**IMMUNOFLUORESCENCE AND BIOCHEMICAL
CHARACTERIZATION OF THE NUCLEAR ENVELOPE
ANTIGEN, PI2**

by

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the requirements for the degree of

Master of Science

Department Biology

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Immunofluorescence and Biochemical Characterization
of the Nuclear Envelope Antigen, PI2

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ABSTRACT

Previously, monoclonal antibody PI2 was shown to label interphase animal cells primarily at the nuclear rim, and mitotic cells throughout the cytoplasm. The antigen was tightly associated with the nuclear envelope, resisting extraction during nuclear matrix preparation. In this study, the nature of this association has been investigated by immunoblotting and/or immunofluorescence microscopy of mouse 3T3 fibroblast samples permeabilized with digitonin, or differentially extracted with 8 M urea, 0.1 M NaOH, or 1% TX-100 and up to 500 mM NaCl, and probed for PI2 or for known proteins of the nuclear membrane (LAP2, emerin) and lamina (lamin B). The results indicate that PI2 is highly insoluble and behaves like a protein of the INM. Further, double immunolabelling of cycling cells suggests that PI2 is not involved in initiating post-mitotic envelope reassembly, as PI2 and lamin B were recruited to the envelope at the same time, and after LAP2 and emerin.

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LIST OF ABBREVIATIONS

3T3	mouse fibroblast cell line
AKAP149	A-kinase anchoring protein 149
BAF	barrier-to-autointegration factor
CY3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
EDMD	Emery-Dreifuss muscular dystrophy
EL4	mouse lymphoma cell line
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HeLa	cervical carcinoma cell line derived from Henrietta Lacks
HP1	heterochromatin protein 1
INM	inner nuclear membrane
kDa	kilodalton
LAP	lamina-associated polypeptide
LBR	lamin B receptor
LEM	LAP2, emerin, and MAN1
LMNA	lamin A gene
MAb	monoclonal antibody
MARs	matrix attachment regions
NE	nuclear envelope

NEBD	nuclear envelope breakdown
NPCs	nuclear pore complexes
Nups	nucleoporins
ONM	outer nuclear membrane
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PP1	protein phosphatase 1
RIPA	radioimmunoprecipitation assay
SDS	sodium dodecyl sulfate
TBST	Tris-buffered saline with Tween 20
TX-100	Triton X-100

Chapter 1: LITERATURE REVIEW

The nuclear envelope (NE) separates the nucleus from the cytoplasm in eukaryotic cells. It acts as a selective barrier to molecules moving into and out of the nucleus. The NE also allows for the spatial and temporal separation of such cellular functions as gene transcription and DNA replication. The three constituents of the NE are: (1) the inner (INM) and outer (ONM) nuclear membranes, which are continuous at nuclear pores, together with the luminal content; (2) the nuclear lamina, a proteinaceous layer located between the INM and the peripheral chromatin; and (3) the nuclear pore complexes (NPCs) (Fig. 1) (Collas, 1998; Collas and Courvalin, 2000; reviewed in Gruenbaum *et al.*, 2000; Georgatos, 2001; Hutchison *et al.*, 2001; Vasu and Forbes, 2001).

The ONM, which faces the cytoplasm, is continuous with the endoplasmic reticulum (ER), while the INM, which faces the nucleoplasm, is covered by the nuclear lamina. The nuclear lamina is an irregular protein meshwork composed of a family of intermediate filament proteins known as the lamins (Stuurman *et al.*, 1998) as well as non-lamin components (Chaly and Stochaj, 1999). The function of the nuclear lamina is not fully understood but it is believed to provide structural support for the nuclear membrane and to participate in post-mitotic nuclear reassembly (Gant and Wilson, 1997). It also appears to be required for DNA replication, chromatin organization, cell differentiation, and apoptosis (Moir *et al.*, 1995; Stuurman *et al.*, 1998; Gruenbaum *et al.*, 2000). The NE is also bound to a discontinuous layer of heterochromatin found directly under and coincident with the lamina (Fig. 1) (Stuurman *et al.*, 1998; Hutchison *et al.*, 2001).

Figure 1: Schematic view of the NE.

ONM, outer nuclear membrane; INM, inner nuclear membrane; NPC, nuclear pore complex; LAP, lamina-associated polypeptide; YA, young arrest. UNC-84 is placed in both the INM and the ONM, as its topology in the nuclear envelope is unknown. It is also not clear yet whether it forms dimers. In addition, the topology of emerin, MAN1, and nurim with respect to the INM is hypothesized from their primary sequence. (Gruenbaum *et al.*, 2000).

CYTOPLASM

chromatin→

NUCLEUS

The nuclear pores are found at fusions between the INM and ONM. The NPC is embedded in the NE and includes three major stacked rings, which each have an eight-fold symmetry and unique protein composition (Fig. 1). The cytoplasmic ring has eight cytoplasmic filaments that extend into the cytoplasm. From the nucleoplasmic ring emanate eight nuclear filaments, which form a basket-like structure, while the spoke-ring complex is embedded in the NE and provides the basic framework for the central channel (Burke and Ellenberg, 2002). The NPCs mediate the bi-directional molecular trafficking between the cytoplasm and the nucleus. Small molecules (20-40 kilodalton (kDa)) diffuse passively through the NPC while larger molecules use signal- and temperature-dependent mechanisms for entry into the nucleus (Bagley *et al.*, 2000).

The NPCs are assembled from multiple copies of NPC proteins, which are also known as nucleoporins (Nups). Nups include two transmembrane proteins, gp120 and POM121, as well as more than 20 soluble proteins. In addition to the role that NPCs play in nucleo-cytoplasmic transport, they may also be involved in nuclear organization, as NPCs are important structural units within the NE. Unlike INM proteins, the transmembrane Nups interact only with other NPCs and not with lamins or chromosomes. However, NPC proteins of the nuclear basket bind to both lamins and chromatin, and the lamina directly connects to the nucleoplasmic face of the spoke-ring complex (Vasu and Forbes, 2001).

Since the ER and nuclear membrane are continuous, INM proteins synthesized in the ER can access the INM through the membrane continuities. This was demonstrated using Fluorescence Recovery after Photobleaching studies of African green monkey COS-7 cells expressing green fluorescent protein (GFP) tagged lamin B receptor (LBR)

(Ellenberg *et al.*, 1997), emerin (Ostlund *et al.*, 1999) or MAN1 (Wu *et al.*, 2002). These integral proteins of the INM were shown to be synthesized on the rough ER and targeted to the INM by lateral diffusion in the connected ER and nuclear membranes. Associations with nuclear ligands retained them in the INM (Holmer and Worman, 2001). Consequently, the ONM and the ER are similar biochemically, while the INM is distinctive due to several integral membrane proteins that are found almost exclusively in the INM (Fig. 1) (Burke and Ellenberg, 2002).

The emphasis of the literature review is on the properties of proteins of the inner nuclear membrane, including their interactions and functions.

1A. Nuclear Envelope proteins

I. Integral Proteins of the INM

The best-characterized integral membrane proteins include the Lamina-Associated Polypeptides (LAPs), emerin, MAN1, and LBR (Fig. 1).

(a) LAPs

The LAPs include two families of INM proteins, LAP1 and LAP2 (Gant and Wilson, 1997). They contain long N-terminal nucleoplasmic domains, a single transmembrane segment and a short C-terminal luminal tail located in the luminal space between the inner and outer nuclear membranes, but the two families are otherwise unrelated (Dechat *et al.*, 2000a; Hutchison *et al.*, 2001).

The LAP1 isoforms are 1A (~75 kDa), 1B (~68 kDa), and 1C (~55 kDa) (Martin *et al.*, 1995). The isoforms of LAP2 are closely related, and include human α , β and γ ,

and mouse α (~76 kDa), β (~55 kDa), γ (~45 kDa), δ (~42 kDa), ϵ (~38 kDa) and ζ (~25 kDa) (Berger *et al.*, 1996) (Fig. 2).

(i) LAP1 family isoforms

LAPs 1A, 1B, and 1C were initially identified by monoclonal antibodies that had been raised against NE fractions of rat liver nuclei. The three integral membrane proteins were found to be associated with the nuclear lamina and were hence termed Lamina-Associated Polypeptides (Senior and Gerace, 1988). The family is a group of type II INM proteins which are alternatively spliced products of a single gene. LAPs 1A and 1B possess a 27-residue coiled-coil motif that may be involved in LAP1-LAP1 interactions (Martin *et al.*, 1995).

Immunoelectron microscopy shows that LAP1 forms clusters, which are periodically arranged along the NE (Maison *et al.*, 1997). Correspondingly, the isoforms have been found to have a putative role in membrane attachment and assembly of the nuclear lamina (Martin *et al.*, 1995).

(ii) LAP2 family isoforms

The first member of the LAP2 family was identified by a monoclonal antibody that reacted with a ~55-kDa INM protein which was highly enriched in 1% Triton X-100 (TX-100)/500 mM salt-resistant nuclear lamina fractions (Foisner and Gerace, 1993). Subsequent cloning of the protein and analysis of the human and rat LAP2 cDNAs and genes revealed that the protein originally identified represented the β -isoform (Harris *et al.*, 1994; Furukawa *et al.*, 1995).

The LAP2 family of proteins comprises at least six alternatively spliced isoforms, α , β , γ , δ , ϵ , and ζ , derived from a single gene (Berger *et al.*, 1996) (Fig. 2). Only three

Figure 2: Structural organization and functional domains of LAP2 isoforms, emerin, and MAN1.

A: Six alternatively spliced LAP2 isoforms have been identified in mammals.

Most isoforms are structurally related and differ only by small insertions in the nucleoplasmic domain (shown by differently shaded green boxes), but contain a transmembrane region spanning the INM. LAP2 α is more distantly related, containing a common N-terminus (yellow) and a unique C-terminus (blue) without a putative transmembrane region. The common LAP2 N-terminus contains a conserved domain (LEM region, hatched box), which shows significant homology to a region in emerin and MAN1.

B: Regions in the proteins found to contain specific interaction sites and/or functional domains, are indicated by gray bars. Numbers denote position of amino acids in human protein sequences; IM, inner nuclear membrane; OM, outer membrane; NLS, nuclear localization sequence; TM, transmembrane domain. (Dechat *et al.*, 2000a).

LAP2 isoforms, α , β , and γ , have been described in humans on the protein and mRNA level (Harris *et al.*, 1994), but all six LAP2 mRNA transcripts have been reported in mice (Berger *et al.*, 1996). As well, three isoforms have been identified in *Xenopus*, but LAP2 orthologues have not yet been found in invertebrates (Dechat *et al.*, 2000a).

The LAP2 isoforms are type II transmembrane proteins, with the exception of LAP2 α , which is a soluble protein (Foisner and Gerace, 1993; Harris *et al.*, 1994; Furukawa *et al.*, 1995; Berger *et al.*, 1996; Dechat *et al.*, 1998). As well, LAP2 ζ also appears to be a nonmembrane protein (Fig. 2), but the localization of the protein in the cell has not been investigated yet. Consequently, LAP2 α is least closely related to the other isoforms of the LAP2 family. It only shares its 187-residue amino terminus with the other LAP2 isoforms and has a unique, functionally different C terminus, 506 amino acids long (Fig. 2). Immunofluorescence microscopy found LAP2 β to be restricted to the nuclear rim, co-localizing with the nuclear lamina (Foisner and Gerace, 1993), while LAP2 α was distributed throughout the nucleus, excluding the nucleoli (Dechat *et al.*, 1998). The three slightly smaller isoforms, LAPs 2 γ , 2 δ , and 2 ϵ , are very closely related to LAP2 β on the structural level and only lack short regions of 40, 72, and 109 amino acids, respectively, in the N-terminal nucleoplasmic domain of LAP2 β (Fig. 2). As well, they all have the same transmembrane and C-terminal regions.

The LAP2 isoforms, emerin, and MAN1 have been found to share a ~40 amino acid long highly homologous motif called the “LEM” domain (Lee *et al.*, 2000; Lin *et al.*, 2000). The LEM domain is located in the N-terminus in each of the proteins (Morris and Manilal, 1999) (Fig. 2). The function(s) of this conserved region remains unclear. However, the LEM domain in the LAP2 isoforms overlaps with a region known to

interact with the chromosomal protein barrier-to-autointegration factor (BAF) (Furukawa, 1999). Furthermore, LAP2 β , emerin, and MAN1, as well as intranuclear LAP2 α , have been shown to be resistant to extraction upon treatment of nuclei or NE fractions with TX-100 and high molarity salt (Foisner and Gerace, 1993; Paulin-Levasseur *et al.*, 1996; Dechat *et al.*, 1998; Ellis *et al.*, 1998). On this basis, it has been suggested that there is a tight association of the LEM proteins with the nuclear matrix/lamina fractions, perhaps dependent upon the LEM domain (Dechat *et al.*, 2000a).

(b) *Emerin*

The protein emerin has recently received much attention due to its implication in the disease, Emery-Dreifuss muscular dystrophy (EDMD). EDMD is inherited in both an autosomal dominant and an X-linked recessive manner. The X-linked recessive form is caused by loss, or defects in, the *STA* gene that encodes emerin while the autosomal dominant form of EDMD is caused by mutations in the lamin A gene (*LMNA*) that encodes lamins A and C (see section II. (a)) (Wilson, 2000).

Subcellular fractionation demonstrated that emerin localizes to the NE in normal muscle, and that its expression is not restricted to the tissues that are clinically affected in patients with EDMD (Manilal *et al.*, 1996). It has been found in all other tissues studied so far, including brain, liver, leucocytes and skin, with the highest expression in skeletal and cardiac muscle (Bione *et al.*, 1994; Manilal *et al.*, 1996, 1997; Mora *et al.*, 1997). However, emerin is absent from EDMD muscle samples. Since most of the mutations described to date lead to a premature translational stop, no functional protein is being synthesized in EDMD patients (Nagano *et al.*, 1996). Despite this, emerin expression

does not appear to be essential for the function of most organs because, when mutated or absent, the phenotype of EDMD is restricted to muscle tissue (Manilal *et al.*, 1996; Nagano *et al.*, 1996).

Emerin has been detected by immunocytochemistry and immunoblotting in the NE of all vertebrates studied, including human, mouse, rat, hamster, marsupial, and *Xenopus*, as well as in *Caenorhabditis elegans*, suggesting that it is evolutionarily conserved (reviewed in Dabauvalle *et al.*, 1999; Lee *et al.*, 2000).

Emerin was found to migrate at 34 kDa using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and, based on the ability of translated emerin to insert into microsomal membranes in a type II orientation *in vitro*, emerin was predicted to be a type II integral membrane protein (Ellis *et al.*, 1998). Structural analysis predicts a protein with a hydrophilic N-terminal domain oriented towards the nucleoplasm and a transmembrane region 11 amino acids from the C-terminus (Manilal *et al.*, 1996; Ellis *et al.*, 1998) (Fig. 2). Emerin shares two regions of sequence similarity with LAP2. One is a region located at the C-terminus and includes a hydrophobic transmembrane domain, while the other is the LEM motif in the N-terminal domain of the protein (Bione *et al.*, 1994; Furukawa *et al.*, 1995; Manilal *et al.*, 1996). Confocal immunofluorescence microscopy of COS-7 cells expressing GFP-tagged truncated forms of emerin showed that the nucleoplasmic, amino-terminal domain was necessary and sufficient for nuclear retention (Ostlund *et al.*, 1999).

Subcellular fractionation of human lymphoblastoid cell lines confirmed that emerin was localized to the nuclear fraction and showed that emerin was resistant to extraction with 0.1 M sodium carbonate, or with 1% TX-100 and 500 mM NaCl (Ellis *et*

al., 1998). The insolubility of emerin by 8 M urea extractions of purified NEs further confirmed that the protein possesses properties characteristic of integral membrane proteins (Dabauvalle *et al.*, 1999).

Emerin was found to be inaccessible to anti-emerin antibodies in digitonin-treated cells, in which antibodies cannot enter the nucleus to access the nucleoplasmic face of the NE. However, when HEp-2 cells were formaldehyde-fixed after extraction with TX-100, staining of the NE was seen. Hence, the immunolocalization of emerin in differentially permeabilized cells indicates that it is a protein of the INM (Dabauvalle *et al.*, 1999). Immunogold electron microscopy supports these observations, showing that emerin is localized along the INM on the nucleoplasmic surface, but is not detected on the NPC of human skeletal muscle and HeLa cells (Yorifuji *et al.*, 1997). In African green monkey COS cells, emerin was not only detected at the nuclear rim but was also found to coincide with nuclear lamins B1, B2 and A/C at intranuclear sites, probably corresponding to invaginations of the NE (Manilal *et al.*, 1998).

In addition to the nuclear staining, rat kangaroo PtK2 and *Xenopus* A6 cells also show some labelling for emerin throughout the cytoplasm (Dabauvalle *et al.*, 1999). A subcellular fraction of emerin associated with cytoplasmic membranes, possibly the ER, has also been reported in human cells (Ellis *et al.*, 1998). It has been suggested that targeting of integral membrane proteins to the NE by lateral diffusion from the ER may account for the detection of emerin in the cytoplasm (Furukawa *et al.*, 1995; Ellenberg *et al.*, 1997). Some overexpressed emerin-GFP also reached the plasma membrane of transfected cells, where its diffusion was similar to that in the INM, suggesting that emerin may also associate with non-nuclear structures (Ostlund *et al.*, 1999). Recent

findings suggest the existence of cytoplasmic emerin, as the protein was detected in human platelets, which lack nuclear membranes (Squarzoni *et al.*, 2000).

(c) *MAN1*

The “MAN antigens” are polypeptides first identified by autoantibodies from a patient with a collagen vascular disease (Paulin-Levasseur *et al.*, 1996). They are localized to the NE in all vertebrate cell types examined, including human, mouse, and fish (Georgatos, 2001). Experiments in which cells were extracted with either 0.1 M NaOH or 8 M urea have identified one of these MAN antigens termed, MAN1, as an integral membrane protein (Lin *et al.*, 2000). MAN1 migrates at 82.3 kDa in SDS-PAGE and contains two transmembrane segments, a large N-terminal domain, and a relatively short C-terminal domain projecting into the nucleoplasm (Lin *et al.*, 2000) (Fig. 2). Confocal immunofluorescence microscopy studies in COS-7 cells using FLAG-tagged truncated forms of MAN1 showed that the N-terminal domain and the first transmembrane segment are necessary for INM retention and are sufficient for INM targeting (Wu *et al.*, 2002).

(d) *LBR*

LBR, also known as p58, is an integral protein that migrates at about 60 kDa and is expressed in all metazoans. It acts as a lamin B receptor functioning in the anchorage of the lamina, specifically lamin B, to the INM. Immunofluorescence of digitonin-permeabilized cells shows that LBR is distributed equally between the inner and outer nuclear membranes (Simos *et al.*, 1996). The protein consists of a hydrophilic N-

terminal domain of 204 amino acids, which projects into the nucleoplasm, and a hydrophobic C-terminal part that is predicted to have eight transmembrane segments (Worman *et al.*, 1990). The N-terminal domain contains multiple serine/arginine motifs, which are targets of site-specific phosphorylation. A hinge region in the N-terminus has also been found to bind double-stranded DNA *in vitro*. In the absence of lamins, LBR can still localize to the nuclear membrane, presumably through its chromatin-binding interactions (Chu *et al.*, 1998).

In avian erythrocytes, LBR is known to form a multimeric complex, which includes a specific LBR kinase, nuclear lamins, and two other nuclear polypeptides termed p18 and p34 (Simos *et al.*, 1996). It is postulated that large protein complexes such as this may create subdomains in the INM with distinct functions arising from their differing interactions with lamins and chromatin.

(e) *Other INM proteins*

Additional INM proteins have been identified whose properties are still poorly known. These include otefin, a *Drosophila* protein with a weakly conserved LEM domain and the highest level of homology to human MAN1 (Padan *et al.*, 1990). UNC-84 is a newly identified *C. elegans* nuclear protein whose exact location within the NE has not been determined (Malone *et al.*, 1999), while nurim is a putative transmembrane protein that interacts with the nuclear scaffold (Rolls *et al.*, 1999). As well, a recent proteomics study using 16-benzyl dimethyl hexadecyl ammonium chloride gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry

identified 19 new integral membrane proteins ranging in molecular weight from 23 to 200 kDa (Dreger *et al.*, 2001). These proteins remain to be further characterized.

II. Lamina proteins

(a) *Lamins*

Lamins were initially identified by detergent and salt fractionation of NEs from rat liver (Aaronson and Blobel, 1975), and were shown by electron microscopic immunolabelling to be localized at the NE, underlying the INM (Gerace *et al.*, 1978).

Nuclear lamins are type V intermediate filaments proteins that are the major building blocks of the lamina (Stuurman *et al.*, 1998). Like other intermediate filament proteins, lamins have a tripartite structure consisting of non-helical amino- 'head' and carboxy-terminal 'tail' regions that flank a central α -helical coiled-coil "rod" domain. The α -helical rod domain contains two short segments at either end that are highly conserved among all intermediate filament proteins and are the regions of strongest homology among lamins (Stuurman *et al.*, 1998; Gruenbaum *et al.*, 2000).

Most cytoplasmic intermediate filament proteins can self-assemble into 10-13 nm filaments *in vitro*. Electron microscopy analysis and chemical cross-linking suggest that lamins assemble *in vitro* by lateral interaction between two lamin monomers to form parallel homodimers through coiled-coil association via their α -helical rod domain. These dimers then form 10 nm filaments by associating longitudinally to form head-to-tail polymers (Heitlinger *et al.*, 1991; Moir *et al.*, 1991). The mechanism of formation of lamin filaments *in vivo*, however, still remains speculative (Stuurman *et al.*, 1998; Hutchison *et al.*, 2001).

The lamins differ from other intermediate filament proteins in that they possess such characteristics as cdc2 kinase phosphorylation sites flanking the rod domain and a nuclear localization signal in the tail domain (Georgatos *et al.*, 1994). The nuclear localization signal is required for nuclear import, and it has been suggested that phosphorylation of lamins at sites adjacent to the nuclear localization signal by protein kinase C may influence the rate at which lamins enter the nucleus, affecting lamin transport and assembly during interphase (Hennekes *et al.*, 1993).

The lamins are classified as A- or B-type based on sequence homology, expression pattern, biochemical properties, and their cellular localization in mitosis. Vertebrate genomes contain two B-type lamin genes and one A-type lamin gene (Gruenbaum *et al.*, 2000). Sequence analysis has shown vertebrate A-type lamins differ from B-type lamins in that they have a longer C-terminal tail domain, which contains a characteristic polyhistidine stretch (Peter *et al.*, 1989; Hoyer *et al.*, 1990). In mammals, B-type lamins (~65-70 kDa) are ubiquitously expressed in somatic cells while A-type lamins, are found only in differentiated cells with the exception of cells from the erythropoietic lineage (Stuurman *et al.*, 1998).

In vertebrates, there are four A-type lamins: A, C, A Δ 10, and C2, which are alternatively spliced products from the lamin A gene, LMNA. Lamins A and C are the most abundant forms of the A-type lamins. Lamin C and lamin A Δ 10 proteins differ from each other and from lamin A in their C-terminal tail domains. Lamin C does not have the C-terminal CaaX sequence, a signal for isoprenylation, while lamin A Δ 10 contains the CaaX box as well as the site where proteolytic processing of the C-terminal end of lamin A takes place. However, lamin A Δ 10 differs from lamin A in that it lacks

90 nucleotides from the 5' part of exon 10 (Weber *et al.*, 1989; Machiels *et al.*, 1996). The predicted protein sequence of lamin C2 is identical to that of lamin C except for the head domain and the N-terminal part of the rod domain, which are replaced by a short nonhelical segment (Furukawa *et al.*, 1994).

All vertebrate species studied have two B-type lamin genes, B1 and B2 (Wydner *et al.*, 1996). There are no conspicuous differences in sequence between them, except for the presence of two tetrapeptides (FHRS and DKAA) in the rod domain of all B2 lamins sequenced so far (Hoger *et al.*, 1990). In the germ cell-specific splice variant of lamin B2, called lamin B3 (52 kDa) (Furukawa and Hotta, 1993), the head domain and N-terminal part of the rod are replaced by alternate, nonhelical sequences. No such lamin splice variants have been reported yet from species other than mammals.

The C-terminal ends of all the lamins except lamin C contain a CaaX (C, cysteine; a, any aliphatic amino acid; X, any amino acid) sequence motif (Stuurman *et al.*, 1998). The motif is modified by farnesylation of the C-terminal cysteine residue, followed by cleavage of the three N-terminal residues and carboxy-methylation of the cysteine. It appears that the added hydrophobic prenyl moiety targets (and anchors) lamins to the nuclear membrane. Indirect immunofluorescence studies showed that inhibiting the processing of the lamin A precursor blocked assembly of lamin A. Non-prenylated pre-lamin A accumulated as nucleoplasmic particles, indicating that the carboxyl-terminal peptide of pre-lamin A blocks its proper assembly into the nuclear lamina (Lutz *et al.*, 1992). The hydrophobic farnesyl side group is removed by proteolytic cleavage in the A-type lamins to yield the mature proteins (Sasseville and Raymond, 1995), but B-type lamins remain farnesylated throughout their lifetime (Moir *et al.*, 1995). Hence, B-type

lamins have a tight association with membranes, likely stemming from their post-translational prenylation at the C-terminus. Since mature A-type lamins are not prenylated, they bind membranes less tightly (Stuurman *et al.*, 1998).

However, both A-type and B-type lamins have been found as intranuclear foci (Stuurman *et al.*, 1998). The lamin-containing foci are distinct from the peripheral lamina, with the foci varying in their patterns of distribution as well as their composition in a cell cycle-dependent manner. Nuclei of many cell types contain NE-derived invaginations extending into the nucleoplasm, some of which even cut all the way through the nucleus (Fricker *et al.*, 1997). These channels are lined with NPCs and nuclear lamins. However, it is unlikely that all of the intranuclear lamin spots are associated with NE-derived channels, as many do not co-localize with membranes (Bridger *et al.*, 1993) or with NPCs (Moir *et al.*, 1994).

The intranuclear foci of A-type lamins are most prominent during the G1 phase of the cell cycle and align closely with condensed chromatin. These structures have been proposed to represent pools of newly synthesized lamins, which are necessary for nuclear growth in daughter cells. They may also represent sites where intermediate assembly of the lamin protein takes place before it is incorporated into the peripheral lamina (Bridger *et al.*, 1993). The B-type lamin foci are most prominent during S-phase and co-localize with sites of DNA replication as visualized by BrdU labelling (Moir *et al.*, 1994). Furthermore, immunolocalization studies found lamin A foci, with which lamin B1 was also associated, to co-localize with RNA splicing factors, suggesting that intranuclear lamins may also play a structural role in RNA splicing (Jagatheesan *et al.*, 1999).

The acquisition of lamins A and C is a stage- and tissue-specific event during development (Stewart and Burke, 1987; Rober *et al.*, 1989). However, some cell types, most notably from the hematopoietic cell lineage, never express A-type lamins (Rober *et al.*, 1989; Guilly *et al.*, 1990). The A-type lamin expression levels increase with terminal differentiation and growth arrest, with the latter suggesting a role for A-type lamins in cellular quiescence (Coates *et al.*, 1996; Pugh *et al.*, 1997). Lamin B2, on the other hand, is expressed at relatively constant levels throughout development, while lamin B1 is expressed mainly in early development, and its expression correlates with proliferation (Broers *et al.*, 1997). Available data indicate that the expression patterns of the somatic lamins A, B1, and B2 are very similar in all vertebrate species, with the only exception arising in the strategies used to express lamins in germ cells, as described above.

Mice lacking A-type lamins develop to term with no overt abnormalities. However, their postnatal growth is severely retarded and they exhibit tissue-specific alterations to their NE integrity and to the distribution of emerin, primarily in muscle tissue (Sullivan *et al.*, 1999). Two distinct diseases have been identified stemming from mutations in LMNA, EDMD and Dunnigan-type familial partial lipodystrophy, which causes the selective loss of subcutaneous fat from the extremities, trunk, and gluteal region (Sullivan *et al.*, 1999; Gruenbaum *et al.*, 2000; Wilson *et al.*, 2001).

Based on clinical, pathologic, and genetic data, it appears that mutations in the rod domain of the lamin A/C gene can cause the full clinical spectrum of EDMD (Felice *et al.*, 2000). A model for this disease was created by knocking out the gene that encodes lamin A/C in mice (Sullivan *et al.*, 1999). The lack of lamin A/C causes the

mislocalization of emerin, rendering the protein nonfunctional and leading to the initial, but erroneous, conclusion that mutations in emerin were the cause of EDMD. It has been proposed that the absence of functional lamins and/or emerin may render muscle cells incapable of withstanding the continual mechanical stress that they are subjected to. Although the NE integrity may be compromised in other tissue, the effects may not be apparent as they do not experience the same contractile forces. Hence, the loss of lamins and subsequent NE integrity may directly produce the muscular dystrophy phenotype. Alternatively, lamin disorganization may cause downstream effects on chromatin structure or gene expression resulting in the clinical symptoms and the tissue-specific pathology of the disease (Wilson *et al.*, 2001).

Lipodystrophy cannot be accounted for by the “mechanical stress” models of EDMD, as fat cells are not contractile. However, fat cells and muscle cells are both derived from the same progenitor, the mesenchymal stem cells. Hence, it has been proposed that mutations in lamin A/C and emerin may affect the gene expression of cells from the mesenchymal stem cell lineage resulting in a selective effect on differentiation, maintenance, repair or regulation of these cells (Wilson, 2000).

(b) Non-lamins

There also exist non-lamin components in the lamina such as statin and the young arrest protein (see Chaly and Stochaj, 1999, for review). Statin is a 57 kDa non-glycosylated protein that shows peripheral nuclear envelope staining. Statin has been found to be absent from proliferating cells but appears when the cells become postmitotic and/or begin to differentiate (Chaly and Stochaj, 1999). Perhaps the best characterized

non-lamin component of the lamina is the young arrest protein, YA (Fig. 1). It is a peripheral membrane protein identified so far only in *Drosophila* that is required in embryos for the transition from meiosis to mitosis (Gruenbaum *et al.*, 2000).

1B. NE protein organization and function

I. NE protein-protein interactions

Direct and indirect protein-protein interactions play a major role in the structural organization as well as function of the NE (Fig. 1, Table 1).

Lamin assembly and association with the NE may be stabilized by integral INM proteins. As well, lamins may also play a role in the sorting and anchoring of INM proteins themselves (Gruenbaum *et al.*, 2000; Foisner, 2001; Hutchison *et al.*, 2001). The interaction between the INM and B-type lamins is mediated by very specific interactions with the nucleoplasmic N-termini of the INM proteins in cases where their binding domains are known (Chu *et al.*, 1998; Dechat *et al.*, 2000b; Wilson, 2000; Hutchison *et al.*, 2001; Georgatos, 2001). Lamin B associates with LBR and LAP2 β via its head and tail domains, respectively (Hutchison *et al.*, 2001).

Table 1: Proteins that organize the nuclear envelope

<i>Protein class</i>	<i>Name</i>	<i>Interacts with</i>
Lamins	Lamin A and B	LAP1 and 2 families, histones, DNA
	Lamin A	Emerin
	Lamin B	LBR, LAP2 β
Integral INM	LBR	Lamin B, DNA, HP1
	LAP1 α , β and γ	Lamins
	LAP2 β	Lamin B, DNA, BAF
	LAP2 γ , δ and ϵ	Lamins
	Emerin	Lamin A, Lamin B, BAF
	MAN1	BAF?
NPC	Nup153	Lamin B
	gp120	Nuclear membranes
	POM121	Nuclear membranes
Chromatin	BAF	*(DNA), emerin, MAN1, LAP2 β
	HP1 α , β and γ	LBR
	LAP2 α	(DNA), Lamin A

*Parentheses denote interactions that group a protein into one of the four classes of NE proteins. These interactions do not link two structural elements of the NE (Burke and Ellenberg, 2002).

The lamin B-binding domain of LAP2 β has been localized between amino acids 298-370 (Furukawa and Kondo, 1998). Since the association of the protein with the lamina is mediated by direct interactions, it was suggested that LAP2 β might control nuclear lamina dynamics. This hypothesis was supported by the findings that microinjection of recombinant LAP2 β fragments containing the lamin B-binding region into G1 cells (Yang *et al.*, 1997), or addition of the fragment to *in vitro* nuclear assembly reactions (Gant *et al.*, 1999), inhibit nuclear growth.

The lamin B-binding domain is also found in its entire length in LAP2 ϵ and LAP2 δ , and in parts in LAP2 γ and LAP2 ζ , but is completely absent from LAP2 α (Dechat *et al.*, 2000a) (Fig. 2). However, LAP2 α appears to organize higher order chromatin structure by interacting with A-type lamins and chromosomes (Dechat *et al.*, 2000b). LAP2 α was found to co-localize specifically with intranuclear lamin A/C foci in early G1 cells using confocal immunofluorescence microscopy (Dechat *et al.*, 2000b). Co-immunoprecipitation and blot overlay assays showed further that the C-terminal domain of LAP2 α binds directly to the C terminus of lamin A/C (Dechat *et al.*, 2000b). Hence, the LAP2 family of proteins interacts with either A- or B-type lamins, with the LAP2 membrane isoforms binding B-type lamins whereas the LAP2 α isoform binds A-type lamins.

As well, *in vitro* studies found LAPs 1A and 1B to bind specifically to A- and B-type lamins (Foisner and Gerace, 1993), with LAP1C binding only to lamins A/C (Chu *et al.*, 1998). Emerin also binds to both A- and B-type lamins *in vitro* but, *in vivo*, appears to interact primarily with lamin A. The localization of emerin at the INM has been

shown to depend upon the presence of A-type lamins, specifically lamin A (Clements *et al.*, 2000; Hutchison *et al.*, 2001).

The interactions between lamins and nurim or MAN1 have not yet been investigated (Foisner, 2001). Furthermore, although these *in vitro* studies have identified multiple potential binding partners for lamins, the role of protein-protein interactions *in vivo* requires further study, for instance, to determine if these interactions are competitive or cooperative in nature.

II. NE protein-chromatin interactions

(a) INM protein-chromatin interactions

The molecular interactions between INM proteins and chromatin are significant as morphological and biochemical studies suggest that they provide the driving force for NE reassembly at the end of mitosis (Georgatos, 2001). Further, genetic and cell biological experiments imply that the INM attracts and “concentrates” specific silencers and remodelling factors (Wilson, 2001). As well, two NPC proteins, Nup98 and Nup96, have recently been shown to reverse chromatin silencing (Enninga *et al.*, 2002). Hence, the coupling of chromatin domains to the NE may alter chromatin structure resulting in a change in transcriptional activity.

Three different proteins have been proposed so far to connect the INM to the underlying chromatin network: heterochromatin protein 1 (HP1), BAF and HA95 (Georgatos, 2001) (Table 1). In addition, chromatin may also bind indirectly to the INM proteins through lamina proteins like lamins, which are known to bind DNA and INM proteins (Burke and Ellenberg, 2002).

HP1 is a component of heterochromatin and has been implicated in gene silencing. It consists of two structural domains, the chromodomain and the chromoshadow domain. It has three distinct variants HP1 α , HP1 β , and HP1 γ , which are products of distinct genes (Jones *et al.*, 2000). Two-hybrid screens and biochemical studies suggest that the α and γ forms bind specifically with LBR (Ye and Worman, 1996). HP1 also appears to participate in post-mitotic NE assembly, facilitating the recruitment of INM proteins to the surfaces of chromosomes (Haraguchi *et al.*, 2000; Georgatos, 2001).

BAF is a protein identified for its ability to protect retroviral DNA against autointegration, which is the often self-destructive insertion of DNA into its own genome (Furukawa, 1999). It is a 10 kDa highly conserved protein that forms a dodecamer with DNA. The interactions between BAF and DNA are non-specific with respect to the DNA sequence (Zheng *et al.*, 2000), but mutagenesis studies suggest that BAF changes conformation when complexed with DNA (Shumaker *et al.*, 2001). BAF forms high-order structures with DNA bound at multiple sites in the complex, which compacts the DNA and suggests that BAF may play a role in chromosome organization.

BAF was also found to bind to LAP2 β by the LEM domain (Shumaker *et al.*, 2001), suggesting that all LEM proteins have the potential to interact with BAF. In addition, the LAP2 polypeptides appear to prefer to bind to BAF complexed with DNA than to BAF dimers. This is suggestive that LAP2 binds to chromatin *in vivo* and implies that LAP2 isoforms might interact with BAF-DNA complexes and with lamins, thus anchoring chromatin to the lamina and INM (Shumaker *et al.*, 2001).

HA95, also called HAP95 or NAKA95, is a nuclear protein homologous to the nuclear A-kinase anchoring protein 95 (Georgatos, 2001). It associates with interphase

chromatin and metaphase chromosomes. HA95 associates with B-type lamins and potentially forms a complex with the INM proteins LBR, LAP2 β and emerin (Martins *et al.*, 2000).

(b) Peripheral NE protein-chromatin interactions

Based on the studies to date, it appears that both A- and B-type lamins bind to DNA. Vertebrate A- and B-type lamins bind to specific DNA sequences called matrix attachment regions (MARs) (Luderus *et al.*, 1992, 1994). MARs are sequences 300-1000 bp long that have a high A-T content and remain attached, or bind *in vitro*, to the nuclear matrix or nuclear scaffold. Lamins A/C and B have also been shown to bind core histones, H2A and H2B, *in vitro* (Taniura *et al.*, 1995; Goldberg *et al.*, 1999). In addition, the tail domains of lamin A/C, along with LBR and LAP2 β also bind to chromosomes; and all the lamins also interact with chromatin (Moir *et al.*, 1995; Stuurman *et al.*, 1998). The spatial organization of lamins relative to chromatin at the nuclear periphery in interphase nuclei has been determined using anti-lamin antibodies along with three-dimensional light and electron microscopy (Paddy *et al.*, 1990). It has been qualitatively and quantitatively, observed using *Drosophila* early embryos, *Drosophila* Kc cells, and human HeLa cells, that the lamin network is highly discontinuous at the NE, with alternating patches of high and low lamin concentrations. The areas of high lamin concentration are coincident with regions concentrated in DNA (Belmont *et al.*, 1993; Stuurman *et al.*, 1998). Based on these data it has been proposed that lamins may anchor chromatin at the NE and affect higher order chromatin organization.

The interactions between chromatin and proteins of the INM and lamina may also play a role in gene expression. The direct binding of LBR to HP1 and of LAP2 β to BAF has the potential to alter the ordered structure of chromatin and gene activity at the nuclear periphery (Wilson *et al.*, 2001). As well, lamin B, lamin A/C, and LAP2 β have been shown to interact specifically with transcription factors, like the retinoblastoma protein, at the nuclear rim, enabling INM proteins to have a more direct effect on gene expression through the regulated release of the factors (Cohen *et al.*, 2001; Wilson *et al.*, 2001). Furthermore, the elucidation of a role of lamin A/C in diseases such as EDMD also suggests that the interactions between the NE and chromatin may function as a structural tether for the chromatin (Wilson *et al.*, 2001).

III. NE dynamics in mitosis

The NE is a dynamic structure. It grows in interphase, disassembles during mitosis in higher eukaryotes, and reassembles around the nuclei of the two new daughter cells that are formed.

(a) Mitosis-specific changes in NE protein organization

The moment at which the NE breaks down has been defined as the end of prophase and the beginning of prometaphase. Some NE proteins are reversibly phosphorylated during mitosis by protein kinase C and cdc2 (Foisner and Gerace, 1993; Dechat *et al.*, 1998; Ellis *et al.*, 1998; Burke and Ellenberg, 2002). This alters the interactions of NE components with one another and with chromatin, and is believed to abolish protein-protein interactions necessary for NE integrity, resulting in its disassembly.

Phosphorylation of lamins A, B, and C by cdc 2 kinase has been shown to be necessary for the disassembly of the nuclear lamina in mitosis through structural mutagenesis studies (Heald and McKeon, 1990). Conserved serine and threonine residues outside the ends of the α -helical domain of lamins were phosphorylated both *in vitro* and *in vivo* by cdc2-kinase and protein kinase C. Mitotic phosphorylation of lamins by cdc2 kinases results in their depolymerization, reversible dissociation from chromosomes, and dispersal into the cytoplasm. On the other hand, mutations within protein kinase C motifs inhibit transport of mutant lamin A to the nucleus and the proper assembly of lamin A into the perinuclear lamina. The phenotype showed discontinuous and patch-like aggregates of the mutant protein in the nucleus. Phosphorylation by cyclic-AMP-dependent kinase, however, facilitates the incorporation of new lamin subunits into the assembled lamina structure as the nucleus grows (reviewed in Foisner, 1997; Gant and Wilson, 1997; Marshall and Wilson, 1997). Hence, phosphorylation of lamins is necessary for both assembly and disassembly of the nuclear lamina.

LBR (Courvalin *et al.*, 1992), LAP1 and LAP2 (Foisner and Gerace, 1993), emerin (Ellis *et al.*, 1998), and some Nups (Vasu and Forbes, 2001) also show such phosphorylation, as do chromatin proteins such as histones and HP1 during mitosis (Minc *et al.*, 1999). Phosphorylation disrupts the binding of LAP1 to lamins, causing the release of LAP1 from its lamina anchor and partitioning with mitotic membranes (Foisner and Gerace, 1993; Yang *et al.*, 1997), while the phosphorylated form of LAP2 was found to no longer bind lamin B (Foisner and Gerace, 1993). Hence, it has been proposed that the phosphorylation of INM proteins may abolish their ability to interact with lamins and/or chromatin, allowing for their detachment from chromosomes (Burke and

Ellenberg, 2002). The phosphorylation of Nups is similarly believed to cause their release from NPCs, resulting in the disassembly of the NPC, and subsequent dispersion in mitotic cells (Macaulay *et al.*, 1995; Favreau *et al.*, 1996).

Upon the disassembly of the nucleus at prophase, the lamins and all other NE proteins studied so far are localized in the cytoplasm (Gerace and Blobel, 1980; Gant and Wilson, 1997; Dabauvalle *et al.*, 1999; Haraguchi *et al.*, 2000). It was long believed that the NE and ER become vesiculated during mitosis, but it now appears that the ER remains continuous in most cells (Ellenberg *et al.*, 1997; Burke and Ellenberg, 2002) and that the integral proteins of the nuclear membrane, such as lamin receptors and integral nuclear pore proteins, are dispersed throughout the ER (Ellenberg *et al.*, 1997; Yang *et al.*, 1997; Ostlund *et al.*, 1999; Wu *et al.*, 2002).

(b) Models of Nuclear Envelope Breakdown and re-assembly

Two different models have been proposed to explain the disassembly and reformation of nuclear membranes during mitosis. The traditional view was based on subcellular fraction studies of *Xenopus laevis* oocytes, in which isolated vesicles enriched in NE proteins were able to assemble the NE *in vitro*. This model implies the release of nuclear membrane proteins into specific NE-derived vesicles distinct from bulk ER membranes as a result of NE disassembly. Reassembly of the nuclear proteins into the NE would occur by selective targeting of the NE-specific vesicles to the chromosome surfaces (Wilson and Newport, 1988; Chaudhary and Courvalin, 1993; Maison *et al.*, 1993). On the other hand, recent studies using direct visualization of intact mammalian mitotic cells expressing fluorescent envelope proteins suggest that nuclear membrane

proteins become dispersed throughout all ER membranes during mitosis and are sorted to the reforming NE by diffusion through a functionally continuous ER, coupled with binding to specific sites at the chromosome surfaces (Gerace and Foisner, 1994; Ellenberg *et al.*, 1997; Yang *et al.*, 1997; Collas and Courvalin, 2000).

The apparent contradiction between the vesiculation and ER dispersion models has been proposed to arise from cell-type-specific differences between the embryonic (*Xenopus* oocytes) and somatic (mammalian culture cells) systems used. However, evidence of intact ER in echinoderm embryos (Terasaki, 2000) and of vesiculation in fractionated HeLa cells (Buendia and Courvalin, 1997) argue against this explanation. A more likely reason for the discrepancy may be that the extraction procedures used to obtain pure membrane fractions *in vitro* result in vesiculation of the ER network in intact cells. This induced fragmentation would result in distinct membrane fractions of transmembrane proteins and lipids with different properties (Burke and Ellenberg, 2002). The notion that a membrane network is the precursor for NE assembly is strengthened by the recent finding of membrane homogenates forming networks prior to NE formation in *Xenopus* extracts (Hetzer *et al.*, 2000, 2001).

However, a model in which the ER is passively dispersed cannot account for the speed with which the NE needs to be removed from the vicinity of the chromosomes in order to allow for spindle apparatus assembly and function, as diffusion into the ER takes time. Two recent complementary studies of living cells may have provided the solution for this problem.

The mechanism of nuclear envelope breakdown (NEBD) was investigated by multicolor confocal 4D imaging of live NRK cells expressing fluorescently tagged LBR,

lamin B1, LAP2 β , POM121, and α -tubulin (Beaudouin *et al.*, 2002). The behaviour of the tagged proteins and chromosomes were analyzed during mitosis. Mechanical tension in the nuclear lamina was created by early spindle microtubules causing folds and invaginations in the NE. Using similar approaches, Salina *et al.* (2002) showed further that, during prophase, centrosomes localize to deep invaginations in the NE in a microtubule-dependent process. The concentrating of dynein, a molecular motor protein, and dynactin, an associated regulatory complex, on the NE preceded changes in NE organization. The first gap in the NE appeared before lamin B depolymerization at the site of maximal tension, in a region of the NE not in contact with chromosomes and distal from centrosomes. Gap formation relaxed this tension and dramatically accelerated the rate of chromosome condensation. The hole produced in the NE then rapidly expanded over the nuclear surface. NE fragments remaining on chromosomes were removed toward the centrosomes in a microtubule-dependent manner. These findings suggest a model in which nuclear membrane detachment is the result of dynein pulling nuclear membranes and associated proteins poleward along astral microtubules. The discovery that interference with dynein function dramatically alters the nuclear membrane dynamics in prophase and prometaphase, provided further support for this model. (Salina *et al.*, 2002).

These data form the basis for a new model for NEBD, in which NEBD is initiated by a tearing process driven by forces exerted by dynein, with dynein also quickly transporting pieces of the envelope still continuous with the ER away from chromosomes. This process is carried out along microtubules and results in NE lipids

and proteins being mixed with the ER components (Beaudoin *et al.*, 2002; Burke and Ellenberg, 2002; Salina *et al.*, 2002) (Fig. 3).

Reassembly of the NE begins at anaphase and continues through telophase and early G1, presumably as a result of the removal of the mitotic phosphorylations. Evidence for this is available only for lamins so far. Mitotic lamin phosphatase activity was found to co-fractionate with protein phosphatase 1 (PP1) after ion exchange chromatography (Thompson *et al.*, 1997). This suggests that PP1 is the major mitotic lamin phosphatase responsible for removal of mitotic phosphates from lamin B, a process required for nuclear lamina reassembly. Hence, the rate of dephosphorylation by PP1 may influence the initial rate of lamin filament assembly at telophase.

It has also been shown that PP1 is targeted to the NE by A-kinase anchoring protein 149 (AKAP149), an ER-membrane protein, and that this targeting correlates with assembly of B-type lamins into nuclei in HeLa cells *in vitro* (Steen *et al.*, 2000). It was further demonstrated that the failure of AKAP149-mediated assembly of B-type lamins into the nuclear lamina at the end of mitosis is followed by apoptosis within hours after nuclear reformation, and that it also induced expression of the gene encoding A-type lamins in cells that normally do not express lamins A/C. Inhibition of the association of PP1 with the NE was found to be mediated by a peptide containing the PP1-binding domain of AKAP149. B-type lamins fail to assemble in these cells and are rapidly degraded by caspase-dependent proteolysis, but the assembly of lamins A/C is unaffected (Steen *et al.*, 2000). In a parallel *in vivo* system, inhibition of B-type lamin assembly, in human lymphoid KE37 cells which do not express lamins A/C, triggers rapid synthesis

Figure 3: Model of nuclear envelope breakdown in mammalian cells.

Schematic of a 'half-cut-open' nucleus cleared from most of the surrounding ER for clarity. Depicted are chromosomes (purple), lamina (green), nuclear membranes (yellow), microtubules (red) and centrosomes (orange). Nuclear pores are omitted for simplicity.

a: Interphase. Decondensed chromosomes are surrounded by the smooth and elastic lamina and the double membrane layer of the NE. Connections to the ER are truncated. Centrosomes nucleate long and stable interphase microtubules.

b: Prophase. About 30 minutes before NE breakdown, microtubule-filled folds appear in the NE close to the centrosomes, followed by the appearance of condensed chromosomes along the NE. As prophase proceeds, folds in the NE increase in size. At this time, the NE becomes stretched on the side that is opposite to the centrosomes due to pulling forces of minus-end-directed microtubule motor proteins on its surface.

c: NE breakdown occurs when the tension has reached a critical point and tears the lamina and the associated membranes, which produces a hole in the NE. Hole formation is followed by nuclear collapse as the tension is released.

d: Prometaphase. The initial hole spreads rapidly and fragments the NE. However, large pieces of NE are still attached to chromosomes.

e: Metaphase. NE-associated minus-end-directed motors remove NE remnants from the chromosomes towards the centrosomes. (Burke and Ellenberg, 2002).

and nuclear assembly of both lamins A and C before apoptosis takes place (Steen and Collas, 2001). These results indicate that nuclear assembly of B-type lamins is essential for cell survival. They also suggest that mistargeting of B-type lamins at the end of mitosis elicits a tentative rescue process to assemble a nuclear lamina in lymphoid cells that normally do not express lamins A/C (Steen and Collas, 2001).

NE formation around demembranated sperm chromatin in *Xenopus* egg extracts and *in vivo* studies using GFP-LBR suggest that there are several phases in the re-establishment of nuclear membranes in telophase (reviewed in Burke and Ellenberg, 2002). In metaphase, the NE is completely disassembled and GFP-LBR diffuses freely within an intact interconnected ER network that surrounds the spindle apparatus and the condensed chromatin. In late anaphase, binding sites for LBR become available, trapping the LBR molecules and consequently forcing the ER membranes to wrap around the chromatin. This progressive immobilization and wrapping results in the rapid enclosure of nuclear material by ER membranes, which are enriched in LBR, forming the new NE (Ellenberg *et al.*, 1997). From telophase to early interphase, the NE expands slowly into a sphere surrounding the chromatin. This process is dependent on GTP hydrolysis by Ran, a small Ras-related protein that has a regulatory function in nucleo-cytoplasmic transport and in mitotic spindle assembly (Hetzer *et al.*, 2000). The precise role of Ran in the reformation of the chromatin-associated membrane network is unclear. However, it is interesting to note that Ran-coated beads can substitute *in vitro* for chromatin as a template for NE assembly (Zhang and Clarke, 2000). After the appearance of the membrane network, the two final phases include the sealing of the NE and the expansion of the NE to accommodate decondensing chromatin (Burke and Ellenberg, 2002).

(c) *Sequence of protein assembly and disassembly from the NE of mitotic cells*

(i) NE disassembly

At mitosis, the NE dissociates from condensing chromosomes and disassembles. LBR has been shown to be redistributed into the ER before NEBD, with the majority of the protein already residing in the ER at the time of NE “permeabilization”, which is defined as the point in the cell cycle when the NE no longer represents a barrier to nucleocytoplasmic exchange (Beaudouin *et al.*, 2002). The same study showed, however that LAP2 β , POM121, a NPC membrane protein, and lamin B1 were still concentrated at the nuclear rim when the first gap appeared in the NE, and dispersed only after the nucleus was permeabilized. After NEBD, lamin B1 partitions with membranes as the lamina depolymerizes, while lamins A/C become soluble in the cytoplasm (Gerace and Blobel, 1980), and the transmembrane proteins POM121 and LAP2 β equilibrate with the ER over a similar time frame (Beaudouin *et al.*, 2002). The only NE protein that behaved differently was LBR, which concomitant with NE permeabilization, started to diffuse into the ER in prophase, possibly due to its loss of affinity for the lamina due to mitotic phosphorylation (Courvalin *et al.*, 1992). Hence, except for LBR, all the NE proteins examined so far disperse only after permeabilization. This suggests that interactions among these proteins persist in prophase and that NE permeabilization itself may trigger the dismantling of the stable interphase NE protein-protein interactions. However, by prometaphase, the other NE proteins were equilibrated with the ER. Thus, the ER appears to act as a reservoir and means of partitioning for the NE proteins during mitosis (Beaudouin *et al.*, 2002).

(ii) NE assembly

Studies in mammalian systems have shown that the reassociation of proteins with the chromosome surface after chromatid segregation occurs in a stepwise manner (Chaudhary and Courvalin, 1993; Bodoor *et al.*, 1999; Haraguchi *et al.*, 2000). The earliest events in NE assembly that have been characterized involve the association of certain soluble NPC proteins, including Nup153 and Nup133, with the chromatid surfaces in mid-anaphase (Bodoor *et al.*, 1999; Burke and Ellenberg, 2002), but NPC function is not detected until the NE is sealed (Vasu and Forbes, 2001).

Following the association of these soluble Nups, membranes begin to associate with the polar and lateral faces of the chromatids. INM proteins such as LAP2, LBR, LAP1, and emerin concentrate at the membrane-chromatin interface in late anaphase (Foisner and Gerace, 1993; Ellenberg *et al.*, 1997; Maison *et al.*, 1997; Bodoor *et al.*, 1999; Dabauvalle *et al.*, 1999; Haraguchi *et al.*, 2000), followed by B-type lamins and then A-type lamins (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Broers *et al.*, 1999).

In anaphase, emerin was found around the surfaces of the chromosomes as focal concentrations in areas of the mitotic spindle and in the midbody of mitotic cells (Dabauvalle *et al.*, 1999; Haraguchi *et al.*, 2000). This was in contrast to LAP2, which was redistributed uniformly at the nuclear periphery. It was also noted that in late telophase, emerin co-localizes with lamin A/C but not with any of LAP2, LBR or lamin B (Dabauvalle *et al.*, 1999; Haraguchi *et al.*, 2000). Thus interactions with lamin A/C may be involved, or required, for the correct localization of emerin in nuclear reassembly.

The nuclear lamina is essential in the maintenance of NE integrity but the function of the individual lamins in NE reassembly is not clear. Since A-type lamins are not expressed in all cell types, they cannot be essential for nuclear re-formation (Moir *et al.*, 2000). In cells, which do express A-type lamins, the bulk of these are re-imported into the nucleus in early G1 after a sealed NE has been formed (Gruenbaum *et al.*, 2000).

Experiments to determine directly whether lamins are involved in NE assembly have yielded conflicting results. In some studies, the immunodepletion of lamins from *Xenopus* nuclear assembly extracts does not inhibit NE assembly (Newport *et al.*, 1990; Meier *et al.*, 1991; Hutchison *et al.*, 1994), whereas other studies using *Drosophila*, mammalian, and *Xenopus* nuclear assembly extracts indicate that the immunoabsorption of lamins does inhibit NE assembly (Burke and Gerace, 1986; Dabauvalle *et al.*, 1991; Ulitzur *et al.*, 1992). Anti-lamin antibodies microinjected into cultured rat kangaroo PtK2 cells during mitosis, expected to decrease the soluble pool of lamins, were also shown to inhibit specifically the reformation of daughter nuclei. The antibody injected, together with the lamins, was detected in cytoplasmic aggregates by immunoelectron microscopy. Although the cells were able to complete cytokinesis, the chromatin of their daughter nuclei remained arrested in a telophase-like configuration and inactive. These results indicate that lamins and the nuclear lamina structure are involved in the functional organization of the interphase chromatin (Benavente and Krohne, 1986). The different results and conclusions drawn from these studies may be due to difficulties inherent in attempts to completely remove lamins by immunodepletion (Lourim and Krohne, 1994; Moir *et al.*, 1995). For example, small amounts of lamin can be detected in nuclei assembled in lamin-depleted *Xenopus* extracts (Jenkins *et al.*, 1993). It is possible that in

the cases in which envelope assembly is not inhibited, the amount of lamin remaining after immunodepletion, while insufficient to assemble a lamina, may be adequate for normal NE formation (Lourim and Krohne, 1993).

The reassembly of B-type lamins in mammalian cells has been shown to be under the control of PP1 (Steen *et al.*, 2000), as described in section III (b). PP1 dephosphorylates B-type lamins, which allows them to associate to form the nuclear lamina. Lamin fragments, however, can interfere with the assembly of artificial nuclei in *Xenopus* extracts (Lopez-Soler *et al.*, 2001). In mature nuclei, reduced lamin content is associated with extreme mechanical fragility of the NE as well as mislocalization of certain INM proteins, such as emerin (Newport *et al.*, 1990; Burke and Ellenberg, 2002).

As well, LAP2 α was shown to co-localize with A-type lamins during nuclear formation and may specifically regulate the assembly of lamin A (Dechat *et al.*, 1998). The α - and β -isoforms of LAP2 both possess the same putative chromatin interaction domains in their N-terminal regions. However, double immunofluorescence confocal microscopy experiments using transfected HeLa cells expressing LAP2 α mutants demonstrated that LAP2 α associates with chromosomes via an α -specific domain clearly before LAP2 β -containing membrane structures accumulate around chromosomes (Vlcek *et al.*, 1999).

In summary, lamina assembly appears to involve first the recruitment of lamin B to the INM by interactions with LAP2 β and LBR. Lamin A/C then associates with lamin B, and together they recruit emerin and, perhaps, LAP1C. Binding of lamins to LBR, LAPs and emerin may act to anchor them at the INM (Ostlund *et al.*, 1999; Wilson, 2000; Hutchison *et al.*, 2001).

1C. The PI2 Antigen

Antibody PI2 is a monoclonal IgM produced from mice immunized with nuclear matrices from resting mouse splenic lymphocytes (Chaly *et al.*, 1984; N. Chaly, personal communication). Immunofluorescence labelling of mouse 3T3 fibroblasts with the antibody PI2 showed a fine granular staining at the periphery of the nucleus as well as sparse internal speckling and some internal streaks (Chaly *et al.*, 1984). The internal localization of PI2 was neither centromere-associated nor correlated with specific interchromatic structures (Chaly *et al.*, 1989). Since many cell types contain NE-derived invaginations extending into the nucleoplasm, the internal nuclear labelling noted, may be at least partly attributable to NE invaginations.

At the electron microscope level, the internal labelling component of the PI2 antigen in 3T3 fibroblasts was found to consist of uneven spots distributed in a seemingly random manner in the interchromatic regions (Chaly *et al.*, 1989). Peripheral staining was shown to be continuous at the nuclear rim except where interrupted by nuclear pores, suggesting that the protein is not associated with NPCs. The peripheral staining pattern indicated that the antigen was localized at the NE, but it could not be determined whether it was on either the nucleoplasmic or cytoplasmic face, or on both faces.

Immunofluorescence microscopy of different cell types detected the PI2 antigen in HeLa, Chinese Hamster Ovary (CHO), rat kangaroo PtK2 and *Drosophila melanogaster* KcA311 cells, and in mouse, bovine and human lymphocytes. However, MAb PI2 was found not to label the plant cells *Vicia hajistana*, the fungus *Entomophthora aulicae*, and the unicellular algae *Polytomella agilis*. The labelling pattern also showed some tissue and/or species-specific variability, as the intranuclear

labelling was not detected in CHO cells or in mouse lymphocytes (Chaly *et al.*, 1986).

Immunoblotting of lymphocyte matrices with MAb PI2 identified three bands with relative molecular weights of 35, 70, and 140 kDa. It was suggested that they might be related subunits or degradation products (Chaly *et al.*, 1984). Subsequent immunoblotting results from nuclear matrix preparations of dexamethasone-treated rat thymocytes detected two bands at 80 and 140 kDa (Weaver *et al.*, 1996) while, in heat-treated mouse lymphocytes, protein bands at 70 and 35 kDa were obtained (Sodja *et al.*, 1997). These results suggest that apoptosis does not induce the cleavage of the PI2 antigen.

During mitosis, as the NE disassembled at prometaphase, the PI2 antigen gradually dispersed into the cytoplasm in immunofluorescence-labelled 3T3 fibroblasts. During metaphase through telophase, the cytoplasm was diffusely labelled, but the chromosomes remained unstained. However, during these stages, two spots were labelled at sites apparently corresponding to the spindle poles. At telophase, PI2 reappeared at the periphery of the chromatin, apparently in parallel with the NE. The cytoplasmic staining seen during mitosis was fainter than the nuclear staining seen in interphase (Chaly *et al.*, 1984).

The nuclear matrix is a residual nuclear fraction that consists of non-histone nuclear proteins and small quantities of DNA and RNA. Structurally, the nuclear matrix comprises relatively insoluble components of the NPC and lamina, an internal fibrogranular network, and residual nucleoli (Berezney *et al.*, 1995). Nuclear matrices were prepared by subjecting isolated morphologically intact nuclei from mouse 3T3 fibroblasts to a series of treatments involving detergent (TX-100) and salt extractions,

and nuclease digestion. The PI2 antigen was found not to be redistributed during nuclear matrix preparation from mouse 3T3 fibroblasts, but was partly extracted. The internal component of the protein was more labile, as it was progressively removed from nuclei treated with hypotonic buffer. However, the peripheral component was proportionately less solubilized since it was present in DNase and RNase matrices, although staining less brightly. The residual peripheral staining closely resembled the localization of lamins (Chaly *et al.*, 1985). Based on the relative insolubility of the PI2 antigen, it was suggested that the protein might function as a structural component throughout nuclei, perhaps in conjunction with lamins.

Studies to date suggest that PI2 does not appear to play a role in physiological processes. No reorganization or change in PI2 intensity was seen in stimulated lymphocytes as compared with that of resting lymphocytes. Similar results were obtained with samples labelled with anti-lamin B, as no change was detected during stimulation (Chaly *et al.*, 1988). Thus, it has been proposed that these antigens may be present at the nuclear periphery in amounts sufficient to keep pace with the growth in the nuclear lamina/envelope.

It was also observed that the PI2 antigen did not redistribute during the massive changes in the nuclear structure and function induced by adenovirus infection (Chaly *et al.*, 1988). As well, PI2 showed no change in localization during L6E9 differentiation from myoblasts to myotubes (E. Jones-Villeneuve, N. Chaly and D.L. Brown, unpublished data) or during blastogenesis. It was therefore suggested that PI2 does not play a role in metabolic processes.

1D. Working Hypothesis

Based on the data obtained for the antigen PI2 to date, it appears that it is not a NPC protein. Nor does it appear to be any of the previously characterized nuclear proteins due to the unique molecular weight banding pattern detected. However, because of the localization of the antigen at the nuclear periphery in immunofluorescently labelled samples and at the electron microscope level, it is postulated that the PI2 antigen is a protein of the nuclear membrane. Therefore, the protein may participate in nuclear envelope disassembly and reassembly during mitosis. Consequently, immunofluorescence and biochemical characterization of the PI2 antigen were carried out in relation to the known NE proteins lamin B, LAP2, and emerin.

Chapter 2: MATERIALS AND METHODS

2A. Cell Culture

Human HeLa cervical carcinoma cells and mouse 3T3 fibroblast cells were routinely cultured in Alpha-Modified Minimum Essential Medium, supplemented with 10% Controlled Process Serum Replacement-type 1 (Sigma) and 1% penicillin-streptomycin-Fungizone (100 X stock: penicillin G 10,000 U/mL, streptomycin sulphate 10 mg/mL, Fungizone 25 µgm/mL (Sigma)) at 37°C and 5% CO₂. Mouse EL4 lymphoma cells were routinely cultured at 37°C and 8% CO₂ in Dulbecco's Modified Eagle Medium, supplemented as above.

2B. The PI2 Hybridoma

The antibody PI2 is a monoclonal IgM produced by the PI2 hybridoma, which was derived from mice immunized with nuclear matrices from resting mouse splenic lymphocytes (Chaly *et al.*, 1984). At the start of these studies, the PI2 hybridoma was sub-cloned in soft agar, and eight clones were picked for assaying. Of these, supernatants from three, PI2(A3), PI2(A5) and PI2(A7), were positive in initial immunofluorescence microscopy tests on mouse 3T3 cells, *i.e.* the nuclear periphery and the nucleoplasm were labelled as described by Chaly *et al.* (1984). The supernatant fractions of the medium from these clones were then tested further using 3T3, HeLa, and mouse EL4 cells to determine which clone produced the brightest staining intensity of the nucleus, with the least amount of cytoplasmic labelling. The clones had varying amounts of internal labelling and different intensities at the periphery. After discussion with Dr. Chaly, it was decided that the clone showing the most restricted labelling, *i.e.* confined to the

nuclear periphery, would be selected. Of the three clones, PI2(A7) had the most nucleoplasmic labelling while the peripheral labelling of PI2(A3) was less intense than that of PI2(A5). The clone best fitting the defined criteria was PI2(A5), which resulted in the brightest labelling at the nuclear periphery and no internal nuclear labelling in all of the three cell lines tested. Two hundred milliliters of the tissue culture medium supernatant from this clone was then collected, concentrated by ammonium sulphate precipitation and lyophilized (Harlow and Lane, 1988). All microscopy experiments for this thesis were performed using this antibody preparation. Immunoblotting and immunoprecipitation were performed using an antibody preparation made from ascites fluid purified by hydroxyapatite chromatography (J. E. Little, D. L. Brown, and N. Chaly, unpublished data).

2C. Conventional Immunofluorescence Microscopy

Cells were seeded onto coverslips at 0.5×10^6 cells per 100 mm tissue culture dish and grown overnight. For labelling, they were fixed for 5 min in 3% paraformaldehyde in phosphate-buffered saline (0.13 M NaCl, 5.1 mM Na_2HPO_4 , 1.6 mM $\text{K}(\text{H}_2\text{PO}_4)_2$, pH 7.0) (PBS), rinsed with PBS, and permeabilized in 0.2% TX-100 in PBS for 20 min as previously described (Chaly *et al.*, 1984, 1988). Samples were then incubated sequentially in PBS-diluted primary and secondary antibodies, stained for 2 min with 0.2 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI), and mounted using Vectashield mounting medium (Vector Laboratories).

Samples to be double-labelled for two antigens were similarly fixed and permeabilized; they were then incubated sequentially in the first set of primary and

secondary antibodies and then with the second set of primary and secondary antibodies.

The samples were stained with DAPI and mounted as described above.

Samples were viewed on a Zeiss Axiophot I epifluorescence microscope equipped with a 63 X oil (N.A. 1.3) Neofluar objective and a 50-watt mercury burner. Images were digitally captured using a Hamamatsu C-5985 integrating chilled CCD camera and MetaMorph® v.4.0 software (Universal Imaging Corporation). Tagged image file format images were processed in MetaMorph® v.4.0 and Adobe Photoshop v.5.0.2.

2D. SDS-PAGE and Immunoblotting

For preparation of lysates, cells were seeded in 100 mm plates at 4×10^6 cells/dish and cultured overnight as described in section 2A. They were released from the 100 mm culture dishes by trypsinization, with the action of the trypsin stopped by addition of complete medium. Cell counts were done using a haemocytometer after trypsinization. The cells were then harvested by centrifugation at 1000 rpm for 5 min in a Centra 4 (rotor 215) (International Equipment Company) at room temperature. The pellet was washed once in ice-cold PBS and pelleted at 1500 rpm for 5 min in an Eppendorf Centrifuge 5415 (rotor A11) (Brinkmann). Pellets were lysed by incubation in high radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, and 1% TX-100) with protease inhibitors (1 mM Pefabloc (Boehringer-Mannheim), 1 mM benzamidine (Sigma), 50 µg/mL leupeptin (Sigma), and 10 µg/mL aprotinin (Sigma)) on ice for 15 min. After centrifugation in an Eppendorf Centrifuge 5415 (rotor A11) at 14,000 rpm for 20 min at 4°C, the volume of the supernatant, *i.e.* lysate, was

measured and transferred to a microfuge tube. The concentration of protein in the lysate was determined using the Bio-Rad Protein Assay.

Gel electrophoresis was performed according to standard protocols with a 4% stacking gel and a 10% separating gel, using the Bio-Rad Mini Protein II apparatus. Samples were diluted in a 4X sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 7.5% glycerol, 0.005% bromophenol blue, 22.5% β -mercaptoethanol) (Laemmli, 1970) and loaded on the gels after boiling for 5 min. Samples were loaded by equal cell number, usually 3×10^4 cells, or by equal amount of protein, usually 20 μ g. The gels were run at 120 V for 1-1.5 h until the dye front had run off the bottom of the gel.

For immunoblotting, proteins were electrophoretically transferred onto nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% (w/v) methanol) using the Bio-Rad Mini Protein II blotting apparatus at 30 V overnight at 4°C with stirring. After the transfer, the nitrocellulose membrane was rinsed several times with distilled water, stained with Ponceau Red (0.5% Ponceau Red (Sigma), 3% trichloroacetic acid) for 5 min and scanned. The membrane was destained in PBS for 10 min and washed 3 X 10 min in Tris-buffered saline with Tween 20 (50 mM Tris base, 150 mM NaCl, pH 7.5 with 0.12% Tween 20) (TBST). The membrane was then blocked for 2 h on a Belly Dancer (Stovall Life Science, Inc.) with 5 % skim milk in TBST, incubated with primary antibody in 1% skim milk in TBST for 1 h on a Nutator (Adams), washed 8 X 5 min with TBST, incubated with secondary antibody in 1% skim milk in TBST for 1 h on a Nutator, and washed again for 8 X 5 min in TBST. All of the aforementioned steps were carried out at room temperature and all washes were performed on a Belly Dancer. The nitrocellulose membrane was then treated with the

chemiluminescence kit Lumi-Light Plus (Roche) and the reaction detected using Kodak X-OMAT Blue film and Kodak Bio-Max intensifying screen. The film was developed using the GBX developing kit (Kodak), air-dried, and scanned.

Migration of the proteins was measured and interpolated into standard curves for all of the immunoblots. Each calculation represents results from at least 15 blots.

2E. Antibodies

Primary antibodies: Monoclonal antibody (MAb) PI2, a mouse monoclonal IgM (lyophilized supernatant; 1:50 for IFS) (Chaly *et al.*, 1984); MAb PI2, a mouse monoclonal IgM, ascites fluid purified by hydroxyapatite chromatography and lyophilized (1:500 for blotting) (see section **2B**); rabbit anti-lamin B, an antiserum produced against a peptide containing residues 411-425 (NH₂-TTRGKRKRVDVEESC-COOH) which are found in both human and mouse (1:500 for IFS, 1:6,000 for blotting) (gift of N. Chaudhary) (Chaudhary and Courvalin, 1993); anti-LAP2, a mouse monoclonal IgG produced against a peptide containing residues 1-187 that binds to at least 6 known isoforms of LAP2 in mouse, (1:500 for IFS, 1:7000 for blotting) (gift of K. Wilson, Johns Hopkins University, Baltimore); anti-emerin, a mouse monoclonal IgG produced against a 222 amino acid fragment near the N-terminus of the emerin protein (lyophilized supernatant, 1:50 for IFS, 1:200 for blotting) (Novocastra); antibody Y12, a mouse monoclonal anti-Sm IgG produced by fusing spleen cells from mice with a lupus erythematosus-like syndrome and the mouse myeloma SP 2/0 cell line (lyophilized supernatant, 1:10 for IFS) (Lerner *et al.*, 1981).

Secondary antibodies: All were purchased from Jackson ImmunoResearch Laboratories, Inc.

For immunofluorescence: Cyanine 3 (CY3)-conjugated goat anti-mouse IgM (μ chain-specific) (1:200); CY3-conjugated donkey anti-rabbit IgG (heavy- and light-chain specific) (1:200); CY3-conjugated goat anti-mouse IgG (Fc fragment-specific) (1:400); fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Fc fragment specific) (1:100); FITC-conjugated goat anti-mouse IgM (μ chain-specific) (1:200).

For immunoblotting: horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (μ chain-specific) (1:1000); HRP-conjugated goat anti-rabbit IgG (1:10,000) (Promega); HRP-conjugated goat anti-mouse IgG (heavy- and light-chain specific) (1:4000).

2F. Digitonin Permeabilization

Coverslips of 3T3 or HeLa cells were prepared as described in section 2C. Cells were permeabilized with 0.004% digitonin (Sigma) in PBS for 10 min on ice, washed in PBS (2 X 30 sec) and fixed in 3% paraformaldehyde in PBS for 5 min as previously described (Simos *et al.*, 1996). The coverslips were then washed 3 X 4 min in PBS and incubated sequentially in PBS-diluted primary antibodies and the appropriate secondary antibodies, stained with DAPI, and mounted as described in section 2C.

An alternate method for digitonin permeabilization as described by Pante *et al.* (1994) was also carried out, in which the samples were first fixed in 3% paraformaldehyde in PBS for 20 min, washed in PBS (2 X 30 sec) and only then

permeabilized in 0.004% digitonin in PBS for 15 min on ice. The coverslips were then washed in PBS (3 X 4 min) and immunolabelled as described above.

2G. Triton X-100 Extractions

I. Immunofluorescence

3T3 cells growing on coverslips were incubated on ice for 30 min in PBS, in 1% TX-100 in PBS, or in 1% TX-100 with 350 mM or 500 mM NaCl in PBS, with each supplemented with 1mM dithiothreitol, 1 mM Pefabloc, 1 mM benzamidine, 50 µg/mL leupeptin, and 10 µg/mL aprotinin. They were then washed in PBS (2 X 30 sec) and fixed in 3% paraformaldehyde in PBS for 5 min as previously described (Rolls *et al.*, 1999). The coverslips were then washed in PBS (3 X 4 min) and processed for immunofluorescence labelling as in section 2C.

II. Immunoblotting

Overnight cultures of 3T3 cells grown in 100 mm tissue culture dishes, as in section 2A, were trypsinized, counted, and aliquots containing 3×10^6 cells harvested by centrifugation at 1000 rpm in an Eppendorf Centrifuge 5810 (rotor A-4-62) (Brinkmann) at room temperature for 5 min. The pellets were washed once in ice-cold PBS and pelleted at 1500 rpm in an Eppendorf Centrifuge 5810 (rotor F 45-30-11) for 5 min. The pellets were then resuspended in ice-cold PBS, in 1% TX-100 in PBS, or in 1% TX-100 in PBS with 350 mM, or 500 mM NaCl, with each supplemented with 1mM dithiothreitol, 1 mM Pefabloc, 1 mM benzamidine, 50 µg/mL leupeptin, and 10 µg/mL aprotinin, and incubated on ice for 30 min.

The samples were then centrifuged at 6500 rpm in an Eppendorf Centrifuge 5810 (rotor F 45-30-11) for 10 min at room temperature. The pellet fractions were washed once in PBS, pelleted at 6500 rpm for 10 min as above and solubilized directly in 20 μ l of 4X sample buffer. Proteins in the supernatant fractions were precipitated with 15% trichloroacetic acid and pelleted at 14,000 rpm in an Eppendorf Centrifuge Model 5810 (rotor F 45-30-11) for 10 min; the pellets were washed twice with acetone and solubilized in 20 μ l of 4X sample buffer. The pellet and supernatant samples were then processed for SDS-PAGE and immunoblotting as in section **2D**.

2H. 8 M Urea Extractions

Overnight cultures of mouse 3T3 cells grown in 100 mm tissue culture dishes, as in section **2A**, were trypsinized, counted, and aliquots containing 3×10^6 cells harvested by centrifugation at 1000 rpm in an Eppendorf Centrifuge 5415 (rotor A11) at room temperature for 5 min. The pellet was resuspended in a solution of 20 mM Tris-HCl (pH 8.8), 25 mM NaCl, 8 M urea, 1 mM dithiothreitol, 1 mM Pefabloc, 1 mM benzamidine, 50 μ g/mL leupeptin, and 10 μ g/mL aprotinin, and incubated for 30 min at room temperature, as previously described (Lin *et al.*, 2000). The sample was then centrifuged at 19,200 rpm in an MR 22i ultracentrifuge (rotor AM2.19) (Jouan) at 18°C for 60 min. Proteins in the supernatant fractions were precipitated with 15% trichloroacetic acid as in section **2H**, and were solubilized in 40 μ l of 4X sample buffer. The pellets were solubilized directly in 40 μ l of 4X sample buffer, and each sample was then processed for SDS-PAGE and immunoblotting as above.

2I. 0.1 M NaOH Extractions

Overnight cultures of mouse 3T3 cells grown in 100 mm tissue culture dishes, as in section **2A**, were trypsinized, counted, and aliquots containing 3×10^6 cells harvested by centrifugation at 1000 rpm in an Eppendorf Centrifuge 5415 (rotor A11) at room temperature for 5 min. The pellet was resuspended in a solution of 0.1 M NaOH, 10 mM dithiothreitol, 1 mM Pefabloc, 1 mM benzamidine, 50 $\mu\text{g/mL}$ leupeptin, and 10 $\mu\text{g/mL}$ aprotinin, and centrifuged at 19,200 rpm in an MR 22i ultracentrifuge (rotor AM2.19) (Jouan) at 4°C for 60 min, as previously described (Lin *et al.*, 2000). Proteins in the supernatant fractions were precipitated with 15% trichloroacetic acid as in section **2H**, and were solubilized in 40 μl of 4X sample buffer. The pellets were solubilized directly in 40 μl of 4X sample buffer, and each sample was then processed for SDS-PAGE and immunoblotting as above.

Chapter 3: RESULTS

The data presented below are all derived from at least three repeats of the experiment with consistent results.

3A. The PI2 Hybridoma

I. Immunofluorescence

To compare the PI2(A5) labelling produced by the subcloned PI2(A5) with the labelling originally reported for PI2 in Chaly *et al.* (1984), immunofluorescence microscopy with the antibody PI2(A5) was carried out using several cell types. In interphase mouse 3T3 fibroblasts and human HeLa cells, a fine granular staining was observed at the periphery of the nucleus (Fig. 4A, a-a', b-b') as well as nucleoplasmic streaks, as previously described (Chaly *et al.*, 1984, 1985, 1986). A similar labelling pattern was also noted in interphase mouse EL4 lymphoma cells (Fig. 4A, c-c'). However, the internal nuclear speckles and the stained granules in the cytoplasm near the nuclear surface previously reported by Chaly *et al.* (1984, 1985) were not detected with PI2(A5). The remainder of the microscopy studies were carried out using the antibody produced by the A5 subclone of PI2.

II. Immunoblotting

It was originally intended that all work for this thesis would be conducted using MAb PI2(A5). Immunoblotting with the lyophilised MAb PI2(A5) initially consistently only detected a band at 140 ± 2.5 kDa. However, the antibody preparation lost potency over time, presumably as a result of refrigeration problems during the moves of

Figure 4: Immunofluorescence microscopy and immunoblotting analysis of the PI2 antigen in mouse 3T3 fibroblasts, human HeLa cells, and mouse EL4 lymphoma cells.

A: Immunofluorescence microscopy of mouse 3T3 fibroblasts (a-a'), human HeLa cells (b-b'), and mouse EL4 lymphoma cells (c-c') were labelled with MAb PI2(A5) (a-c) and counterstained with DAPI (a'-c'). Arrowhead points to internal streak. Magnification X 2700.

B: Immunoblotting analysis with MAb PI2. Immunoblotting was carried out on a 10% SDS-PAGE gel loaded with 20 µg of protein per lane. Samples were whole cell lysates prepared from 3T3, HeLa, and EL4 cells. Migration of molecular weight standards is shown in kDa.

the laboratory from ELBA to Tory and then to Nesbitt. Immunoblotting with this antibody preparation after the two moves gave inconsistent results where either a single band at 70 kDa or an additional band at 35 kDa was also seen. Due to time constraints rather than subcloning the hybridoma once again and preparing fresh supernatants, a preparation of MAb PI2 made previously from ascites fluid was tested. This antibody preparation consistently detected three bands at 138 ± 3.2 kDa, 70 ± 2.2 kDa, and 35 ± 1.5 kDa during immunoblotting.

Immunofluorescence labelling with this antibody preparation was essentially identical to that described by Chaly *et al.* in 1984 for the PI2 antibody. It was also compared to MAb PI2(A5) and in addition to the immunofluorescence labelling at the nuclear periphery and the internal streaks detected by PI2(A5), the more concentrated MAb preparation also produced a fine granular nucleoplasmic staining that appeared to exclude the nucleoli. Within the cytoplasm there was scattered granular staining as well as one or more brighter foci detected close to the nuclear membrane.

The differences noted between the MAb PI2(A5) preparation and that of the ascites fluid in immunofluorescence and immunoblotting are likely due to the higher antibody concentration in the latter, which was therefore used for all of the immunoblotting experiments. It will be referred to as MAb PI2.

In this study, immunoblotting of whole cell lysates prepared from 3T3, HeLa and EL4 cells with MAb PI2 consistently detected three bands migrating with the relative molecular masses of 138, 70, and 35 kDa in all three cell types (Fig. 4B). Of the three bands detected, the 138 kDa band was always of the highest intensity with the 35 kDa band being the least intense. This observation was consistent across the three species.

These results are also consistent with the original immunoblotting studies which found three bands at 140, 70, and 35 kDa in samples of mouse lymphocyte nuclear matrices (Chaly *et al.*, 1984).

The molecular weights and banding pattern detected for the PI2 antigen are distinct from other known NE proteins, indicating that further investigation of the antigen was warranted.

3B. Dynamics of the PI2 antigen during mitotic disassembly and assembly of the nuclear envelope

As discussed in the Introduction, the NE disassembles at prophase and reforms around segregated chromosomes beginning in late anaphase. Studies show that NE proteins leave the nuclear periphery and reappear sequentially (Chaudhary and Courvalin, 1993; Ellenberg *et al.*, 1997; Bodoor *et al.*, 1999; Haraguchi *et al.*, 2000). Though it was reported that the PI2 antigen disperses into the cytoplasm at prometaphase and reappears around the periphery of chromatin at telophase (Chaly *et al.*, 1984), the timing of these events in relation to the dynamics of known nuclear proteins was not examined. To characterize the behaviour of PI2 during the cell cycle in greater detail, double-labelling experiments were therefore carried out on 3T3 cells, using MAb PI2 and the available antibodies against the peripheral protein of the inner NE, lamin B, and the integral NE proteins, LAP2, and emerin. The stage of mitosis was determined by counterstaining with DAPI.

With respect to the PI2 antigen, the results were consistent with and extend the initial description of its cell cycle behaviour in 3T3, HeLa, Chinese Hamster Ovary, rat kangaroo PtK2 and *Drosophila melanogaster* KcA311 cells, and in mouse, bovine and

human lymphocytes (Chaly *et al.*, 1984, 1985, 1986). During interphase (Figs. 4a-a', 5a-a', 7a-a', 9a-a'), MAb PI2 labelling was noted at the periphery of the nucleus, and occasional internal streaks were seen in the nucleoplasm (Figs. 4c). During prophase, as the NE starts to disassemble, the PI2 antigen was still detected at the periphery of the condensing chromosomes but at a slightly lesser intensity (Figs. 5b-b', 7b-b', 9b-b'). In late prophase (Figs. 7c-c', 9c-c') and early prometaphase (Figs. 5c-c'), however, the labelling became discontinuous at the rim of the chromosome mass. As prometaphase progressed, the antigen PI2 gradually dispersed from the periphery of the chromosomes into the cytoplasm (Figs. 5d-d', 5e-e'), and at metaphase, the labelling was throughout the cytoplasm (Figs. 5f-f', 7d-d', 9d-d'). Depending on the plane of focus at which the images were captured, a band of label in the cytoplasm outlining the unstained chromosomes was sometimes visible (Fig. 5f-f').

Throughout anaphase, MAb PI2 labelling was excluded from the regions occupied by the chromosomes (Figs. 6a-a', 6b-b', 8a-a', 8b-b', 10a-a', 10b-b'). The staining was largely diffuse, with regions of higher intensity labelling which were apparently random in localization seen in some cells (Fig. 6b'). The antigen was first detected at the periphery of the decondensing daughter chromosomes in early telophase (Figs. 6c-c', 8c-c', 10c-c'). By late telophase (Figs. 6d-d', 8d-d', 10d-d'), the PI2 antigen showed uniform distribution around the decondensing chromosomes, with cytoplasmic labelling no longer being detected at early G1 (Figs. 6e-e', 8e-e', 10e-e').

Figure 5: Immunofluorescence microscopy of the distribution of the PI2 antigen in relation to lamin B at interphase through metaphase.

3T3 cells were labelled with MAb PI2 (a'-f') and anti-lamin B (a''-f''), and counterstained with DAPI (a-f). The corresponding overlayed images of PI2 and lamin B are shown in a'''-f'''; PI2 is shown in green, and lamin B is shown in red, with regions of overlays in yellow. Magnification X 2600.

Figure 6: Immunofluorescence microscopy of the distribution of the PI2 antigen in relation to lamin B at early anaphase through early G1.

3T3 cells were labelled with MAb PI2 (a'-e') and anti-lamin B (a''-e''), and counterstained with DAPI (a-e). The corresponding merged images of PI2 and lamin B are shown in a'''-e'''; PI2 is shown in green, and lamin B is shown in red, with regions of overlays in yellow. Magnification X 2600.

Figure 7: Immunofluorescence microscopy of the distribution of the PI2 antigen in relation to LAP2 at interphase through metaphase.

3T3 cells were labelled with MAb PI2 (a'-d') and anti-LAP2 (a''-d''), and counterstained with DAPI (a-d). The corresponding overlayed images of PI2 and LAP2 are shown in a'''-d'''; PI2 is shown in green, and LAP2 is shown in red, with regions of overlays in yellow. Magnification X 2600.

Figure 8: Immunofluorescence microscopy of the distribution of the PI2 antigen in relation to LAP2 at early anaphase through early G1.

3T3 cells were labelled with MAb PI2 (a'-e') and anti-LAP2 (a''-e''), and counterstained with DAPI (a-e). The corresponding merged images of PI2 and LAP2 are shown in a'''-e''''. Magnification X 2600.

Figure 9: Immunofluorescence microscopy of the distribution of the PI2 antigen in relation to emerin at interphase through metaphase.

3T3 cells were labelled with MAb PI2 (a'-d') and anti-emerin (a''-d''), and counterstained with DAPI (a-d). The corresponding overlayed images of PI2 and emerin are shown in a'''-d'''; PI2 is shown in green, and emerin is shown in red, with regions of overlays in yellow. Magnification X 2600.

Figure 10: Immunofluorescence microscopy of the distribution of the PI2 antigen in relation to emerlin at early anaphase through early G1.

3T3 cells were labelled with MAb PI2 (a'-e') and anti-emerin (a''-e''), and counterstained with DAPI (a-e). The corresponding merged images of PI2 and emerlin are shown in a'''-e'''; PI2 is shown in green, and emerlin is shown in red, with regions of overlays in yellow. Magnification X 2600.

I. Antigen PI2 and lamin B

The results of the double labelling experiments with anti-lamin B and MAb PI2 are illustrated in Figures 5 and 6. The lamin B distribution detected during mitosis was in accordance with previously published reports using HeLa, NRK, and mouse epidermal cells (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Moir *et al.*, 2000).

Overall, the labelling of lamin B and MAb PI2 were found to co-localize throughout the mitotic cycle (Figs. 5, 6). During interphase and prophase, lamin B and MAb PI2 were both detected at the nuclear periphery and inside the nucleus and as nucleoplasmic streaks. However, the streaks seen with MAb PI2 were fewer and perhaps a subset of those seen with lamin B (Figs. 5a-a''', 5b-b'''). The organization of MAb PI2 and lamin B during the early stages of mitosis from prophase through to late prometaphase were closely compared, and showed no significant differences in the labelling patterns (Figs. 5c-e'''). In late prometaphase and metaphase, the labelling of the two antigens dispersed into the cytoplasm, with each showing enrichment in the area immediately surrounding the chromosome mass (Figs. 5e-f'''). This observation was emphasized in the overlay by the presence of a bright yellow band (Figs. 5e'', 5f''). This enrichment outside the metaphase plate for lamin B is consistent with reports by Chaudhary and Courvalin (1993), but has not been reported previously for the PI2 antigen.

From early anaphase through to late telophase, there was no temporal displacement detected in the reassociation of lamin B and the antigen PI2 with the chromosome surfaces. Throughout anaphase, the labelling for both remained cytoplasmic, excluding the chromosomes (Figs. 6a-a''', 6b-b'''), with reassociation at the

periphery of the decondensing daughter chromosomes evident early in telophase (Figs. 6c-c'''). By late telophase (Figs. 6d-d'''), lamin B and MAb PI2 both were uniform in their localization around the periphery of the reforming nuclei and were co-localized, with no apparent cytoplasmic staining detected by early G1 (Figs. 6e-e''').

These observations indicate that the dynamics of the two proteins during NE assembly and disassembly are very similar, and may be the same.

II. Antigen PI2 and LAP2

Figures 7 and 8 show results from the double-labelling experiments with MAb PI2 and anti-LAP2. The labelling patterns detected for LAP2 throughout mitosis were consistent with the published reports for rat FRSK, NRK, and HeLa cells (Foisner and Gerace, 1993; Furukawa *et al.*, 1995; Dechat *et al.*, 2000). At interphase and prophase, anti-LAP2 labelling was detected at the nuclear periphery as well as in the nucleoplasm (Figs. 7a, 7a'', 7b, 7b''). The nucleoplasmic staining consisted of speckles, which were uniformly distributed throughout the nucleus. The anti-LAP2 antibody was raised against a peptide common to the 6 isoforms of LAP2 found in mouse, including the LAP2 α isoform, which is nucleoplasmic. Therefore, even though 5 of the 6 LAP2 isoforms are integral membrane proteins, the labelling detected in the nucleoplasm is most likely due to the LAP2 α isoform, which lacks the transmembrane domain (Dechat *et al.*, 2000a).

In very late prophase, the LAP2 staining was consistently fainter and more diffuse at the periphery of the chromosomes than that of MAb PI2, which was still punctate in certain regions along the periphery (Figs. 7c-c'''). This suggests a slight temporal displacement between when the two proteins disassemble from the nuclear periphery,

with LAP2 leaving before the PI2 antigen. During metaphase and anaphase, the labelling with the two antibodies was indistinguishable, showing the antigens dispersed in the cytoplasm excluding the chromosomes (Figs. 7d-d'', 8a-a'', and 8b-b''). However, at early telophase, as the two proteins started to reassociate with the decondensing chromosomes, anti-LAP2 consistently showed brighter and more uniform labelling than MAb PI2; a substantial amount of staining in the cytoplasm was still visible with both antibodies (Figs. 8c-c''). By late telophase, the two antigens were essentially uniform at the periphery (Figs. 8d-d''), with no cytoplasmic labelling detected at early G1 (Figs. 8e-e'').

III. Antigen PI2 and emerin

Next, the localization of PI2 and emerin was compared by double-labelling, as illustrated in Figures 9 and 10. The emerin distribution detected for the different stages of mitosis was in agreement with that previously reported by Dabauvalle *et al.* (1999) for a variety of vertebrates, including man, mouse, and *Xenopus*. At interphase and early stages of prophase, emerin was localized to the periphery of the nucleus with some internal focal points (Figs. 9a, 9a'', 9b, 9b''). MAb PI2 showed some similar foci, which were coincident with those of anti-emerin but less numerous. The peripheral labelling pattern of emerin was coincident with that of MAb PI2 (Figs. 9a-a'', 9b-b''). At late prophase, however, the anti-emerin labelling was very faint, with only trace amounts left at the periphery (Figs. 9c, 9c''), while MAb PI2 still showed bright, though discontinuous, labelling at the periphery (Fig. 9c'). The relative difference in staining intensity at this stage is underlined in the overlay of the two antibodies (Fig. 9c'''). Both

emerin and antigen PI2 completely dispersed into the cytoplasm during metaphase, with MAb PI2 staining dispersing further away from the metaphase plate than that of anti-emerin (Figs. 9d-d''').

From early through late anaphase, emerin and antigen PI2 were both dispersed in the cytoplasm (Figs. 10a-b'''). However, emerin was absent from the inter-chromosomal region (Figs. 10a, 10a'', 10b, 10b''), while the PI2 antigen was also located between the separating daughter chromosomes (Figs. 10a-a''', 10b-b'''). In telophase, the PI2 antigen showed uniform distribution around the chromosome sets as usual (Figs. 10c-c'). Emerin, however, was concentrated at the two faces of each set of daughter chromosomes, the face closer to the spindle pole and the face towards the cleavage plane, with little or no labelling at the lateral face of the chromosome sets (Fig. 10c''). Both PI2 and emerin labelling was also detected in the cytoplasm surrounding the chromosomes. In late telophase, emerin displayed continuous and more even labelling around the periphery of the chromosomes (Figs. 10d-d'''). By early G1, emerin and the antigen PI2 showed uniform distribution at the nuclear rim, with no cytoplasmic staining (Figs. 10e-e''').

3C. Is PI2 an antigen of the inner or outer nuclear membrane?

Previous reports and this study have shown the PI2 antigen to be localized to the nuclear rim. The peripheral staining pattern of PI2(A5) indicated that the antigen was localized at the NE, but it could not be determined whether it was on the nucleoplasmic face, on the cytoplasmic face, or on both faces. Selective permeabilization experiments were therefore carried out to determine whether the protein faces the cytoplasm or nucleoplasm.

Methods used routinely to prepare samples for immunolabelling of nuclear antigens include permeabilization of the plasma membrane and the dual membranes of the nuclear envelope. Antibodies can then penetrate the nucleus and access epitopes on the inner nuclear membrane as well as in the nucleoplasm. To test whether PI2 is specifically associated with the inner or the outer nuclear membrane, a modified procedure was carried out in which a permeabilization step with a low concentration of the glucoside digitonin, was used prior to fixation. Digitonin permeabilizes the cholesterol-rich plasma membrane but leaves the cholesterol-poor NE essentially intact. This permits antibody access to the cytoplasm while excluding antibodies from the nuclear interior (Simos *et al.*, 1996).

Digitonin-permeabilized 3T3 cells were fixed and processed for immunofluorescence labelling as described in the Materials and Methods. If PI2 were a component of the inner nuclear membrane, it would be unlabelled in such samples. If it is associated with the outer membrane, and faces the cytoplasm, on the other hand, it would be labelled.

To verify that the digitonin-permeabilization method was effective in excluding antibodies from the nuclear interior, control samples were digitonin-permeabilized and labelled for nucleoplasmic snRNPs with the anti-Sm antibody, Y12. Using conventional labelling methods, the antibody Y12 labelled discrete nucleoplasmic foci, in interphase cells (data not shown) as previously reported by Spector and Smith (1986). Fluorescence microscopy of digitonin-permeabilized samples, however, showed no snRNP labelling of interphase nuclei, due to the exclusion of the antibody from the nucleus (Figs. 11a-a'). On the other hand, during prophase, as the nuclear envelope starts to disassemble, snRNP

Figure 11: Immunofluorescence microscopy of digitonin-permeabilized samples.

Mouse 3T3 cells (a-f'') were permeabilized with 0.004% digitonin, fixed in 3% paraformaldehyde and labelled with Y12 (a', a'''), MAb PI2 (b', b'''), anti-lamin B (c', c'''), anti-LAP2 (d', d'''), or anti-emerin (e', e'''). DAPI counterstaining is shown for interphase (a-h) and prophase (a''-h'') cells. HeLa samples which were digitonin permeabilized prior to fixation and labelled with anti-emerin are shown in g' and g'''. Mouse 3T3 (f' and f'') and HeLa (h' and h'') cells were also fixed in 3% paraformaldehyde prior to permeabilization in 0.004% digitonin and labelling with anti-emerin. Arrowheads point to prophase nuclei.

Magnification X 2150.

labelling was detected within the nucleoplasm as discrete foci (Figs. 11a''-a'''). From prophase to telophase, while the NE was disassembled, the snRNP labelling in digitonin-permeabilized samples was overall as previously described using conventional labelling methods (not shown) (Spector and Smith, 1986).

In digitonin-permeabilized samples labelled for lamin B (Figs. 11c-c') or LAP2 (Figs. 11d-d'), there was no staining of the nuclear periphery at interphase. The lamin B results are consistent with earlier reports for digitonin-permeabilized human endometrial adenocarcinoma (Ishikawa) cells, showing that the cells that were not stained with lamin B in interphase but were stained in late prophase/prometaphase (Georgatos *et al.*, 1997; Theodoropoulos *et al.*, 1999). In prophase cells, however, labelling was detected for lamin B (Figs. 11c''-c''') and LAP2 (Figs. 11d''-d''') at the nuclear periphery, as in conventionally prepared samples (Figs. 5b'', 7b''), as the antibodies now had access to the nuclear interior and their antigens, due to the disassembly of the NE. Similarly, from metaphase through telophase, as long as the NE was disassembled, the labelling detected with the two antibodies (not shown) was as previously described with conventional labelling methods (Figs. 5f'', 6a''-d'', 7d'', 8a''-d'').

There was also no staining detected in interphase digitonin-permeabilized samples labelled with MAb PI2 (Figs. 11 b-b'), but the rim of prophase nuclei was stained (Figs. 11b''-b'''). During metaphase through telophase in these samples (data not shown), labelling for MAb PI2 was consistent with the patterns seen with conventional staining methods (Figs. 5f', 6a'-d', 7d', 8a'-d', 9d', 10a'-d').

However, in digitonin-permeabilized samples labelled with anti-emerin, both interphase and prophase cells showed staining at the nuclear periphery, as well as

speckles concentrated in the cytoplasm immediately surrounding the nucleus (Fig. 11e-e’’’). These results were not consistent with previously published reports using digitonin-permeabilization on human HEP-2 cells and African green monkey COS-7 cell line (Dabauvalle *et al.*, 1999; Ostlund *et al.*, 1999). In these studies, the digitonin-permeabilized cells were not labelled by anti-emerin in interphase, and the localization of emerin was assigned to the INM. I postulated that this could be a species-specific observation and that labelling seen at the nuclear periphery during interphase in mouse 3T3 cells might be due to a back-up of emerin in the ER and outer nuclear membrane prior to its incorporation into the inner nuclear membrane (Ellenberg *et al.*, 1997; Ostlund *et al.*, 1999; Sullivan *et al.*, 1999). To test this hypothesis, the experiment was repeated using the available human cell line HeLa. However, emerin labelling at the nuclear periphery, although not uniform, was still detected at interphase in digitonin-permeabilized samples (Figs. 11g-g’), with less labelling seen in the cytoplasm immediately surrounding the NE, and uniform NE labelling as the antigen gained access to the nuclear interior at prophase (Figs. 11g’’-g’’’’).

In further efforts to reproduce published results, another method for digitonin permeabilization was tested in which the cells were fixed prior to permeabilization with digitonin (Pante *et al.*, 1994). This was the method used by Dabauvalle *et al.* (1999) when anti-emerin was shown to be excluded from the nuclear interior in HEP-2 cells. However, labelling of 3T3 and HeLa cells was still detected with the anti-emerin antibody in samples prepared with this post-fixation permeabilization method at the nuclear periphery of both interphase (Figs. 11f-f’, 11h-h’) and prophase (Figs. 11f’’-f’’’, 11h’’-h’’’’). The reason for this discrepancy with published results is unknown.

Nonetheless, the absence of PI2 labelling in digitonin-permeabilized samples suggests that the antigen is a component of the INM.

3D. Triton X-100 Extractions

Extractions using TX-100 detergent and varying concentrations of salt is an approach to characterize the solubility of proteins. When cells are extracted with 1% TX-100 and salt concentrations of 500 mM NaCl or more, most known nuclear membrane proteins, including LBR and LAP2, are solubilized (Foisner and Gerace, 1993; Rolls *et al.*, 1999). Hence, this extraction treatment is another method to determine the solubility properties of nuclear proteins. The solubility of the PI2 antigen was examined in relation to the lamina protein lamin B, and integral NE proteins LAP2 and emerin. Mouse 3T3 cells were incubated in 1% TX-100, 1% TX-100 + 350 mM, or 1% TX-100 + 500 mM NaCl, and then processed for immunofluorescence microscopy or immunoblotting.

I. Immunofluorescence

As a control, untreated samples were labelled conventionally for PI2, lamin B, LAP2, and emerin, and invariably showed the previously described labelling patterns (Figs. 12a-d). All samples were counterstained with DAPI to verify the presence of the cells (Figs. 12a'-p').

Table 2 outlines the overall labelling intensity noted for the antigens where the number of "+" indicates the overall relative labelling intensity of the sample. The labelling intensity of the untreated controls was defined as "++++".

After incubation in 1% TX-100, labelling at the nuclear periphery was still seen for all of the antigens (Figs. 12e-h, respectively), with the overall intensity noted for each

Figure 12: Immunofluorescence microscopy of the solubility of NE antigens in detergent and salt.

Immunofluorescence microscopy of mouse 3T3 cells incubated in 1% TX-100 (e-h'), 1% TX-100 + 350 mM NaCl (i-l'), or 1% TX-100 + 500 mM NaCl (m-p'), fixed in 3% paraformaldehyde and labelled with MAb PI2 (e, i, m), anti-lamin B (f, j, n), anti-LAP2 (g, k, o), or anti-emerin (h, l, p), and counterstained with DAPI (e'-p'). Control untreated samples (a-d) were processed conventionally and labelled with MAb PI2 (a), anti-lamin B (b), anti-LAP2 (c), or anti-emerin (d), and counterstained with DAPI (a'-d'). Magnification 2250X.

antigen being equal to that of the control (Table 2). However, in the LAP2 samples, the nucleoplasmic labelling likely contributed by LAP2 α was no longer detected (Fig. 12g). In samples incubated in 1% TX-100 + 350 mM NaCl, the labelling patterns of PI2 (Fig. 12i), lamin B (Fig. 12j), and emerin (Fig. 12l) were unaffected though the labelling intensity of all was slightly diminished (Table 2). However, LAP2 labelling was no longer detected (Figs. 12k, Table 2).

Extraction with 1% TX-100 + 500 mM NaCl resulted in no labelling detected for lamin B, LAP2, or emerin (Figs. 12m-p, Table 2). However, MAb PI2 labelling was still clearly visible, though it was no longer uniform around the periphery and was considerably fainter than in controls (Fig. 12m, Table 2).

II. Immunoblotting

TX-100 extractions were also carried out on 3T3 fibroblast cells and probed with MAb PI2, anti-lamin B, anti-LAP2, and anti-emerin (Fig. 13).

When the whole cell lysates were probed with MAb PI2, three bands migrating with relative molecular weights of 138, 70, and 35 kDa were detected as before (Fig. 4B; Chaly *et al.*, 1984). Samples probed with anti-lamin B detected a single molecular weight band of about 70 ± 4.2 kDa, which is consistent with previously published data that describe the mass of the lamin B proteins as being between 65-70 kDa (Chaudhary and Courvalin, 1993). Probing whole cell lysates with anti-LAP2 consistently detected three bands and two additional fainter bands were sometimes detected with longer exposure times. Based on the molecular mass of the isoforms of mouse LAP2 listed in the literature (Dechat *et al.*, 2000a), it appears that the major isoforms being visualized are LAP2 β (55 ± 5.1 kDa), LAP2 ϵ (47 ± 4.3 kDa), and LAP2 δ (42 ± 3.2 kDa), with the

Table 2: Relative labelling intensity of the antigens after incubation in detergent and salt ^a

Antigen	Untreated	1% Tx-100	1% Tx-100 + 350 mM NaCl	1% Tx-100 + 500 mM NaCl
PI2	++++ ^b	++++	+++	++
Lamin B	++++	++++	+++	-
LAP2	++++	++++	-	-
Emerin	++++	++++	+++	-

^a Immunofluorescence microscopy of mouse 3T3 cells incubated in 1% TX-100, in 1% TX-100 + 350 mM NaCl, or in 1% TX-100 + 500 mM NaCl, fixed in 3% paraformaldehyde and labelled with MAbPI2, anti-lamin B, anti-LAP2, or anti-emerin

^b the number of “+” indicates overall labelling intensity of the sample, with the intensity of untreated controls set at “++++”; “-” indicates no labelling

Figure 13: Immunoblotting analysis of the solubility of NE antigens in detergent and salt.

Immunoblotting of pellet and supernatant fractions of 3×10^6 mouse 3T3 cells incubated in PBS (PBS), 1% TX-100 (1%), 1% TX-100 + 350 mM NaCl (350), or 1% TX-100 + 500 mM NaCl (500) before processing for immunoblotting with MAbs PI2, anti-lamin B, anti-LAP2, or anti-emerin. Whole cell lysates (lys) of 3×10^6 mouse 3T3 cells were also processed as a positive control. Molecular weight standards are 250, 148, 98, 64, 50 and 36 kDa.

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minor bands corresponding to LAP2 α (75 ± 5.1 kDa) and LAP2 γ (38 ± 2.1 kDa). A band at about 34 ± 2.5 kDa was present in the lysate sample probed with anti-emerin, which is consistent with the previously reported molecular weight for this protein (Manilal *et al.*, 1996).

Upon treatment with 1% TX-100, all of the antigens were substantially solubilized, and bands were now detected in both the pellet and supernatant fractions for each of the proteins. The 55 and 42 kDa isoforms of LAP2 appeared to be proportionately more solubilized than the 47 kDa isoform after treatment with 1% TX-100, as seen by the decrease in intensity of their bands in the pellet fraction. Without quantification of the amount of the isoforms present in each lane, however, it is not possible to rule out the possibility that this disproportionate decrease in intensity stems from the fact that in general the 55 and 42 kDa isoforms are fainter. All three isoforms were detected in the supernatant fraction.

Extraction with 1% TX-100 and 350 mM NaCl solubilized a larger fraction of all of the NE antigens than did 1% TX-100 alone. However, the 35 kDa band of the PI2 antigen appeared to increase in intensity rather than decrease.

In samples treated with 1% TX-100 + 500 mM NaCl, lamin B, emerin, and LAP2 were detected only in the supernatant fraction. However, though the PI2 antigen was found primarily in the supernatant, all three bands were still clearly visible in the pellet fraction.

In control samples processed as above in the absence of detergent and salt (Fig. 13, PBS), the banding pattern for each of the proteins was the same as detected for the whole cell lysates (Fig. 13, lys). Bands were only present in the pellet fraction, which

indicated that the components of the extraction solution themselves did not solubilize the proteins.

3E. 8 M Urea and 0.1 M NaOH Extractions

Upon extraction with 8 M urea or 0.1 M NaOH, integral membrane proteins such as MAN1 and LBR have been shown to remain in the membrane fractions, *i.e.* the pellet (Gerace *et al.*, 1982). On the other hand, peripheral proteins such as lamin B are fractionated into the supernatant (Foisner and Gerace, 1993). To test whether the PI2 antigen is a peripheral or an integral membrane protein, 8 M urea and 0.1 M NaOH extractions were performed on mouse 3T3 cells and the pellet and supernatant fractions processed for immunoblotting (Fig. 14). As positive controls, extracted samples were also immunoblotted using antibodies against the known integral membrane proteins LAP2 and emerin, while the antibody against lamin B was used to characterize the behaviour of peripheral nuclear membrane proteins.

Lamin B was detected only in the supernatant fraction when treated with 8 M urea (Fig. 14A), which is consistent with reports by Foisner and Gerace (1993). In samples treated with 0.1 M NaOH, bands were detected in both the pellet and the supernatant fraction but the band in the pellet fraction was relatively fainter (Fig. 14B).

The 58, 47, and 42 kDa isoforms of LAP2 were retained in the pellet fraction both after treatments with 8 M urea (Fig. 14A) and with 0.1 M NaOH (Fig. 14B). These results are consistent with previously published results (Foisner and Gerace, 1993). Similarly, emerin was also detected only in the pellet fraction for both extraction treatments (Fig. 14), as expected for an integral membrane protein.

Figure 14: Immunoblotting analysis of the solubility of NE antigens in 8 M urea or 0.1 M NaOH.

A: Immunoblotting analysis of pellet (P) and supernatant (S) fractions of 3×10^6 mouse 3T3 fibroblasts extracted with 8 M urea before processing for immunoblotting with MAb PI2, anti-lamin B, anti-LAP2, or anti-emerin. Migration of molecular weight standards is shown in kDa.

B: Immunoblotting analysis of pellet (P) and supernatant (S) fractions of 3×10^6 mouse 3T3 fibroblasts extracted with 0.1 M NaOH before processing for SDS-PAGE, separation on a 10% SDS-PAGE gel and probing with MAb PI2, anti-lamin B, anti-LAP2, or anti-emerin. Migration of molecular weight standards is shown in kDa.

A

B

All three molecular weight bands for the PI2 antigen were found to be located in the pellet fraction when treated with 8 M urea (Fig. 14A). In comparison to whole cell lysates where the band intensity decreased correspondingly with molecular mass, the 70 kDa band was considerably fainter than the other two in the urea-treated samples. Similarly, upon extraction with 0.1 M NaOH, the PI2 antigen was only detected in the pellet fraction. However, the 35 kDa of the PI2 antigen was consistently fainter than the other two (Fig. 14B). The intensities of the 138 and 70 kDa band were almost equal. Hence, the solubility of the different bands of the PI2 antigen may vary depending on the extraction conditions. Overall, this resistance to extraction strongly suggests that the antigen PI2 is an integral protein of the NE rather than a peripheral membrane protein.

Chapter 4: *DISCUSSION*

This study involved the immunofluorescence and biochemical characterization of the PI2 antigen. It was shown that it is an integral protein of the NE, based on the resistance of the protein to be extracted by either 0.1 M NaOH or 8 M urea. Digitonin-permeabilization experiments indicate that the PI2 antigen is a component of the INM. The incomplete solubilization of the antigen when treated with 1%TX-100 and 500 mM NaCl suggests that the protein is associated with the nuclear lamina (Gerace *et al.*, 1982), and is in agreement with the findings of a protein facing the nucleoplasm. Double-immunofluorescence labelling revealed the PI2 antigen dissociates from and reassociates with the nuclear periphery late in NE disassembly and reassembly, respectively, in a temporal manner distinct from emerin and LAP2 but similar to lamin B.

4A. *Molecular Mass(es) of the PI2 Antigen*

In this study, immunoblotting of mouse 3T3 cells detected three bands at 138, 70 and 35 kDa for the PI2 antigen, as did Chaly *et al.* (1984) during the original characterization of the protein. These three bands were also detected in human HeLa and mouse EL4 lymphoma cells, suggesting species and tissue conservation. Work by Sodja *et al.* (1997) found only two bands of 70 and 35 kDa in mouse lymphocytes. Since mouse IgM is present on the surface of these cells, the 70 kDa band may partly be due to the detection of this IgM by the anti-IgM secondary antibody (N. Chaly, personal communication). However, in rat thymocytes two bands of relative molecular weight 140 and 80 kDa were detected.

The variability in the banding pattern detected in the above three studies could arise from differences in the PI2 antibody preparations and detection methods that were used. As well, it is known that expression of the proteins of the NE can be cell type specific as is noted with lamins (see section *1A. II. (a)* in the Introduction; Stuurman *et al.*, 1998). Hence, cell and species differences could have an effect on the expression of the PI2 antigen as Chaly *et al.* (1984) used mouse splenic nuclear matrix preparations, Sodja *et al.* (1996) used whole mouse lymphocytes while Weaver *et al.* (1996) used rat thymus lymphocytes.

Degradation products was the plausible explanation put forth by Chaly *et al.* (1984) for the detection of multiple bands with the PI2 antibody, as only the protease inhibitor phenyl methyl sulphonyl fluoride, which is an irreversible inhibitor of serine proteases, was used. However, this seems unlikely as the three bands have been consistently detected in this study under all conditions despite the presence of the protease inhibitors aprotinin, leupeptin, benzamidine, and Pefabloc, which among them inhibit most known proteases including several categories of serine, cysteine, trypsin and trypsin-like proteases. Furthermore, the crispness of the bands seems to argue against proteolytic degradation, which is usually manifested as a smear on the blot.

The fact that the molecular weights of the bands were near multiples of 35 kDa raised the possibility that the three bands detected were actually the 35 kDa polypeptide, as well as a dimer and tetramer of the 35 kDa protein (Chaly *et al.*, 1984). Polypeptides fold through covalent and non-covalent interactions with themselves and other polypeptides. The strongest of these molecular bonds are covalent linkages of cysteine thiol residues, that is, disulfide bonds. Weaker forces of interaction are attributed to non-

covalent bonds such as hydrogen bonding, ionic bonds, hydrophobic interactions, dipole moments, and van der Waals forces. However, heating of the sample in the presence of the anionic detergent SDS is sufficient to disrupt most non-covalent interactions (reviewed in Molloy, 2000), while disulfide interactions are reduced by β -mercaptoethanol (Jocelyn, 1987). Since the samples were dissolved in a buffer containing SDS and β -mercaptoethanol prior to loading a gel, even though multimerization is suggested by the arithmetic of the molecular weights, it is highly unlikely.

It is also possible that the lower bands arise by post-translational cleavage. However, it is unusual that both the pre- and post-cleavage products would be consistently found in almost equal amounts (Weaver *et al.*, 1996). Another possibility is that these bands could arise as separate translation products from mRNAs produced by differential splicing of a single gene, or could even be products of three separate genes, with their multimeric molecular weights being purely coincidental. The significance of the banding pattern of the PI2 antigen cannot be resolved definitively until the DNA sequence of the protein is obtained.

4B. The PI2 Antigen as an Integral Membrane Protein

Cell membranes are composed of dynamic lipid bilayers and their associated proteins. These proteins include integral and peripheral membrane proteins. Integral proteins either penetrate or span the bilayer entirely, while peripheral proteins are associated with only one face of the membrane and are linked to other membrane proteins by non-covalent interactions (Alberts *et al.*, 2002).

The two types of integral membrane proteins are transmembrane proteins, which can either span the membrane once or multiple times, or covalently tethered membrane proteins. The latter group of proteins may be largely exposed to either face of the bilayer and is covalently linked to membrane phospholipids or glycolipids. In the case of the NE, which consists of two membranes, the tethered proteins of the INM face either the nucleoplasm or the luminal space between the two membranes of the NE. Integral membrane proteins can only be removed from membranes by disrupting the phospholipid bilayer. In contrast, peripheral proteins can be dissociated from membranes under conditions that disrupt protein-protein interactions, without disrupting membrane integrity (reviewed in Silviu, 1992). Thus, the method of solubilization can be diagnostic as to whether a protein is an integral or peripheral membrane protein. Peripheral proteins can be extracted from the membrane by treating with high salt, alkaline pH, or chaotropic agents such as urea. High salt concentrations and extreme pH disrupt ionic interactions and hydrogen bonds. The relative degree of “tightness” with which a protein is bound to the membrane can be inferred from the concentration of the salt required to cause extraction (reviewed in Silviu, 1992). Chaotropic agents can disrupt the hydrogen bonding structure of water and, in concentrated solutions, they can denature proteins because they reduce the hydrophobic effect. High concentrations of chaotropic agents like 8 M urea cause the unfolding of the protein, destroying any protein-protein interactions, but permit the protein to refold upon removal of the chaotrope (Walker, 1984). Consequently, all of the above treatments will cause peripheral proteins to be solubilized, but integral proteins embedded in the membrane will remain resistant to extraction.

The non-ionic detergent TX-100 binds to proteins in proportion to their hydrophobicity and acts as a denaturant. It is used to study integral membrane proteins as the detergent binds to membranes, causing lysis and formation of detergent-lipid-protein complexes. These complexes are further solubilized to give detergent-protein complexes and detergent-lipid complexes (Neugebauer, 1988). High salt after incubation with TX-100 is used to extract the peripheral membrane proteins that may be associated with the membrane. When NEs are incubated with 1% TX-100 and then 500 mM NaCl, NPCs as well as nuclear membranes are solubilized, with the nuclear lamina being the only structure that remains insoluble (Gerace *et al.*, 1982). Therefore, resistance to extraction with 8 M urea or 0.1 M NaOH is considered diagnostic of integral membrane proteins (Gerace *et al.*, 1982), while resistance to extraction with 1%TX-100 and 500 mM NaCl is considered to be indicative of associations with the nuclear lamina.

The integral membrane proteins emerin, LAP1, LAP2, LBR, and MAN1 have all been shown to be insoluble when treated with 8 M urea or 0.1 M NaOH (Senior and Gerace, 1988; Foisner and Gerace, 1993; Lee *et al.*, 2000; Lin *et al.*, 2000). Similar results were obtained in this study for emerin and LAP2, which were only detected in the pellet fractions when samples were treated with 8 M urea or 0.1 M NaOH.

On the other hand, the peripheral membrane proteins lamins A, B1, and C were previously reported to be solubilized with 8 M urea (Foisner and Gerace, 1993). However, in this work, urea and NaOH extractions probed with anti-lamin B, gave somewhat conflicting results. As anticipated for a peripheral membrane protein, lamin B was detected in the supernatant fraction in samples treated with 8 M urea, but was detected in both supernatant and pellet fractions when treated with 0.1 M NaOH, though

the latter band was relatively fainter. However, due to the tight association of lamin B with INM proteins and chromatin (Simos *et al.*, 1996), the protein does not always behave in a manner consistent with a peripheral membrane protein. This may account for the faint band detected in the pellet fraction of the 0.1 M NaOH extractions.

The antigen PI2 was found to be insoluble when extracted with 8 M urea or 0.1 M NaOH and was detected only in the pellet fraction. These results indicate that the PI2 antigen is an integral membrane protein of the NE.

Extractions using 1% TX-100 and varying salt concentrations suggest that emerin, nurim, and the LAPs, to some degree, have associations with the nuclear lamina (Foisner and Gerace, 1993; Dechat *et al.*, 1998; Ellis *et al.*, 1998; Rolls *et al.*, 1999), which has also been indicated by other data (as reviewed in Stuurman *et al.*, 1998; Dechat *et al.*, 2000a; Gruenbaum *et al.*, 2000; Wilson, 2000; Foisner, 2001; Georgatos, 2001).

In earlier studies using isolated NE from rat liver, LAP2 was found not to be solubilized by 1% TX-100 and only half-solubilized by 1% TX-100 and 250 mM NaCl, but completely solubilized by 1% TX-100 with 500 mM NaCl (Foisner and Gerace, 1993). These data are congruent with the results in this study in that they are identical for the 1% TX-100 and for the 1% TX-100 and 500 mM NaCl. However, only partial solubilization was reported for LAP2 with 250 mM NaCl, whereas it appeared that LAP2 was completely solubilized in this study with 350 mM NaCl. The latter condition is a stronger salt concentration closer to 500 mM, which could have been strong enough to solubilize LAP2. Furthermore, Dechat *et al.* (1998) reported that, whereas LAP2 α is not solubilized from rat liver nuclei by extraction with 1% TX-100 and 250 mM NaCl, the majority of both LAP2 α and LAP2 β in isolated rat kidney nuclei were soluble under

these conditions. The difference in results between the two tissues was attributed to LAP2 being more tightly bound to karyoskeletal structures in rat liver than in rat kidney cells (Dechat *et al.*, 1998).

Similar work was done by Rolls *et al.* (1999) with a truncated LAP2 construct, lacking the chromatin- but not the lamin-binding domain, fused with GFP called LAP2-S. Using immunofluorescence microscopy, GFP-LAP2-S expressed in baby hamster kidney cells were shown to remain at the nuclear periphery after treatment with 1% TX-100 but was removed after extraction with 1% TX-100 and 500 mM NaCl. Further, it was also demonstrated by immunofluorescence that GFP-LAP2-S expressed in chicken fibroblasts is also resistant to 1% TX-100. Hence, the results of this study are in agreement with published data for LAP2.

It is known that LAP2 β is the predominant isoform in humans (Harris *et al.*, 1994). However, immunoblotting of all the mouse LAP2 isoforms has not been previously performed. My results appear to indicate that LAP2 ϵ is most predominant followed by LAP2 β .

Nuclear lamins A, B1 and C were found mainly in the pellet fraction, although there was some protein visible in the supernatant lane, after extraction of isolated rat liver NE with 1% TX-100 and 500 mM NaCl (Foisner and Gerace, 1993). However, in this work, lamin B was found to be completely solubilized when treated with 1% TX-100 and 500 mM NaCl.

Emerin was also found to be resistant to extraction with 1% TX-100 and 500 mM NaCl using isolated nuclei from lymphoblastoid cells (Ellis *et al.*, 1998). The species and cell type differ from that of this study, which found emerin to be soluble at 1% TX-100

and 500 mM NaCl. However, Ellis *et al.* (1998) found differences in the solubility of emerin with 1% TX-100 and 500 mM NaCl treatments when examining tissue from human individuals suffering from EDMD depending on the patient being examined. Further, differences in emerin expression levels were found among different tissues. The varying amount of protein may be indicative of differences in NE organization and the properties of NE proteins in the different tissues, which could possibly affect the solubility of emerin and its detection.

The PI2 antigen was also only partially solubilized when treated with 1% TX-100 and 500 mM NaCl, suggesting that the protein has associations at the NE such that it remains bound upon treatment with detergent and high salt.

The reason for the discrepancy between the results for lamin B and emerin in this study and in the published data may lie in differences in methodology. In the Foisner and Gerace study (1993), extractions were performed on isolated NEs and Ellis *et al.* (1998) used isolated nuclei, while in this work, the extractions were performed on intact cells. As well, Foisner and Gerace (1993) isolated NE using detergent, and then treated with salt. Similarly, Chaly *et al.* (1985) prepared nuclear matrices from 3T3 fibroblasts by extracting sequentially with 0.5% TX-100 and 2 M NaCl. Lamins as well as the PI2 antigen were found to be insoluble under these conditions. However, in this work the procedure was carried out on intact cells treated with detergent and salt simultaneously. The methods for the TX-100 and salt extractions in this study were drawn up based on the protocol from Rolls *et al.* (1999), who performed the detergent and salt extractions simultaneously. This suggests that lamin solubility depends on whether detergent and salt extractions are done simultaneously or sequentially. It is noteworthy that the PI2

antigen was insoluble both in this study and in Chaly *et al.* (1985) with sequential detergent and salt treatment. This implies that the PI2 antigen is an insoluble component of the NE.

To determine whether the antigen PI2 is on the cytoplasmic face, nucleoplasmic face, or on both faces, digitonin-permeabilization was carried out on cells, which were then immunolabelled with MAb PI2. No labelling was detected at the nuclear rim in interphase cells indicating that MAb PI2 did not have access to its antigen. Lack of labelling at the nuclear periphery was also noted in interphase cells for lamin B and LAP2. The results obtained for lamin B are in agreement with previous work (Georgatos *et al.*, 1997; Theodoropoulos *et al.*, 1999). This is the first report of digitonin-permeabilization experiments using anti-LAP2. The results obtained here confirm the predictions that LAP2 is an integral membrane protein based on previous immunofluorescence and cDNA data (Foisner and Gerace, 1993; Berger *et al.*, 1996). The lack of nuclear peripheral staining at interphase in samples labelled with MAb PI2 indicates that the PI2 antigen is an INM protein.

The observation of nuclear peripheral labelling with anti-emerin in digitonin-permeabilized interphase 3T3 cells was inconsistent with previously published reports (Dabauvalle *et al.*, 1999; Ostlund *et al.*, 1999). However, these results were consistently obtained in both mouse 3T3 and HeLa cells with digitonin-permeabilized samples suggesting that the emerin visualized at the NE at interphase may be due to detection of the lateral diffusion of emerin in the ER.

As well, cytoplasmic emerin was also noted in the digitonin-permeabilized samples. Even at prophase, when the nuclear membrane began to disassemble, and the antigen had access to the nuclear interior, anti-emerin labelling was still detected in the cytoplasm immediately surrounding the nucleus in addition to its normal localization at the NE.

A plausible reason for previous studies not detecting cytoplasmic emerin in interphase using the same antibody preparation, could be that the fixation methods used to prepare the cells were developed to preserve nuclear antigens. Consequently, the cytoplasmic epitopes of emerin may have not been conserved, resulting in their lack of detection. Even in this study, the cytoplasmic emerin was more prominent in digitonin-permeabilized samples than in samples treated for nuclear antigen fixation.

Previous reports using PtK2, *Xenopus* A6, and human lymphoblastoid cells have shown some labelling of emerin throughout the cytoplasm (Ellis *et al.*, 1998; Dabauvalle *et al.*, 1999). These authors suggested that a subcellular fraction of emerin was associated with cytoplasmic membranes, possibly the ER, as it has been suggested that integral membrane proteins are targeted to the NE by lateral diffusion in the ER (Furukawa *et al.*, 1995; Ellenberg *et al.*, 1997). A recent study by Squarzoni *et al.* (2001) found emerin in human platelet cells, which lack nuclei, giving support for the existence of non-nuclear emerin.

Initial characterization of the PI2 antigen by Chaly *et al.* (1984) of the PI2 antigen detected labelling at the nuclear periphery, as well as in the cytoplasm in the vicinity immediately surrounding the nucleus. Similar labelling was noted in this study with the immunofluorescence experiments carried out with the concentrated acites fluid antibody

preparation, MAb PI2 (data not shown). It is known that integral protein synthesis occurs in the rough ER and that the proteins are then targeted to the INM by lateral diffusion through the connected ER and nuclear membranes (Holmer and Worman, 2001).

Peripheral nuclear proteins, however, are made on free ribosomes in the cytoplasm and are targeted to the nucleus by a nuclear localization signal and imported into the nucleus via the NPCs (Gant and Wilson, 1997). Hence the cytoplasmic labelling near the NE for the PI2 antigen may represent PI2 protein present in the ER prior to its incorporation in the INM. This observation would be consistent with the hypothesis that the protein is synthesized and transported in a manner similar to that of integral membrane proteins of the NE. The labelling at the nuclear periphery would arise from the localization of the PI2 antigen at the NE in the INM.

A nucleoplasmic component of the PI2 antigen was also reported initially by Chaly *et al.* (1984) and was observed in this study with MAb PI2. Since many cell types have been shown to contain NE-derived invaginations that extend into the nucleoplasm (Fricker *et al.*, 1997), the internal nuclear labelling noted with MAb PI2 could be partly attributable to NE invaginations. However, if these extensions are found not to be associated with corresponding nuclear membranes, these internal streaks may suggest that the antigen PI2 constitutes part of the nuclear matrix (Gant and Wilson, 1997). Previous immunofluorescence studies of nuclear matrix preparations labelled with the PI2 antibody found that the PI2 antigen was not redistributed but was partly extracted with the internal component being quite labile (Chaly *et al.*, 1985). Further work to determine whether the PI2 antigen is a nucleoplasmic component can be done using confocal microscopy and cells double-labelled with a fluorochrome-conjugated

concanavalin A, which labels the ER and nuclear membrane, and MAb PI2, to test for co-localization. If the internal component of the PI2 antigen is localized to areas only labelled with concanavalin A, then this suggests that the nucleoplasmic component of the protein is due to NE invaginations in the nucleus. If not, then this indicates that the antigen may have other functions within the nucleoplasm and more work would need to be done to find out what co-localizes with PI2 antigen in these internal locations.

4C. Dynamics of the PI2 antigen during the disassembly and assembly of the nuclear envelope at mitosis

NE disassembly at mitosis involves the dismantling of interphase interactions between chromatin and other interphase components of the nucleus. As the DNA condenses, INM proteins become distributed over the ER, while the nuclear lamina depolymerizes. The loss of the tethering interactions between NPCs, INM proteins, lamina, and chromatin result in the breakdown of the NE (Beaudouin *et al.*, 2002; Burke and Ellenberg, 2002).

During NEBD, LBR is the only protein of those tested so far that appears to dissociate from the NE before its permeabilization (Beaudouin *et al.*, 2002). The other nuclear proteins examined, such as LAP2 β , the Nup POM121, and lamins, disperse only after permeabilization, suggesting that NE permeabilization itself may trigger the disassembly of the NE protein-protein interactions (Beaudouin *et al.*, 2002).

At the end of mitosis, the reassociation of proteins with the chromosome surface occurs in a stepwise manner (Chaudhary and Courvalin, 1993; Bodoor *et al.*, 1999; Haraguchi *et al.*, 2000). During reassembly of the NE, the nucleoporins Nup153 and Nup133, are the first to associate with chromatid surfaces in mid-anaphase (Bodoor *et al.*,

1999; Burke and Ellenberg, 2002) (see section 1B.III (c) (ii)). INM proteins such as LAP2, LBR, LAP1, and emerin concentrate at the membrane-chromatin interface in late anaphase (Foisner and Gerace, 1993; Maison *et al.*, 1997; Ellenberg *et al.*, 1997; Bodoor *et al.*, 1999; Dabauvalle *et al.*, 1999; Haraguchi *et al.*, 2000), followed by B-type lamins and then A-type lamins (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Broers *et al.*, 1999). In current models of NE assembly, the binding of INM proteins to the chromatin surface immobilizes the proteins and consequently forces the ER membranes in which they are distributed to wrap around the chromatin, forming the new NE. From telophase to early interphase, the NE expands and seals, surrounding the decondensed chromatin (Burke and Ellenberg, 2002). On the other hand, lamina assembly appears to involve first the recruitment of lamin B dimers to the INM by interactions with LAP2 β and LBR. Lamin A/C tetramers then associate with lamin B and together recruit emerin and, perhaps, LAP1C, while the binding of lamins to LBR, LAPs and emerin may act to anchor them at the INM (Ostlund *et al.*, 1999; Wilson, 2000; Hutchison *et al.*, 2001).

Double-labelling immunofluorescence microscopy was carried out to examine the behaviour of the PI2 antigen during the cell cycle, specifically at the time of disassembly and reassembly of the NE, in relation to the behaviour of the known NE proteins lamin B, LAP2, and emerin. At mitosis, emerin was the first to be lost from early prometaphase nuclei, followed by LAP2. The last to leave were the PI2 antigen and lamin B, which were temporally indistinguishable in their dissociation from the NE. Overall, these results indicate that the PI2 antigen dissociates from the NE after nuclear permabilization. The order of disassembly from the NE for LAP2, emerin, and lamin B is consistent with

the sequence of NE protein loss previously reported (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Bodoor *et al.*, 1999; Haraguchi *et al.*, 2000).

During NE reassembly, emerin was the first to reappear at mid-anaphase followed by LAP2 at early telophase. Lamin B and the PI2 antigen reappeared during telophase, apparently at the same time. The order of reassembly was also consistent with previously published data (Foisner and Gerace, 1993; Dabauvalle *et al.*, 1999). These data suggest that the PI2 antigen behaves in a manner distinct from the integral membrane proteins LAP2 and emerin during nuclear assembly and disassembly, but in a manner temporally similar to the peripheral protein lamin B. It appears that lamin B may be responsible for chromatin organization (Stuurman *et al.*, 1998). Likewise, the PI2 antigen may also play a role in organizing the proteins of the nuclear membrane that are already present in the reforming NE as well as peripheral components of the NE providing structural support for the reforming membrane.

4D. Putative Functions of the PI2 Antigen

How a membrane-bound protein is associated with the lipid bilayer usually reflects the function of the protein. Peripheral membrane proteins function on only one side of the bilayer and are often associated exclusively with the lipid monolayer and/or a protein domain on that side. Functions of the peripheral proteins of the NE that face the nucleoplasm include linking INM proteins to non-membrane structures such as DNA and maintaining NE integrity. The functions of the integral proteins of the NE, however, are less clear and may include attachment and assembly of the lamina and NE during mitosis, as well as regulation of gene expression (Wilson, 2000).

Electron microscopy has shown peripheral staining of the PI2 antigen to be continuous at the nuclear rim, except at the nuclear pores (Chaly *et al.*, 1989). This suggests that the protein is not associated with NPCs, but it does not rule out the possibility that the PI2 antigen may play a role in organizing the NPCs during NEBD and reformation.

Lamina proteins are peripheral membrane proteins (Chaly and Stochaj, 1999). Thus, although the data from this study suggest that the PI2 antigen may have associations with the nuclear lamina, it is not a lamina protein. However, temporally it was found to behave in a manner similar to lamin B, arriving late during NE reassembly. This observation seems to imply that the protein PI2 may not play a role in NE assembly, but it could be involved in the maturation of the NE and in maintaining its integrity.

Chapter 5: SUMMARY

In summary, this study has shown that the PI2 antigen is an integral membrane protein of the NE. The protein appears to be relatively insoluble compared to the other INM proteins examined. During mitosis, the PI2 antigen dissociates from and reassociates with the nuclear periphery late in NEBD and reassembly, respectively. This is in a manner temporally distinct from emerin and LAP2 but similar to lamin B. The lateness of the PI2 antigen in dissociating from the NE indicates that it does so after NE permeabilization. Similarly, since it does not reappear until late in reassembly, it is unlikely that the PI2 antigen plays a role in the initial organization of the proteins arriving at the reforming NE. However, the protein could be involved in the maturation of the NE and in maintaining its integrity. In addition, in interphase it may also play a role in regulating gene expression, in light of the recent findings suggesting that integral NE proteins may influence chromatin silencing.

Obtaining the cDNA sequence of the PI2 antigen, however, will be the best tool in unraveling the functions of the protein. Once the sequence of the protein is obtained, it will allow for the construction of mutants in order to discover the role of the antigen PI2 in nuclear structure and function. Consequently, immunoprecipitation of the PI2 antigen was attempted. However, immunoprecipitation of the PI2 antigen using a biotinylated secondary antibody and streptavidin-coated magnetic beads was unsuccessful (see Appendix I for method). Attempts to isolate nuclei from 3T3 cells, in order to enrich the initial starting sample for the PI2 antigen, still did not yield detectable amounts of the

protein by immunoblotting or silver staining of the gel. One plausible explanation for the difficulty encountered in isolating the protein may be that its associations with the nuclear membrane and other nuclear proteins interfere with the precipitation of the protein onto the beads. Thus, possible future attempts at purifying the protein may include the use of different solubilization treatments of the isolated nuclei, in order to weaken the interactions between the antigen and other proteins. Furthermore, to facilitate the binding of the antibody to the antigen, a lower stringency RIPA buffer could be utilized, and variables in the method, such as protein and antibody concentrations and incubation times, may need to be optimized. Alternatively, immunoaffinity purification of the protein could be attempted using columns containing the MAb PI2 covalently attached to a solid matrix, such as Sepharose beads (Harlow and Lane, 1988). Another approach for obtaining the cDNA sequence of the PI2 antigen that may be tried is the screening of expression libraries using the PI2 antibody.

Further characterization of the PI2 antigen should include studies of post-translational modifications. For instance, many integral proteins are glycoproteins (Lewin, 1998). Glycosylation begins in lumen of the ER and the oligosaccharides are modified in Golgi. Hence, further work could also entail the use of deglycosylation kits to determine if all or only some of the bands detected for the PI2 antigen are glycosylated. It is also known that NE proteins are phosphorylated during mitosis (Foisner and Gerace, 1993). Thus, it could be tested to see if the PI2 antigen is phosphorylated using phosphatases and running the samples on a two-dimensional gel, since changes in mobility due to dephosphorylation are not detectable by one-dimensional SDS-PAGE (N. Chaly, personal communication).

APPENDIX I: Immunoprecipitation of the PI2 Antigen

The PI2 antigen was immunoprecipitated using a biotinylated secondary antibody and streptavidin-coated magnetic beads. The protocol was adapted from that obtained from C. Sodja (Division of Biological Sciences, National Research Council, Ottawa).

Mouse 3T3 lysates were prepared as in section **2D**. To form antigen-antibody complexes, a volume of cell lysate (~300-400 μ L) containing 500 μ g of protein was added to 0.5 mL Wash Buffer (0.1% polyvinylpyrrolidone (PVP) in RIPA buffer) in a 1.5 mL microfuge tube and incubated with 5 μ L of antibody PI2 (see section **2B**) at 4°C for 2 h on a rotator.

To prepare the beads for use, 0.5 mL Wash Buffer was added to 20 μ L of Streptavidin-coated Dynabeads M-280 (DynaL Biotech); the suspension was vortexed, mixed on a rotator for 2 min, and the beads recovered with a Magnesphere Technology Magnetic Separation Stand (Promega). This procedure was carried out three times. The beads were then incubated with 10 μ g of biotinylated goat anti-mouse IgM (μ -chain specific) (Jackson ImmunoResearch Laboratories, Inc.) in 0.5 mL of Wash Buffer for 1 h at room temperature on a rotator, washed 5 X 2 min as above in 0.5 mL Wash Buffer, and incubated with 0.5 mL Blocking Buffer (0.5 % PVP in RIPA buffer) for 30 min at room temperature on a rotator. The beads were then washed again for 5 X 2 min in 0.5 mL Wash Buffer as described above.

To perform the immunoprecipitation, the mixture of cell lysate and primary antibody prepared above was added to the prepared beads and incubated on a rotator

overnight at 4°C. The beads were then washed for 5 X 2 min in 0.5 mL Wash Buffer, resuspended in 40 µL of 4X sample buffer, and boiled for 5 min. The tubes were then placed on the magnetic stand and the supernatants were recovered. Controls included samples processed similarly but using no primary antibody, no secondary antibody, or no lysate.

Half of each sample was then loaded in the same order in duplicate on a single SDS-PAGE gel, and separated as in section **2D**. The gel was then cut in half, with one half processed for silver staining and the other half transferred overnight onto a nitrocellulose membrane and processed for immunoblotting as described in section **2D**.

For silver staining, gels were processed according to a protocol obtained from J. Kelly (Division of Biological Sciences, National Research Council, Ottawa). All solutions were prepared fresh just before use. The gel was placed in fixer (50% ethanol, 5% acetic acid in Nanopure water (Millipure)) for 30 min, washed for 10 min in 50% methanol, washed for 10 min in water and placed in 0.02% sodium thiosulphate in water for 2 min. The gel was then washed 2 X 5 min in water, incubated in stain (0.1% silver nitrate in water) at 4°C for 30 min, rinsed in water for 2 min and developed using 0.04% formalin in 2% sodium carbonate. The developing solution was discarded, and the gel was rinsed briefly in water and placed in stop solution (5% acetic acid in water) for 5 min. The gel was then scanned and stored in 1% acetic acid in water at 4°C.

In an alternate approach, 500 µg of isolated nuclei, rather than protein from whole cell lysates, was used in an attempt to enrich the starting protein sample for the PI2 antigen. Nuclei were isolated as previously described by Belgrader *et al.* (1991).

Overnight cultures of 3T3 cells grown in 30 X 500 mm tissue culture dishes, as in section 24, were trypsinized, and harvested by centrifugation at 1000 rpm in a Centra 4 (rotor 215) (International Equipment Company) at room temperature for 5 min. The pellets were washed twice in ice-cold PBS and pelleted at 1500 rpm for 5 min. The cell pellet was warmed for 45s at room temperature, then resuspended in TM-2 buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 1 mM Pefabloc, 1 mM benzamidine, 50 µg/mL leupeptin, and 10 µg/mL aprotinin) at 10°C to a density of 10⁷ cells/mL and incubated at room temperature for 1 min. The samples were then incubated on ice-water for 5 min, TX-100 was added to 0.5% (v/v), and the samples were incubated on ice-water for an additional 5 min. The cells were sheared by eight passes through a 22 gauge needle and nuclei were separated from the cytosol by centrifuging at 1500 rpm for 6 min. Nuclei were washed twice in TM-2 buffer, at a protein concentration determined using the Bio-Rad Protein Assay, the nuclei were then dissolved and processed for IP as above.

REFERENCES

- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. Molecular Biology of the Cell: Fourth Edition. New York: Garland Science, 2002.
- Aaronson, R. P. and Blobel, G. 1975. Isolation of nuclear pore complexes in association with a lamina. *Proc. Natl Acad. Sci. USA* 72:1007-1011.
- Bagley, S., Goldberg, M. W., Cronshaw, J. M., Rutherford, S. A., and Allen, T. D. 2000. The nuclear pore complex. *J. Cell Sci.* 113:3885-6.
- Beaudouin, J., Gerlich, D., Daigle, N., Eils, R., and Ellenberg, J. 2002. Nuclear envelope breakdown proceeds by microtubule-induced tearing of the lamina. *Cell* 108:83-96.
- Belmont, A. S., Zhai, Y., and Thilenius, A. 1993. Lamin B distribution and association with peripheral chromatin revealed by optical sectioning and electron microscopy tomography. *J. Cell Biol.* 123:1671-1685.
- Benavente, R. and Krohne, G. 1986. Involvement of nuclear lamins in postmitotic reorganization of chromatin as demonstrated by microinjection of lamin antibodies. *J. Cell Biol.* 103:1847-54.
- Berezney, R., Mortillaro, M., Ma, H., Wei, X., and Samarabandu, J. 1995. The Nuclear Matrix: A structural milieu for genomic function. *Int. Rev. Cytol.* 162A:1-65.
- Berger, R., Theodor, L., Shoham, J., Gokkel, E., Brok-Simoni, F., Avraham, K. B., Copeland, N. G., Jenkins, N. A., Rechavi, G., and Simon, A. J. 1996. The characterization and localization of the mouse thymopoietin/lamina-associated polypeptide 2 gene and its alternatively spliced products. *Genome Res.* 6:361-370.
- Bione, S., Maestrini, E., Rivella, S., Mancini, M., Regis, S., Romeo, G., and Toniolo, D. 1994. Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. *Nature Genetics* 8:323-327.
- Bodoor, K., Shaikh, S., Salina, D., Raharjo, W. H., Bastos, R., Lohka, M., and Burke, B. 1999. Sequential recruitment of NPC proteins to the nuclear periphery at the end of mitosis. *J. Cell Sci.* 112:2253-2264.
- Bridger, J. M., Kill, I. R., O'Farrell, M., and Hutchison, C. J. 1993. Internal lamin structures within G1 nuclei of human dermal fibroblasts. *J. Cell Sci.* 104:297-306.
- Broers, J. L., Machiels, B. M., Kuijpers, H. J., Smedts, F., van den Kieboom, R., Raymond, Y., and Ramaekers, F. C. 1997. A- and B-type lamins are differentially expressed in normal human tissues. *Histochem. Cell Biol.* 107:505-517.

- Broers, J. L., Machiels, B. M., van Eys, G. J., Kuijpers, H. J., Manders, E. M., van Driel, R., and Ramaekers, F. C. 1999. Dynamics of the nuclear lamina as monitored by GFP-tagged A-type lamins. *J. Cell Sci.* 112:3463-75.
- Buendia, B. and Courvalin, J. C. 1997. Domain-specific disassembly and reassembly of nuclear membranes during mitosis. *Exp Cell Res.* 230:133-44.
- Burke, B. and Gerace, L. 1986. A cell free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell.* 44:639-652.
- Burke, B. and Ellenberg, J. 2002. Remodelling the walls of the nucleus. *Nature Reviews Mol. Cell Biol.* 3:487-497.
- Chaly, N. and Stochaj, U. 1999. Non-lamin components of the lamina: a paucity of proteins. *Biochem. Cell Biol.* 77:311-319.
- Chaly, N., Bladon, T., Brown, D. L., Setterfield, G., Little, J. E., and Kaplan, J. G. 1984. Changes in distribution of nuclear matrix antigens during the mitotic cell cycle. *J. Cell Biol.* 99: 661-671.
- Chaly, N., Little, J. E., and Brown, D. L. 1985. Localization of nuclear antigens during preparation of nuclear matrices *in situ*. *Can. J. Biochem. Cell Biol.* 63:644-653.
- Chaly, N., Sabour, M. P., Silver, J. C., Aitchison, W. A., Little, J. E., and Brown, D. L. 1986. Monoclonal antibodies against nuclear matrix detect nuclear antigens in mammalian, insect and plant cells: an immunofluorescence study. *Cell Biol. Int. Rep.* 10:421-428.
- Chaly, N., Cadrin, M., Kaplan, J. G., and Brown, D. L. 1988. Modulation of lymphocyte nuclear matrix organization *in vivo* by 5,6-dichloro-1-B-D-ribofuranosyl benzimidazole: an autoradiographic and immunofluorescence study. *Biol. Cell* 6: 9-17.
- Chaly, N., St. Aubin, G. and Brown, D. L. 1989. Ultrastructural localization of nuclear antigens during interphase in mouse 3T3 fibroblasts. *Biochem. Cell Biol.* 67:563-574.
- Chaudhary, N., and Courvalin, J. C. 1993. Stepwise reassembly of the nuclear envelope at the end of mitosis. *J. Cell Biol.* 122:295-306.
- Chu, A., Rassadi, R., and Stochaj, U. 1998. Velcro in the nuclear envelope: LBR and LAPs. *FEBS Lett.* 441:165-169.
- Clements, L. Manilal, S., Love, D. R., and Morris G. E. 2000. Direct interaction between Emerin and Lamin A. *Biochem. Biophys. Res. Comm.* 267:709-714.

- Coates, P. J., Hobbs, R. C., Crocker, J., Rowlands, D.C., Murray, P., Quinlan, R., and Hall, P. A. 1996. Identification of the antigen recognized by the monoclonal antibody BU31 as lamins A and C. *J. Pathol.* 178:21-29.
- Cohen, M., Lee, K. K., Wilson, K. L., and Gruenbaum, Y. 2001. Transcriptional repression, apoptosis, human disease and the functional evolution of the nuclear lamina. *Trends Biochem. Sci.* 26:41-7.
- Collas, P. 1998. Nuclear envelope disassembly in mitotic extract requires functional nuclear pores and a nuclear lamina. *J. Cell Sci.* 111: 1293-1303.
- Collas, P. and Courvalin, J. C. 2000. Sorting nuclear membrane proteins at mitosis. *Trends Cell Biol.* 10:5-8.
- Courvalin, J. C., Segil, N., Blobel, G., and Worman, H. J. 1992. The lamin B receptor of the inner nuclear membrane undergoes mitosis-specific phosphorylation and is a substrate for p34cdc2-type protein kinase. *J. Biol. Chem.* 267:19035-8.
- Dabauvalle, M.C., Loos, K., Merkert, H., and Scheer, U. 1991. Spontaneous assembly of pore complex-containing membranes ("annulate lamellae") in *Xenopus* egg extract in the absence of chromatin. *J. Cell Biol.* 112:1073-1082.
- Dabauvalle, M. C., Muller, E., Ewald, A., Kress, W., Krohne, G., and Muller, C. R. 1999. Distribution of emerin during the cell cycle. *Eur. J. Cell Biol.* 78:749-756.
- Dechat, T., Gotzmann, J., Stockinger, A., Harris, C. A., Talle, M. A., Siekierka J. J., and Foisner, R. 1998. Detergent-salt resistance of LAP2alpha in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. *EMBO J.* 17:4887-4902.
- Dechat, T., Vlcek, S., and Foisner, R. 2000a. Review: lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. *J. Struct. Biol.* 129:335-45.
- Dechat, T., Korbei, B., Vaughan, A., Vlcek, S., Hutchison, C. J., and Foisner, R. 2000b. Lamina-associated polypeptide 2a binds intranuclear A-type lamins. *J. Cell Sci.* 113:3473-3484.
- Dreger, M., Bengtsson, L., Schoneberg, T., Otto, H., and Hucho, F. 2001. Nuclear envelope proteomics: novel integral membrane proteins of the inner nuclear membrane. *Proc. Natl. Acad. Sci. USA* 98:11943-8.
- Ellenberg, J., Siggia, E. D., Moreira, J. E., Smith, C. L., Presley, J. F., Worman, H. J., and Lippincott-Schwartz, J. 1997. Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* 138:1193-1206.

- Ellis, J. A., Craxton, M., Yates, J. R. W., and Kendrick-Jones, J. 1998. Aberrant intracellular targeting and cell cycle-dependent phosphorylation of emerin contribute to the Emery-Dreifuss muscular dystrophy phenotype. *J. Cell Sci.* 111:781-792.
- Enninga, J., Levy, D. E., Blobel, G., and Fontoura, B. M. 2002. Role of nucleoporin induction in releasing an mRNA nuclear export block. *Science* 295:1523-5.
- Favreau, C., Worman, H. J., Wozniak, R. W., Frappier, T., and Courvalin, J. C. 1996. Cell cycle-dependent phosphorylation of nucleoporins and nuclear pore membrane protein Gp210. *Biochem.* 35:8035-44.
- Felice, K. J., Schwartz, R. C., Brown, C. A., Leicher, C. R., and Grunnet, M. L. 2000. Autosomal dominant Emery-Dreifuss dystrophy due to mutations in rod domain of the lamin A/C gene. *Neurology* 55:275-80.
- Foisner, R. 1997. Dynamic organisation of intermediate filaments and associated proteins during the cell cycle. *BioEssays* 19:297-305.
- Foisner, R. 2001. Inner nuclear membrane proteins and the nuclear lamina. *J. Cell Sci.* 114:3791-2.
- Foisner, R. and Gerace, L. 1993. Integral membrane proteins of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. *Cell* 73:1267-1279.
- Fricker, M., Hollinshead, M., White, N., and Vaux, D. 1997. Interphase nuclei of many mammalian cell types contain deep, dynamic, tubular membrane-bound invaginations of the nuclear envelope. *J. Cell Biol.* 136:531-544.
- Furukawa, K. 1999. LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2-chromatin interaction. *J. Cell Sci.* 112:2485-92.
- Furukawa, K., and Hotta, Y. 1993. CDNA cloning of a germ cell specific lamin B3 from mouse spermatocytes and analysis of its function by ectopic expression in somatic cells. *EMBO J.* 12:97-106.
- Furukawa, K. and Kondo, T. 1998. Identification of the lamina-associated-polypeptide-2-binding domain of B-type lamin. *Eur. J. Biochem.* 251:729-733.
- Furukawa, K., Inagaki, H., and Hotta, Y. 1994. Identification and cloning of an mRNA coding for a germ cell-specific A-type lamin in mice. *Exp. Cell Res.* 212:426-430.
- Furukawa, K., Pante, N., Aeby, U., and Gerace, L. 1995. Cloning of cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope. *EMBO J.* 14:1626-1636.

- Gant, T. M. and Wilson, K. L. 1997. Nuclear assembly. *Ann. Rev. Cell Dev. Biol.* 13:669-695.
- Gant, T. M., Harris C. A., and Wilson, K. L. 1999. Roles of LAP2 proteins in nuclear assembly and DNA replication: Truncated LAP2beta proteins alter lamina assembly, envelope formation, nuclear size, and DNA replication efficiency in *Xenopus laevis* extracts. *J. Cell Biol.* 144:1083-1096.
- Georgatos, S. D. 2001. The inner nuclear membrane: simple or very complex? *EMBO J.* 20:2989-94.
- Georgatos, S. D., Meier, J., and Simos, G. 1994. Lamins and lamin-associated proteins. *Curr. Opin. Cell Biol.* 6:347-353.
- Georgatos, S. D., Pyrpassopoulou, A., and Theodoropoulos, P. A. 1997. Nuclear envelope breakdown in mammalian cells involves stepwise lamina disassembly and microtubule-drive deformation of the nuclear membrane. *J. Cell Sci.* 110:2129-40.
- Gerace, L. and Blobel, G. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell* 19:277-287.
- Gerace, L. and Foisner, R. 1994. Integral membrane proteins and dynamic organization of the nuclear envelope. *Trends Cell Biol.* 4:127-131.
- Gerace, L., Blum, A., and Blobel, G. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction: Interphase and mitotic distribution. *J. Cell Biol.* 79:546-566.
- Gerace, L., Ottaviano, Y., and Kondor-Koch, C. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826-37.
- Goldberg, M., Harel, A., Brandeis, M., Rechsteiner, T., Richmond, T. J., Weiss, A. M., and Gruenbaum, Y. 1999. The tail domain of lamin Dm0 binds histones H2A and H2B. *Proc. Natl. Acad. Sci. USA* 96:2852-7.
- Gruenbaum, Y., Wilson, K. L., Harel, A., Goldberg, M., and Cohen, M. 2000. Nuclear lamins – Structural proteins with fundamental functions. *J. Struc. Biol.* 129:313-323.
- Guilly, M. N., Kolb, J. P., Gosti, F., Godeau, F., and Courvalin, J. C. 1990. Lamins A and C are not expressed at early stages of human lymphocyte differentiation. *Exp. Cell Res.* 189:145-147.
- Haraguchi, T., Koujin, T., Hayakawa, T., Kaneda, T., Tsutsumi, C., Imamoto, N., Akazawa, C., Sukegawa, J., Yoneda, Y., and Hiraoka, Y. 2000. Live fluorescence imaging reveals early recruitment of Emerin, LBR, RanBP2, and Nup153 to reforming functional nuclear envelopes. *J. Cell Sci.* 113:779-794.

Harlow, E. and Lane, D. Antibodies: A Laboratory Manual. USA: Cold Spring Harbor Laboratory, 1988.

Harris, C. A., Andryuk, P. J., Cline, S., Chan, H. K., Natarajan, A., Siekierka, J. J., and Goldstein, G. 1994. Three distinct human thymopoietins are derived from alternatively spliced mRNAs. *Proc. Natl. Acad. Sci. USA* 91:6283-6287.

Heald, R. and McKeon, F. 1990. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* 61:579-89.

Heitlinger, E., Peter, M., Haner, M., Lustig, A., Aebi, U., and Nigg, E. A. 1991. Expression of chicken lamin B2 in *Escherichia coli*: Characterization of its structure, assembly, and molecular interactions. *J. Cell Biol.* 113:485-495.

Hennekes, H., Peter, M., Weber, K., and Nigg, E. A. 1993. Phosphorylation on protein kinase C sites inhibits nuclear import of lamin B2. *J. Cell Biol.* 120:1293-1304.

Hetzer, M., Bilbao-Cortes, D., Walther, T. C., Gruss, O. J., and Mattaj, I. W. 2000. GTP hydrolysis by Ran is required for nuclear envelope assembly. *Mol. Cell* 5:1013-24.

Hetzer, M., Meyer, H. H., Walther, T. C., Bilbao-Cortes, D., Warren, G., and Mattaj, I. W. 2001. Distinct AAA-ATPase p97 complexes function in discrete steps of nuclear assembly. *Nat. Cell Biol.* 3:1086-91.

Hoger, T. H., Zatloukal, K., Waizenegger, I., and Krohne, G. 1990. Characterization of a second highly conserved B-type lamin present in cells previously thought to contain only a single B-type lamin. *Chromosoma* 100:67-69.

Holmer, L. and Worman, H. J. 2001. Inner nuclear membrane proteins: functions and targeting. *Cell Mol Life Sci.* 58:1737-40.

Hutchison, C. J., Bridger, J. M., Cox, L. S., and Kill, I. R. 1994. Weaving a pattern from disparate threads: lamin function in nuclear assembly and DNA replication. *J. Cell Sci.* 107:3259-3269.

Hutchison, C. J., Alvarez-Reyes, M., and Vaughan, O. A. 2001. Lamins in disease: why do ubiquitously expressed nuclear envelope proteins give rise to tissue-specific disease phenotypes? *J Cell Sci.* 114:9-19.

Jagatheesan, G., Thanumalayan, S., Muralikrishna, B., Rangaraj, N., Karande, A. A., and Parnaik, V. K. 1999. Co-localization of intranuclear lamin foci with RNA splicing factors. *J. Cell Sci.* 112:4651-4661.

Jenkins, H., Holman, T., Lyon, C., Lane, B., Stick, R., and Hutchison, C. 1993. Nuclei that lack a lamina accumulate karyophilic proteins and assemble a nuclear matrix. *J. Cell Sci.* 106:275-285.

- Jocelyn, P. C. 1987. Chemical reduction of disulfides. *Methods Enzymol.* 143:246-56.
- Jones, D. O., Cowell, I. G., and Singh, P. B. 2000. Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* 22:124-37.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lee, K. K., Gruenbaum, Y., Spann, P., Liu, J., and Wilson, K. L. 2000. *C. elegans* Nuclear envelope proteins Emerin, MAN1, Lamin, and Nucleoporins reveal unique timing of nuclear envelope breakdown during mitosis. *Mol. Biol. Cell* 11:3089-3099.
- Lerner, E. A., Lerner, M. R., Janeway, C. A., and Steitz, J. A. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: Probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA* 78:2737-2741.
- Lewin, B. Genes VI. New York: Oxford University Press Inc, 1998.
- Lin, F., Blake, D. L., Callebaut, I., Skerjanc, I. S., Holmer, L., McBurney, M. W., Paulin-Levasseur, M., and Worman, H. J. 2000. MAN1, an inner nuclear membrane protein that shares the LEM domain with lamina-associated polypeptide 2 and emerin. *J. Biol. Chem.* 275:4840-4847.
- Lopez-Soler, R. I., Moir, R. D., Spann, T. P., Stick, R., and Goldman, R. D. 2001. A role for nuclear lamin in nuclear envelope assembly. *J. Cell Biol.* 154:61-70.
- Lourim, D. and Krohne, G. 1993. Membrane-associated lamins in *Xenopus* egg extracts: identification of two vesicle populations. *J. Cell Biol.* 123:501-512.
- Lourim, D. and Krohne, G. 1994. Lamin-dependent nuclear envelope re-assembly following mitosis. *Trends Cell Biol.* 4:314-318.
- Luderus, M. E., de Graaf, A., Mattia, E., den Blaauwen, J. L., Grande, M. A., de Jong, L., and van Driel, R. 1992. Binding of matrix attachment regions to lamin B1. *Cell* 70:949-959.
- Luderus, M. E., den Blaauwen, J. L., de Smit, O. J. B., Compton, D. A., and van Driel, R. 1994. Binding of matrix attachment regions to lamin polymers involving single-stranded regions and the minor groove. *Mol. Cell. Biol.* 14:6297-6305.
- Lutz, R. J., Trujillo, M. A., Denham, K. S., Wenger, L., and Sinensky, M. 1992. Nucleoplasmic localization of prelamin A: implications for prenylation-dependent lamin A assembly into the nuclear lamina. *Proc. Natl. Acad. Sci. USA* 89:3000-4.
- Macaulay, C., Meier, E., and Forbes, D. J. 1995. Differential mitotic phosphorylation of proteins of the nuclear pore complex. *J. Biol. Chem.* 270:254-62.

- Machiels, B. M., Zorenc, A. H., Endert, J. M., Kuijpers, H. J., van Eys, G. J., Ramaekers, F. C., and Broers, J. L. 1996. An alternative splicing product of the lamin A/C gene lacks exon 10. *J. Biol. Chem.* 271:9249-53.
- Maison, C., Horstmann, H., and Georgatos, S. D. 1993. Regulated docking of nuclear membrane vesicles to vimentin filaments during mitosis. *J. Cell Biol.* 123:1491-1505.
- Maison, C., Pyrpassopoulou, A., Theodoropoulos, P. A., and Georgatos, S. D. 1997. The inner nuclear membrane protein LAP1 forms a native complex with B-type lamins and partitions with spindle-associated mitotic vesicles. *EMBO J.* 16:4839-50.
- Malone, C. J., Fixsen, W. D., Horvitz, H. R., and Han, M. 1999. UNC-84 localizes to the nuclear envelope and is required for nuclear migration and anchoring during *C. elegans* development. *Development* 126:3171-81.
- Manilal, S., Nguyen, T. M., Sewry, C. A., and Morris, G. E. 1996. The Emery-Dreifuss muscular dystrophy protein, emerin, is a nuclear membrane protein. *Hum. Mol. Genet.* 5:801-806.
- Manilal, S., Sewry, C. A., Nguyen, T. M., Mutoni, F., and Morris, G. E. 1997. Diagnosis of X-linked Emery-Dreifuss muscular dystrophy by protein analysis of leucocytes and skin with monoclonal antibodies. *Neuromusc. Disord.* 7:63-66.
- Manilal, S., Nguyen, T. M., and Morris, G. E. 1998. Co-localization of emerin and lamins in interphase nuclei and changes during mitosis. *Biochem. Biophys. Res. Commun.* 249:643-647.
- Marshall, I. C. B. and Wilson, K. L. 1997. Nuclear envelope assembly after mitosis. *Trends Cell Biol.* 7:69-74.
- Martin, L., Crimando, C., and Gerace, L. 1995. cDNA cloning and characterization of lamina-associated polypeptide 1C (LAP1C), an integral protein of the inner nuclear membrane. *J. Biol. Chem.* 270:8822-8828.
- Martins, S. B., Eide, T., Steen, R. L., Jahnsen, T., Skalhegg, B. S., and Collas, P. 2000. HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics. *J. Cell Sci.* 113:3703-13.
- Meier, J., Campbell, K. H., Ford, C. C., Stick, R., and Hutchison, C. J. 1991. The role of lamin LIII in nuclear assembly and DNA replication, in cell-free extracts of *Xenopus* eggs. *J. Cell Sci.* 98:271-279.
- Minc, E., Allory, Y., Worman, H. J., Courvalin, J. C., and Buendia, B. 1999. Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* 108:220-34.

- Moir, R. D., Donaldson, A. D., and Stewart, M. 1991. Expression in *Escherichia coli* of human lamins A and C: Influence of head and tail domains on assembly properties and paracrystal formation. *J. Cell Sci.* 99:363-372.
- Moir, R. D., Montaglowy, M., and Goldman, R. D. 1994. Dynamic properties of nuclear lamins: Lamin B is associated with sites of DNA replication. *J. Cell Biol.* 125:1201-1212.
- Moir, R. D., Spann, T. P., and Goldman, R. D. 1995. The dynamic properties and possible functions of nuclear lamins. *Int. Rev. Cytol.* 162B:141-82.
- Moir, R. D., Yoon, M., Khuon, S., and Goldman, R. D. 2000. Nuclear lamins A and B1: different pathways of assembly during nuclear envelope formation in living cells. *J. Cell Biol.* 151:1155-1168.
- Molloy, M. P. 2000. Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Anal. Biochem.* 280:1-10.
- Mora, M., Cartegni, L., Di Blasi, C., Barresi, R., Bione, S., Raffaele di Bartletta, M., Morandi, L., Merlini, L., Nigro, V., Politano, L., Donati, M. A., Cornelio, F., Cobiauchi, F., and Toniolo, D. 1997. X-linked Emery-Dreifuss muscular dystrophy can be diagnosed from skin biopsy or blood sample. *Ann. Neurol.* 42:249-253.
- Morris, G. E. and Manilal, S. 1999. Heart to heart: from nuclear proteins to Emery-Dreifuss muscular dystrophy. *Hum. Mol. Genet.* 8:1847-51.
- Nagano, A., Koga, R., Ogawa, M., Kurano, Y., Kawada, J., Okada, R., Hayashi, Y. K., Tsukahara, T., and Arahata, K. 1996. Emerin deficiency at the nuclear membrane in patients with Emery-Dreifuss muscular dystrophy. *Nature Genet.* 12:254-259.
- Neugebauer, J. A Guide to the Properties and Uses of Detergents in Biology and Biochemistry. New York: CALBIOCHEM Corporation, 1988.
- Newport, J. W., Wilson, K. L., and Dunphy, W. G. 1990. A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* 111:2247-2259.
- Ostlund, C., Ellenberg, J., Hallberg, E., Lippincott-Schwartz, J., and Worman, H. J. 1999. Intracellular trafficking of emerin, the Emery-Dreifuss muscular dystrophy protein. *J. Cell Sci.* 112:1709-1719.
- Padan, R., Nainudel-Epszteyn, S., Goitein, R., Fainsod, A., and Gruenbaum, Y. 1990. Isolation and characterization of the *Drosophila* nuclear envelope otefin cDNA. *J. Biol. Chem.* 265:7808-13.

- Paddy, M. R., Belmont, A. S., Saumweber, H., Agard, D. A., and Sedat, J. W. 1990. Interphase nuclear envelope lamins form a discontinuous network that interacts with only a fraction of the chromatin in the nuclear periphery. *Cell* 62:89-106.
- Pante, N., Bastos, R., McMorro, I., Burke, B. and Aebi, U. 1994. Interactions of three-dimensional localization of a group of nuclear pore complex proteins. *J. Cell Biol.* 126:603-617.
- Paulin-Levasseur, M., Blake, D. L., Julien, M., and Rouleau, L. 1996. The MAN antigens are non-lamin constituents of the nuclear lamina in vertebrate cells. *Chromosoma* 104:367-379.
- Peter, M., Kitten, G. T., Lehner, C. F., Vorburger, K., Bailer, S. M., Maridor, G., and Nigg, E. A. 1989. Cloning and sequencing of cDNA clones encoding chicken lamins A and B1 and comparison of the primary structures of vertebrate A- and B-type lamins. *J. Mol. Biol.* 208:393-404.
- Pugh, G. E., Coates, P. J., Lane, E. B., Raymond, Y., and Quinlan, R. A. 1997. Distinct nuclear assembly pathways for lamins A and C lead to their increase during quiescence in Swiss 3T3 cells. *J Cell Sci.* 110:2483-93.
- Rober, R. A., Weber, K., and Osborn, M. 1989. Differential timing of nuclear lamin A/C expression in the various organs of the mouse embryo and the young animal: A developmental study. *Development* 105:365-378.
- Rolls, M. M., Steinb, P. A., Taylora, S. S., Haa, E., McKeona, F., and Rapoport, T. A. 1999. A visual screen of a GFP-fusion library Identifies a new type of nuclear envelope membrane protein. *J. Cell Biol.* 146:29-43.
- Salina, D., Bodoor, K., Eckley, D. M., Schroer, T. A., Rattner, J. B., and Burke, B. 2002. Cytoplasmic Dynein as a facilitator of nuclear envelope breakdown. *Cell* 108:97-107.
- Sasseville, A. M. J. and Raymond, Y. 1995. Lamin A precursor is localized to intranuclear foci. *J. Cell Sci.* 106:273-285.
- Senior, A. and Gerace, L. 1988. Integral membrane proteins specific to the inner nuclear membrane and associated with the nuclear lamina. *J. Cell Biol.* 107:2029-2036.
- Shumaker, D. K., Lee, K. K., Tanhehco, Y. C., Craigie, R., and Wilson, K. L. 2001. LAP2 binds to BAF.DNA complexes: requirement for the LEM domain and modulation by variable regions. *EMBO J.* 20:1754-64.
- Silvius, J. R. 1992. Solubilization and functional reconstitution of biomembrane components. *Annu. Rev. Biophys. Biomol. Struct.* 21:323-48.

- Simos, G., Maison, C., and Georgatos, S. D. 1996. Characterization of p18, a component of the lamin B receptor complex and a new integral membrane protein of the avian erythrocyte nuclear envelope. *J. Biol. Chem.* 271:12617-25.
- Sodja, C., Walker, R. P., Brown, D. L. and Chaly, N. 1997. Unique behaviour of NuMA during heat-induced apoptosis of lymphocytes. *Biochem. Cell Biol.* 75:399-414.
- Spector, D. L. and Smith H. C. 1986. Redistribution of U-snRNPs during mitosis. *Exp. Cell Res.* 163:87-94.
- Squarzoni, S., Sabatelli, P., Capanni, C., Petrini, S., Ognibene, A., Toniolo, D., Cobianchi, F., Zauli, G., Bassini, A., Baracca, A., Guarnieri, C., Merlini, L., and Maraldi, N. M. 2000. Emerin presence in platelets. *Acta Neuropathol.* 100:291-8.
- Steen, R. L. and Collas, P. 2001. Mistargeting of B-type lamins at the end of mitosis: Implications on cell survival and regulation of lamins A/C expression. *J. Cell Biol.* 153: 621-626.
- Steen, R. L., Martins, S. B., Tasken, K., and Collas, P. 2000. Recruitment of Protein Phosphatase 1 to the nuclear envelope by A-Kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly. *J. Cell Biol.* 150:1251-1261.
- Stewart, C. and Burke, B. 1987. Teratocarcinoma stem cells and early mouse embryos contain only a single major lamin polypeptide closely resembling lamin B. *Cell* 51:383-392.
- Stuurman, N., Heins, S., and Aebi, U. 1998. Nuclear lamins: their structure, assembly, and interactions. *J. Struct. Biol.* 122:42-66.
- Sullivan, T., Escalante-Alcalde, D., Bhatt, H., Anver, M., Bhat, N., Nagashima, K., Stewart, C. L., and Burke, B. 1999. Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J. Cell Biol.* 147:913-20.
- Taniura, H., Glass, C., and Gerace, L. 1995. A chromatin binding site in the tail domain of nuclear lamins that interacts with core histones. *J. Cell Biol.* 131:33-44.
- Terasaki, M. 2000. Dynamics of the endoplasmic reticulum and golgi apparatus during early sea urchin development. *Mol. Biol. Cell* 11:897-914.
- Theodoropoulos, P. A., Polioudaki, H., Koulentaki, M., Kouroumalis, E., and Georgatos, S. D. 1999. PBC68: a nuclear pore complex protein that associates reversibly with the mitotic spindle. *J. Cell Sci.* 112:3049-59.
- Thompson, L. J., Bollen, M., and Fields, A. P. 1997. Identification of protein phosphatase 1 as a mitotic lamin phosphatase. *J. Biol. Chem.* 272:29693-29697.

- Ulitzur, N., Harel, A., Feinstein, N., and Gruenbaum, Y. 1992. Lamin activity is essential for nuclear envelope assembly in a *Drosophila* embryo cell-free extract. *J. Cell Biol.* 119:17-25.
- Vasu, S. K. and Forbes, D. J. 2001. Nuclear pores and nuclear assembly. *Curr. Opin. Cell Biol.* 13:363-75.
- Vlcek, S. Just, H., Dechat, T., and Foisner, R. 1999. Functional diversity of LAP2 α and LAP2 β in postmitotic chromosome association is caused by an α -specific nuclear targeting region. *EMBO J.* 18:6370-6384.
- Walker, J. M. Methods in Molecular Biology, Volume 1: Proteins. New Jersey: Humana Press, 1984.
- Weaver, V. M., Carson, C. E., Walker, P. R., Chaly, N., Lach, B., Raymond, Y., Brown, D. L., and Sikorska, M. 1996. Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. *J. Cell Sci.* 109:45-56.
- Weber, K., Plessmann, U., and Traub, P. 1989. Maturation of nuclear lamin A involves a specific carboxyl-terminal trimming, which removes the polyisoprenylation site from the precursor: Implications for the structure of the nuclear lamina. *FEBS Lett.* 257:411-414.
- Wilson, K. L. 2000. The nuclear envelope, muscular dystrophy and gene expression. *Trends Cell Biol.* 10:125-9.
- Wilson, K. L. and Newport, J. 1988. A trypsin-sensitive receptor on membrane vesicles is required for nuclear envelope formation *in vitro*. *J. Cell Biol.* 107:57-68.
- Wilson, K.L., Zastrow, M. S., and Lee, K. K. 2001. Lamins and disease: insights into nuclear infrastructure. *Cell* 104:647-50.
- Worman, H. J., Evans, C. D., and Blobel, G. 1990. The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential transmembrane domains. *J. Cell Biol.* 111:1535-1542.
- Wu, W., Lin, F., and Worman, H. J. 2002. Intracellular trafficking of MAN1, an integral protein of the nuclear envelope inner membrane. *J. Cell Sci.* 115:1361-71.
- Wynder, K. L., McNeil, J. A., Lin, F., Worman, H. J., and Lawrence, J. B. 1996. Chromosomal Assignment of Human Nuclear Envelope Protein Genes LMNA, LMNB1, and LBR by Fluorescence *in Situ* Hybridization. *Genomics* 32:474-478.
- Yang, L., Guan, T., and Gerace, L. 1997. Integral membrane proteins of the nuclear envelope are dispersed throughout the endoplasmic reticulum during mitosis. *J. Cell Biol.* 137:1199-1210.

Ye, Q. and Worman, H. J. 1996. Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to *Drosophila* HP1. *J. Biol. Chem.* 271:14653-14656.

Yorifuji, H., Tadano, Y., Tsuchiya, Y., Ogawa, M., Goto, K., Umetani, A., Asaka, Y., and Arahata, K. 1997. Emerin, deficiency of which causes Emery-Dreifuss muscular dystrophy, is localized at the inner nuclear membrane. *Neurogenetics* 1:135-40.

Zhang, C. and Clarke, P. R. 2000. Chromatin-independent nuclear envelope assembly induced by Ran GTPase in *Xenopus* egg extracts. *Science* 288:1429-32.

Zheng, R., Ghirlando, R., Lee, M. S., Mizuuchi, K., Krause, M., and Craigie, R. 2000. Barrier-to-autointegration factor (BAF) bridges DNA in a discrete, higher-order nucleoprotein complex. *Proc. Natl. Acad. Sci. USA* 97:8997-9002.