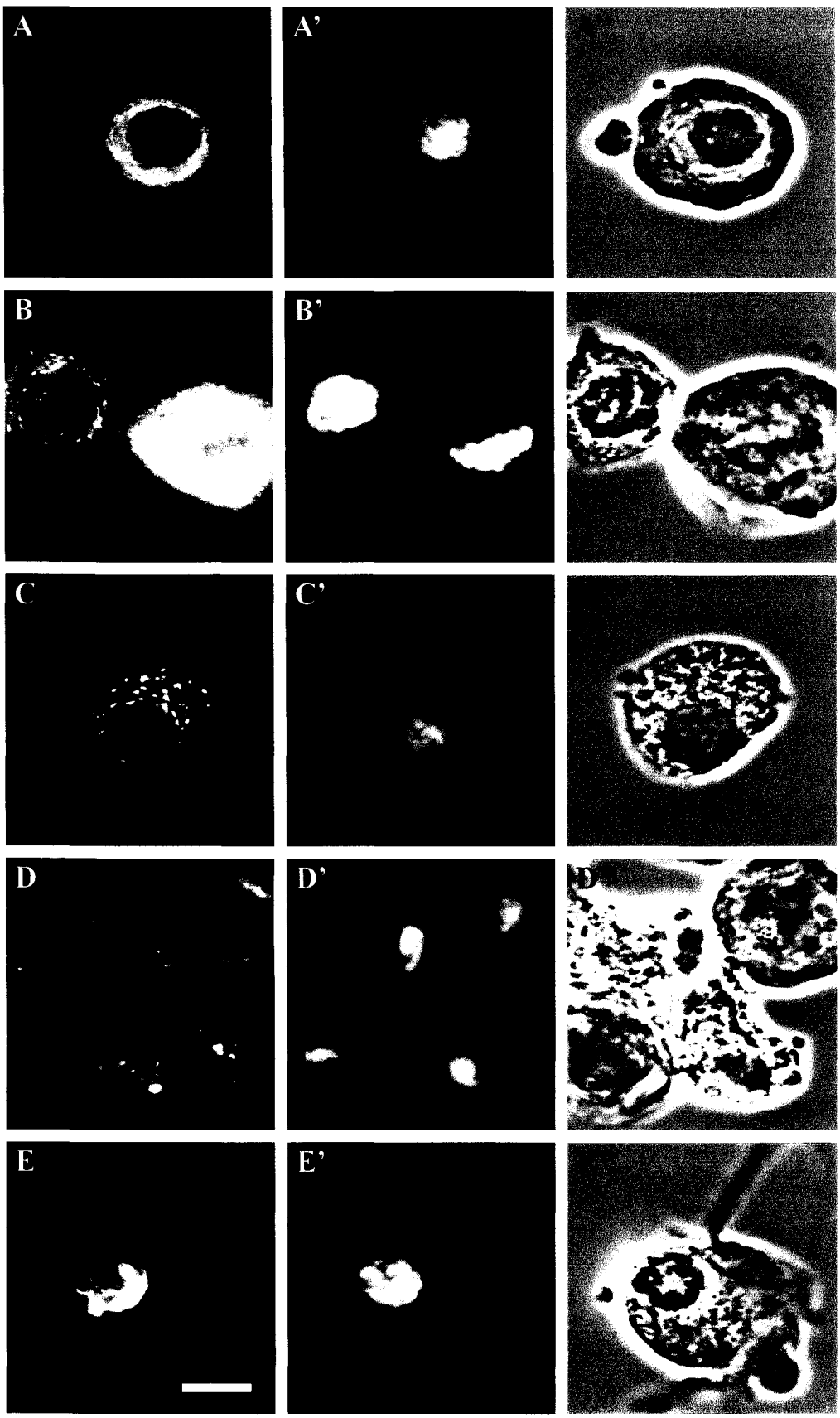

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9
D
1 2 3 4 5 6 7 8 9

Figure 26. Incomplete assembly of LAP2 in nuclei of nocodazole synchronized HeLa cells. Cells were labeled for immunofluorescence with anti-LAP2 (A-E). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-E') and observed by phase-contrast microscopy (A''-E''). Pictures show cells following release from nocodazole block (A-A''), after 10 minutes of incubation (B-B''), after 20 minutes (C-C''), and after 2h (D-D'', E-E''). Bar = 10 μm.


A
A'
B
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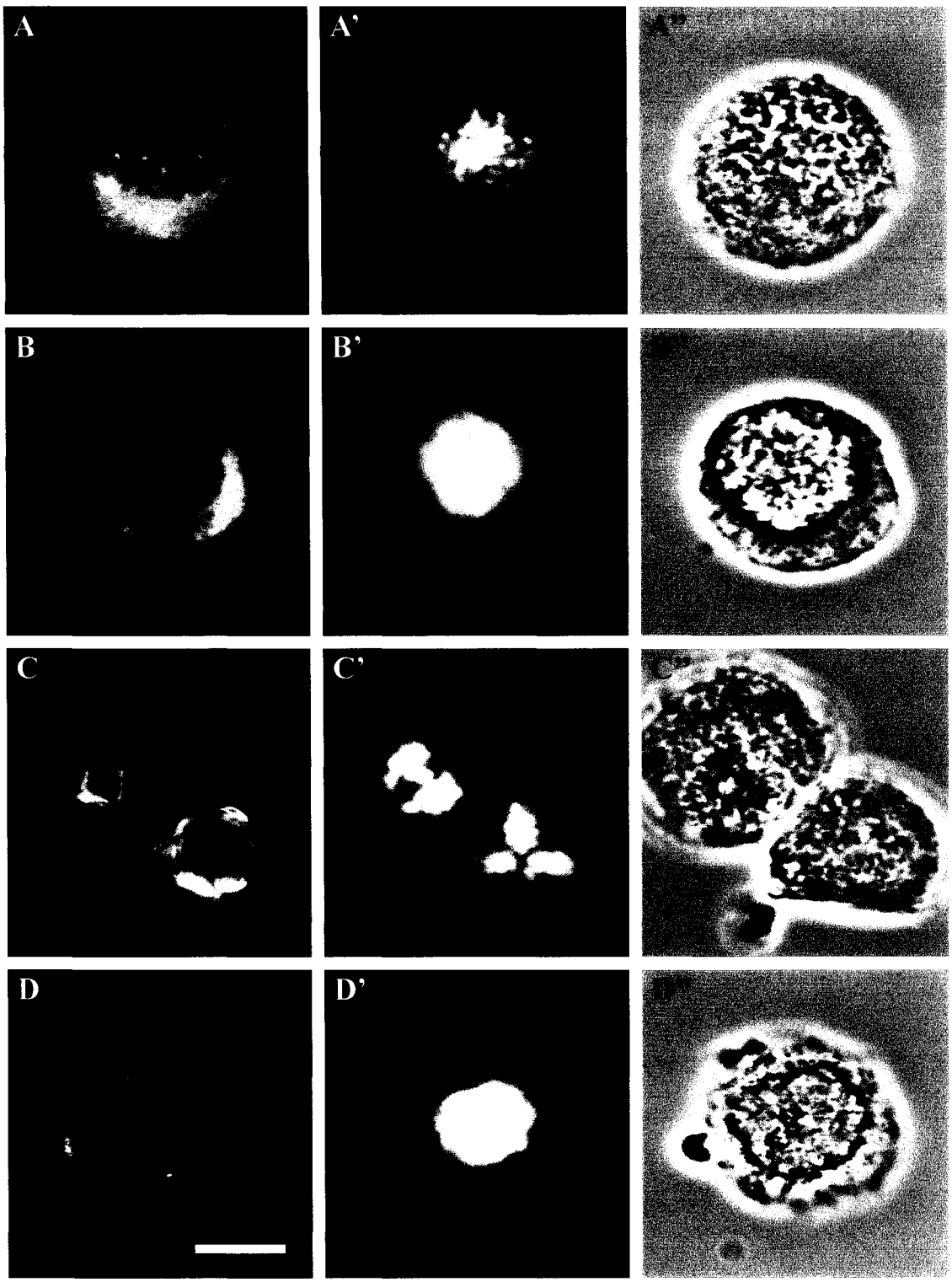
D'
E
E'

Table 2. Nuclear assembly of LAP2 in synchronized HeLa cells. Cells were synchronized 18h with 1μM nocodazole, washed, and released from the block by incubating in complete medium (medium) or in buffer (buffer). Cells were fixed and immunostained either at the start of the release (t=0) or after two hours (t=2h).

	t=0				t=2h			
	medium		buffer		medium		buffer	
Assembly with decondensed DNA	54	24%	26	12%	43	21%	36	16%
Assembly with condensed DNA	0	0%	0	0%	35	17%	43	19%
Incomplete assembly	0	0%	0	0%	75	36%	54	24%
No assembly	174	76%	198	88%	55	26%	93	41%

Figure 27. Tubulin distribution in intact or digitonin permeabilized, nocodazole synchronized HeLa cells. Cells were labeled for immunofluorescence with anti-tubulin (A-D). Preparations were counter-stained with Hoechst 33258 to visualize DNA (A'-D') and observed by phase-contrast microscopy (A''-D''). Pictures show cells following release from nocodazole block either intact (A-A'') or permeabilized with digitonin (B-B''), and after a 45 minute incubation either intact (C-C'') or permeabilized (D-D''). Bar = 10 μm.


A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''

monitored. Cells blocked in prometaphase were rinsed and cultured in fresh medium to resume growth. When healthy HeLa cells are in suspension, either by mitosis or from induced detachment, they will attach to the substrate before they grow. However, after 24h in fresh complete medium and regular culture conditions, only 40% of the released cells attached to the substrate (Table 3).

Table 3. Adherence of nocodazole synchronized HeLa cells. Cells were synchronized by exposure to 1μM nocodazole for 18h, then washed and released from the block by incubating in complete medium for 24h.

# of floating cells	Total # of cells	% of attachment	
3.2×10^5	5×10^5	36%	
2.8×10^5	5×10^5	44%	
		40%	Mean

IV. Discussion

Single and double immunolabelling of unsynchronized HeLa cells confirmed the validity of the immunological tools used in this study. The staining patterns were in agreement with previous published data. INM proteins were localized at the nuclear periphery as previously shown for the LEM proteins (Paulin-Levasseur *et al.*, 1996), LAP2 (Berger *et al.*, 1996), lamins A/C and lamin B (Goldman *et al.*, 1992; Bridger *et al.*, 1993). The nucleocytoplasmic shuttling protein 2A7 was seen to reside within the nucleus as expected (Paulin-Levasseur and Julien, 1999), and LAP2α was dispersed throughout the nucleoplasm (Dechat *et al.*, 2000b). Sequences of mitotic events were also monitored in comparison with the literature. During prometaphase, lamins A/C were the first to be released from the nucleus, followed by LEM proteins, lamin B and LAP2α. In metaphase, all observed antigens were scattered in the cytoplasm. LEM proteins, lamin B and LAP2α reintegrated the nucleus before lamins A/C, which remained detectable in the cytoplasm during late telophase / early G1 (reviewed by Buendia *et al.*, 2001).

1. Nuclear antigens in micronuclei

Some interphase cells contain micronuclei, which are chromatin segments that were left out of the nucleus during mitosis. It is thought that micronuclei preferentially capture extrachromosomal molecules and only infrequently contain chromosomes (Heddle and Carrano, 1977). They can also be created by a budding process to eliminate the ends of abnormally long chromosomes, which seriously impair fertility and viability (Schubert and Oud, 1997). Immunofluorescence microscopy showed that HeLa micronuclei contained various nuclear proteins. Inner nuclear membrane proteins, like LEM and LAP2 proteins, were always found in the envelope of micronuclei.

Lamins A/C were detected in micronuclei, which had also been observed previously in radiation induced micronuclei (Walker *et al.*, 1996). The NE can be considered functional when it is capable of specific import and export of molecules between the cytoplasm and the nucleus, exchanges which are accomplished through

nuclear pores. Since lamins A/C reentry necessitates functional pores (Moir *et al.*, 2000), their presence inside micronuclei seems to imply the presence of a functional envelope capable of nuclear import. Nuclear pore complexes were indeed shown to be present in some micronuclei by single antibody staining immunofluorescence. This partial presence only explains how lamins A/C were able to enter the micronuclei possessing nuclear pores. If all micronuclei contain lamins A/C and only some have nuclear pores, then for micronuclei lacking NPCs, it would indicate a passive trapping of lamins A/C as the nuclear envelope reforms and sequesters nucleoplasmic material. It would be worthwhile to perform double immunolabeling experiments with antibodies directed against nuclear pores and lamins A/C. If functional pores are truly essential for nuclear incorporation of lamins A/C, we expect to see pore complexes whenever lamins A/C are present.

The absence of lamin B from the majority of observed micronuclei was somewhat surprising, since it is commonly believed that lamin B is essential for nuclear assembly (Burke and Gerace, 1986; Lopez-Soler *et al.*, 2001). There is nonetheless evidence that assembly can proceed without lamin B *in vivo*, but this ultimately leads to apoptosis (Steen and Collas, 2001). The fact that cells with assembled nuclei lacking lamin B will undergo apoptosis is compatible with the fate of micronuclei, which will eventually be lost from the cell. The mechanism behind the elimination of micronuclei from the cell is not well understood. Once they are generated, they could be released from the cell, degraded inside the cell, or inefficiently reincorporated into the nucleus at mitosis (Von Hoff *et al.*, 1992). There is at least evidence for the extrusion of micronuclei from cells, since extracellular micronuclei surrounded by a triple membrane were observed (Shimizu *et al.*, 1998).

Beyond eliminating fragments of DNA from the nucleus, micronuclei may also play an important role in cancer. Tumor cells can revert from their malignant phenotype and differentiate by the elimination of oncogenes amplified on the double minutes, which are circular extrachromosomal DNA that adhere to normal chromosomes at mitosis. This expulsion is achieved by selective incorporation of double minutes into micronuclei (Shimizu *et al.*, 2000). The process can be greatly accelerated by treating the cells with drugs such as hydroxyurea (Von Hoff *et al.*, 1992). Micronucleation has also been shown to be induced by dietary curcumin in breast cancer cells (Holy, 2002). These

results suggest that the loss of extrachromosomally amplified genes can be accelerated by some agents, making tools available to moderate the growth of some human neoplasms (Von Hoff *et al.*, 1992). As another example of the importance of micronuclei in medicine, their analysis is gaining popularity as an *in vitro* genotoxicity test and biomonitoring for human genotoxic exposure and effect (Norppa and Falck, 2003).

2. Nuclear access during mitosis

Digitonin permeabilization of cells allowed access to the cytoplasm without affecting the nuclear membranes. It is a useful permeabilization method to determine the moment when the NE becomes "leaky" to the cytoplasm. Staining with the MAN antiserum of a prometaphase cell (Fig. 8 C) showed a deformed nucleus still possessing a NE, but nonetheless "leaky". Although to my knowledge never before done in mammalian cells, access to the nucleus during nuclear disassembly had been previously assessed *in vivo* in Starfish oocytes. Entry of microinjected fluorescent 70 kDa dextran into the nucleus began several minutes before NEBD (Terasaki *et al.*, 2001), which is consistent with my observations on digitonin permeabilized HeLa mitotic cells.

Digitonin permeabilization can also help distinguish if a nuclear protein is present in the inner or the outer membrane of the nucleus, since their staining would look the same. Interphase cells showed no staining with the MAN antiserum (Fig. 8 A), which indicates an inner nuclear localization of the LEM proteins. This observation is consistent with the idea that the family of LEM proteins recognized by the MAN antiserum is constituted in mammalian cells of only integral INM proteins interacting with the nuclear lamina and other nuclear components.

3. Cell synchrony

HeLa cells, like most wild populations of cultured mammalian cell lines, have a limited number of cells in mitosis, namely a proportion of about 5%. To obtain sufficient numbers of mitotic figures for experimentation, cells were synchronized by M-phase block, namely by reversibly disrupting microtubule function. An asynchronous population of cells was exposed to nocodazole, which arrests cells in prometaphase by inhibiting assembly of the microtubule subpopulation necessary for chromosome

separation (Merrill, 1998). The cells were further isolated by mechanical shake-off (Terasima and Tolmach, 1963; Tobey *et al.*, 1967), which selectively detaches rounded M-phase cells. Mammalian cells treated with anti-microtubule drugs progress through interphase but are arrested at mitosis. According to the literature, the HeLa human cell line completes mitosis after release from a nocodazole arrest (4 hours at 0.04μg/ml or 0.13μM, with a preliminary thymidine block) in an average of 80 minutes, with over 80% of the spindles appearing to be bipolar (Zieve *et al.*, 1980). If cells are arrested for extended periods in mitosis, or with higher concentrations of drug, mitotic spindles reform with less efficiency with a large percentage of cells incapable of returning to interphase (Zieve *et al.*, 1980). Prolonged exposures to nocodazole also cause irreversible polyploidization and the formation of micronuclei when some cells exit mitosis without dividing (Miller-Faurès *et al.*, 1981; Nüsse and Egner, 1984). Hence, short exposures to small concentrations of nocodazole seem the best way to go, but it only yields small numbers of mitotic cells. The "best" concentration and time of exposure are therefore the smallest and shortest to obtain enough mitotic cells.

For the experiments in the present study, a conservative exposure of 1 μM nocodazole for 18h was used, as in other published articles (Collas *et al.*, 1999). After the cells were released, aberrant numbers of mitotic spindles (three or more) were observed in more than half of the cells. Furthermore, 60% of cells released from a nocodazole block and cultured in complete medium failed to attach to the substrate. It was also previously reported that a 24h nocodazole block (0.1 μM) of a human malignant glioma cell line resulted in death for the majority of the cells after 24h of recovery (Hueber *et al.*, 1998). Presumably, nocodazole block detrimentally affects cell health. Nonetheless, Klein and colleagues (1997) found that by dye exclusion, nocodazole synchronized (2.5 μM for 24h) HeLa cells had a 95% viability rate.

Nocodazole has nonetheless been used in many studies where the results obtained from synchronized and unsynchronized cells correspond. For example, up-regulated activity of LIM-kinase 1 was monitored in mitotic cells as well as in prometaphase synchronized cells (Sumi *et al.*, 2002). Nucleoplasmic coiled bodies were also analyzed for expression of p80-coilin in cell cultures synchronized by many methods, including nocodazole block, and the results were consistent (Andrade *et al.*, 1993).

A culture is said to be synchronized when all of the cells pass through successive phases of the cell cycle at essentially the same time. There are two experimental approaches to obtain synchronized cell cultures, namely induction and selection (reviewed by Merrill, 1998; Davis *et al.*, 2001). Induction techniques attempt to convert entire growing cultures to a synchronously dividing population. This can be achieved by 1) starvation and replenishment of a required cell culture component; 2) contact inhibition; 3) periodic changes in temperature, nutrition or light; and 4) temporary exposure to blocking drugs. Selection procedures of cell cycle phases entail either collecting the loosely attached mitotic cells from a culture growing on a substrate (Terasima and Tolmach, 1963; Tobey *et al.*, 1967), selecting by size with centrifugal elutriation (Pretlow and Pretlow, 1979), or by any particular cell parameter with a cell sorter (review by Givan, 2001). A relatively new selection method, based on a bacterial system (Helmstetter, 1969; Helmstetter *et al.*, 2001), is membrane-elution, also known as the "baby-machine" (Cooper, 2002). In this method, cells are adhered to a membrane by filtration, which is then inverted and warm media is pumped through it from the top. Bonded cells grow upside-down on the membrane, and at division, one cell remains attached to the membrane, while the other is released into the medium. Cells eluted from the membrane over a short period of time produce a synchronized culture that displays up to four synchronous cell cycles (Thornton *et al.*, 2002). This method is obviously only useful for cells that grow attached to a substrate.

Although the nocodazole "batch" synchronization was necessary in the case of this study to get enough mitotic cells, it suffers many drawbacks. It has been argued that even though batch treatments are extensively used and traditionally considered valid, they simply do not result in a synchronized cell population (Cooper, 1998; Cooper, 2003). Induced G0 arrest, or G1 phase arrest, by starvation or inhibition may be the most utilized approach to cell cycle analysis. The traditional way of defining a G1 cell block is a 2n DNA content. Cells are considered to resume cell cycle synchronously when they are released from the block. However, since growth was inhibited, they have a wide size distribution. Because they need to get to a normal size before entering S phase, the resulting cell population is theoretically no more synchronized than before (Cooper, 1998). The rapid loss of synchrony encountered in induction methods could be due to the

fact that they are in reality not synchronized. This would explain why I was unable to observe a higher mitotic index in HeLa cells synchronized by serum deprivation and replenishment or double thymidine block. A more accurate and useful description of synchronized cells could be that they (1) have a reduced cell mass distribution, (2) have the same DNA content, and (3) progress through the cell cycle simultaneously (Cooper, 1998).

Induction methods can however still be used to obtain a particular cell property, for example, condensed chromatin lacking a nuclear envelope in the case of nocodazole block (Cooper, 2002). But even then, meaningfulness of the result can be arguable. The method of synchronization as well as the cell line can engender different results. This is the case for the cell-cycle effect of DNA-end binding (DEB) activity of Ku, the DNA-binding component of the protein kinase, DNA-PK. While DEB activity varied during the cell cycle of chemically synchronized cells, cells from an unsynchronized population separated by centrifugal elutriation into distinct mitotic phases showed no difference in DEB activity (Chou and Chou, 1999).

In this study, metaphase nocodazole synchronization of HeLa cells resulted in incomplete and aberrant assembly of nuclei compared to an unsynchronized population. Nuclei assembled for two hours from nocodazole synchronized cells showed incomplete nuclear envelopes as well as condensed chromatin. Also, assembly did not appear to proceed in the same way as in unsynchronized cells. During nuclear assembly of nocodazole synchronized cells, cytoplasmic inclusions of INM antigens were observed. These cytoplasmic nuclear antigen inclusions are reminiscent of the inclusions found in "normal" HeLa cells at the end of telophase. However, they were much more prevalent in cells released from a nocodazole block than in unsynchronized HeLa.

4. Validity of cell-free systems of nuclear disassembly and assembly

In this study, it was observed that the HeLa and CHO cell-free systems of nuclear disassembly and assembly give unreliable results. Nuclear disassembly was attempted numerous times with different batches of mitotic extracts and of isolated interphase nuclei, but without success. A personal communication from Dr. Collas revealed that according to his experience, a successful disassembly reaction seemed to depend on the

isolation of nuclei devoid of surrounding material. For unknown reasons, this only happens for some of the isolations. Immunofluorescence showed that the material surrounding isolated nuclei contained vimentin, an intermediate filament. It is unclear how vimentin could have prevented disassembly since intermediate filaments are normally present in cells and do not necessarily disassemble during cell division. During mitosis, vimentin is known to form a "cage" around prometaphase chromosomes and the mitotic spindle (Aubin *et al.*, 1980; Maison *et al.*, 1993). The presence of vimentin around isolated nuclei could therefore hardly account for the lack of disassembly.

This study has also shown that the synchronization of HeLa and CHO cells by nocodazole block results in incomplete and abnormal nuclear assembly of INM proteins like the LEM proteins and LAP2. The mechanism behind the nocodazole block is that mitotic spindles will not form, halting mitosis at prometaphase and preventing subsequent steps of the cell cycle. Since it has become clear that the mitotic spindle plays an important part in disassembly by pulling the NE apart in a specific manner (Beaudouin *et al.*, 2002; Salina *et al.*, 2002), NEBD without mitotic spindles can not happen as *in vivo*. Presumably, an aberrant disassembly will not result in a "normal" reassembly. Furthermore, the abnormal number (3 or more compared to 2) of mitotic spindles could affect chromosome segregation, consequently altering assembly. If the block itself affects assembly, then inferences made from nocodazole synchronized cells will not reflect *in vivo* events.

In cell-free systems of assembly - homogenates as well as digitonin permeabilized mitotic cells - mitotic spindles are totally absent. They are also lacking in cell-free systems of nuclear disassembly, theoretically leading to a NEBD based solely on the weakening associations of nuclear components caused by hyperphosphorylation and not on the specific pattern of tearing initiated by astral microtubules. Since many studies have used cell-free systems of nuclear disassembly and assembly, their relevance to *in vivo* conditions needs to be examined.

Although the many cell-free systems have laid the groundwork for a biochemical dissection of nuclear disassembly and reassembly, evidence from cell-free systems is sometimes contradicted by observations made in whole cells. An *in vivo* electron-spectroscopic imaging study of fixed HeLa seems to show that nuclear envelope cisternae

preassemble in the cytoplasm before docking to the chromatin and gradually forming mature envelopes (Stracke and Martin, 1991). Evidence for the coating of newly segregated chromatids with ER-like cisternae also comes from live cells (Ellenberg *et al.*, 1997). Conflicting with these observations, NE assembly in cell-free systems occurs at the surface of chromosomes (Burke and Gerace, 1986; Collas *et al.*, 1996; Lopez-Soler *et al.*, 2001). However, my experiments with intact (Fig. 24) and digitonin permeabilized (Fig. 18 to 21) synchronized mitotic cells show cytoplasmic inclusions of inner nuclear membrane proteins in great numbers as assembly progresses. These might be nuclear envelope cisternae pre-forming in the cytoplasm, making the digitonin system closer to *in vivo* conditions than cell-free systems based on homogenates.

In another example of the differences between cell-free systems and whole cells, *in vitro* experiments show that vesicles enriched in NE proteins can be isolated from fractionated mitotic cells (Vigers and Lohka, 1991; Chaudary and Courvalin, 1993; Buendia and Courvalin, 1997). This led to the theory that nuclear membranes are fated to vesiculation at nuclear disassembly. In contrast, studies of intact mammalian cells have demonstrated that nuclear membrane proteins are present in the ER during metaphase and that the ER network itself remains intact during division of somatic cells (Ellenberg and Lippincott-Schwartz, 1999). This evidence would indicate that NE proteins are absorbed into the ER at mitosis. The disparity of these results could reflect a difference between cell types. However, another plausible explanation for this apparent contradiction is that the vesicles that arise specifically from the ER and the NE are artifacts of the isolation.

5. Future research

Transfecting cells with GFP-tagged nuclear proteins would permit live observation of assembly and disassembly, as has already been done with the lamin B receptor (Ellenberg and Lippincott-Schwartz, 1999; Haraguchi *et al.*, 2000) as well as with emerin (Haraguchi *et al.*, 2000), a method that also eliminates the possibility of artifacts from fixation. Moreover, the use of different fluorescent proteins would permit real time observation of co-localization and in which order the nuclear proteins leave and rejoin with the nucleus. This could only be done with some proteins since they have not all been sequenced yet, but cloning of remaining nuclear proteins is also feasible.

However, there is always the concern that attaching a peptide as big as GFP to the protein of interest will change its behavior, or that overexpression of the protein will overload the system. Also, unlike classical immunofluorescence, it does not have the wide range of markers available for study in their normal cellular levels or of the possible cell types.

A more appropriate method of synchronization would be to isolate cells already in mitosis. It could be possible with the novel "baby-machine" method (Cooper, 2002), but its major drawback would be obtaining large numbers of mitotic cells, which would necessitate a complex setup and large amounts of cell culture media. Alternatively, isolation of mitotic figures from an unsynchronized population could be achieved by centrifugal elutriation (Davis *et al.*, 2001). However, this method also necessitates specialized equipment and large numbers of cells. To forgo permeabilizing the cells, microinjection could be used to insert the blocking agent inside the cells. This method would still have an effect on the cells, and only a small number of cells could be injected at one time. The study of disassembly would require injection of NLS-conjugated peptides (Martins *et al.*, 2000), this done prior to the expected time of mitosis. To investigate nuclear assembly, blocking peptides would need to be microinjected during mitosis to block a protein function prior to reassembly.

There are other methods of blocking the function of nuclear proteins than cell-free systems. An option is the overexpression of nuclear protein fragments in cells or whole organisms. This has been done previously with the mammalian nucleoporin p62 in HeLa cells, where overexpression of the C-terminal domain tail of p62 induced nuclear fragmentation (Barth, 1999). Another possibility is an *in vivo* block of protein function in cells or whole organisms, either with antisense, short interfering RNA (siRNA), or morpholinos. Antisense molecules and siRNA, which can be delivered by transfection into cell lines, block protein expression by binding to messenger RNA (mRNA). Both methods have been used to specifically target DNA-PK, a nuclear protein crucial for the repair of DNA double-strand breaks (Collis *et al.*, 2005). Morpholino antisense oligos can be used to block mRNA translation on a range of model organisms, from sea urchin, to zebrafish to mouse (reviewed by Heasman, 2002). Achieving overexpression of a protein or getting antisense and siRNA into a cell can be done through transfection, or even using a virus delivery system. Morpholinos on the other hand have to be

microinjected. Although having their own advantages, these options would not specifically block the function of nuclear proteins during nuclear disassembly or reassembly, but throughout the cell cycle. Hence, despite their drawbacks, cell-free systems remain an invaluable tool for the study of nuclear breakdown and assembly because they are still the only way to isolate these processes for study.

On a more fundamental level, if cell-free systems are here to stay, the effects of cell synchronization, particularly nocodazole block, on nuclear assembly need to be thoroughly examined.

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