INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Protein-Protein and Protein-Chromatin Interactions at the Nuclear Envelope

Qian Ye

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1996

UMI Number: 9630529

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

This Manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

3/11/96

Mary R. Rifkin, Ph.D.
Chairman of Examining Committee

3/25/96

Terry A. Krulwich, Ph.D.
Executive Officer

Selina Chen-Kiang, Ph.D.

Jean-Claude Courvalin, M.D., Ph.D.

Manfred Frasch, Ph.D.

Howard Worman, M.D.

Supervisory Committee

The City University of New York

Format of Thesis

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter 3 contains results published as Ye and Worman in *Journal of Biological Chemistry* (1994). 369: 11306-11311. Chapter 4 contains results published as Ye and Worman in *Experimental Cell Research* (1995). 219: 292-298. Chapter 3, 4, 5 has an introduction and discussion specific for each section. A general introduction and a general discussion are placed at the beginning and end of the thesis. To reduce redundancy, "Materials and Methods" and "References " have been consolidated. Result from Fig. 5-9 was obtained from collaborative efforts with Dr. Jean-Claude Courvalin's laboratory. Nothern blot analysis in Fig. 3-4 and Fig. 3-10 was performed using filters provided by Dr. Selina Chen-Kiang.

Acknowledgments

I would like to thank my mentor, Dr. Howard J. Worman, for his guidance and training during my graduate study. He has helped me to look into the edge of the nuclear envelope and has encouraged my development as a scientist. What he has taught me will be of great benefit in the furture. I am also grateful that he has brought me into a new academic environment, where I have contacted more people and learned more.

I would like to thank Dr. Selina Chen-Kiang, Dr. Manfred Frasch and Dr. Mary R. Rifkin for earnest instructions and help during my study at the Brookdale Center. I thank all members of the Brookdale Center for creating a warm and encouraging environment in which to study and work. I also thank my colleagues in Dr. Worman's laboratory for valuable assistance and friendship. I should extend my special thanks to Dr. Selina Chen-Kiang, her lab has been my night and weekend stay on the 25th floor for the the past one year. I also thank the members of Dr. Chen-Kiang's laboratory.

I must give the deepest and warmest thanks to my wife Xiaokui Zhang. Her support, encouragement and dedication to my study can be traced back to the Shanghai Institute of Cell Biology in China in 1989, where and when we studied together. I could not have reached where I am now without her. I appreciate her help in preparing figures and making slides of this thesis.

To my grandparents, my parents, my wife, and my daughter

Table of Contents

Chapter 1.	Introduction	1
Chapter 2.	Materials and Methods	18
Chapter 3.	Primary Structure Analysis and Lamin B and DNA Binding of Human LBR, an Integral Protein of the Nuclear Envelope Inner Membrane	31
	Abstract	31
	Introduction	32
	Results	33
	Discussion	52
Chapter 4.	Protein-Protein Interactions between Human Nuclear Lamins Expressed in Yeast	56
	Abstract	56
	Introduction	57
	Results	60
	Discussion	66
Chapter 5.	Interactions between Heterochromatin Proteins and LBR, an Integral Protein of the Nuclear Envelope Inner Membrane	71
	Abstract	71
	Introduction	72
	Results	75
	Discussion	94
Chapter 6.	General Discussion	99
Chapter 7.	References	104

List of Figures and Tables

Fig.3-1. DNA sequences, deduced amino acid sequences and hydropathy analysis of human LBR. 35

Fig.3-2. Alignment of the amino acid sequences of human LBR and chicken LBR. 37

Fig.3-3. Northern blot using ^{32}P-labeled human LBR cDNA probe QY1. 39

Fig.3-4. Autoantibodies from two patients with PBC react with GST fusion protein containing amino-terminal domain of human LBR. 41

Fig.3-5. Immunofluorescence micrographs showing nuclear envelope labeling by autoantibodies that recognize the amino-terminal domain of human LBR. 42

Fig.3-6. LBR amino-terminal domain GST fusion protein binds to lamin B1. 44

Fig.3-7. Human LBR amino-terminal domain fusion protein retards migration of M13 double-stranded DNA in agarose gel electrophoresis. 47

Fig.3-8. ^{32}P-labled bacteriophage lambda-DNA binds to amino-terminal domain of human LBR immobilized on nitrocellulose. 48

Fig.3-9. Thr^{200} of the amino-terminal domain of human LBR is a phosphorylation site for p34^{cdc2}. 49

Fig.3-10. Decreased concentrations of human LBR mRNA induced by IL-6. 51

Fig.4-1. Interactions between human nuclear lamins expressed in yeast detected using the yeast two-hybrid assay. 62

Fig.4-2. Interactions between the various domains of human lamin B1 detected in the yeast two-hybrid assay. 65

Fig.5-1. Nucleotide sequences and deduced amino acid sequences of ATBP115 and ATBP8132. 81

Fig.5-2. Alignment of the amino acid sequences of $HP1^{Hs\gamma}$, $HP1^{Hs\alpha}$ and *Drosophila melanogaster* (Dm) HP1. 79

Fig.5-3. The amino-terminal domain of LBR binds to $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$ *in vitro*. 81

Fig.5-4. Effect of ionic strength and detergent on the *in vitro* binding of $HP1^{Hs\alpha}$ to the amino-terminal domain of LBR. 82

Fig.5-5. Binding of HP1 proteins in cell extracts to the amino-terminal domain of LBR. 84

Fig.5-6. Co-immunoprecipitation of HP1 proteins from cell extracts by anti-LBR antibodies. 87

Fig.5-7. Co-localization of $HP1^{Hs\alpha}$ and LBR in cells. 88

Fig.5-8. The carboxyl-terminal half of $HP1^{Hs\alpha}$ is necessary and sufficient for HP1-LBR interactions. 92

Fig.5-9. The second half of amino-terminal domain of LBR is necessary and sufficient for HP1-LBR interactions. 93

Fig.5-10. Diagram showing the interactions of LBR with the lamina and heterochromatin at the nuclear envelope. 97

Table 3-1. Comparision of human LBR and chicken LBR. 38

Table 4-1. β-Galactosidase activities in the two-hybrid assay for yeast cotransformed with plasmids that express GAL4 fusion proteins of human nuclear lamins. 63

Table 5-1. β-Galactosidase activities for yeast cotransformed with LBR amino-terminal domain GAL4 DNA binding domain fusion protein and cDNA clones isolated in the two-hybrid screen and controls. 76

Chapter 1. Introduction

The nuclear envelope consists of the nuclear membranes, the nuclear pore complexes (NPCs) and the nuclear lamina. The nuclear membranes are divided into three domains: the outer nuclear membrane continuous with the rough endoplasmic reticulum (ER), the inner nuclear membrane adjacent to the nuclear lamina, and the nuclear pore membranes connecting the outer and inner nuclear membranes at the NPCs. Each nuclear membrane domain has unique integral membrane proteins with different functions. The NPCs are the channels and machinery for both passive and active bidirectional transport between the nucleus and the cytoplasm. Underlying the inner nuclear membrane is the nuclear lamina, a meshwork of intermediate filament proteins called lamins. The nuclear lamina provides a framework to support and organize the structure of nuclear envelope (Goldberg, 1995).

Eukaryotic cells undergo dramatic structural changes in the transition from interphase to mitosis during cell division. The most evident is the condensation of chromatin to form mitotic chromosomes. In most eukaryotic cells, this event is accompanied by the breakdown of the nuclear envelope. The cytoplasmic structures are also altered when the cell enters mitosis. The changes include the vesicularization of ER and Golgi stacks, the disassembly of actin, the rearrangement of intermediate filaments, and the formation of mitotic spindles. At the end of mitosis, two nuclear envelopes are reassembled around the separated two sets of chromosomes. All other cellular structures are also reformed and the cell is divided into two daughter cells (Alberts *et al.*, 1989).

The development of the nuclear envelope in eukaryotic life has two major biological implications. First, the process of gene expression is temporally and spatially segregated by the nuclear envelope. The transcription and maturation of mRNA occur in the nucleus while the translation of mRNA into protein is restricted in the cytoplasm. Second, the

nuclear envelope encloses and organizes chromatin three-dimensionally in the nucleus. The highly condensed, transcriptionally inactive heterochromatin localizes adjacent to the nuclear membrane and in the nuclear core while the less condensed and transcription-capable euchromatin is localized close to the NPCs or the interior of the nucleus. Both of these aspects are thought to be critical for the complicated regulation of eukaryotic gene expression (Blobel, 1985; Darnell *et al.*, 1990).

A. The Nuclear Pore Complexes

1. The Morphology and Proteins of the NPC

Early electron micrographs of the nuclear envelope revealed that NPCs have cytoplasmic, nuclear, and luminal aspects. This tripartite structure has been found evolutionarily conserved in all eukaryotic cells examined (Muller, 1977). The NPC is a symmetric structure with a central cylindrical body sandwiched between a cytoplasmic ring and a nucleoplasmic ring. This core structure is 120 nm in diameter and 70 nm in length. The central body consists of a plug and eight radiating spokes connected with the eight globular components of both the cytoplasmic and nucleoplasmic rings. Between the spokes there are10 nm-diameter channels that could allow for passive diffusion. There are some NPC peripheral structures including short cytoplasmic filaments and cytoplasmic particles associated with the cytoplasmic ring and a basket-like structure connected to the nucleoplasmic ring (Forbes, 1992; Farbe and Hurt, 1994).

The vertebrate NPC has an estimated size of 125 MDa, containing more than one thousand total and more than one hundred different proteins (Forbes, 1992). The protein gp210 was the first major pore complex protein to be identified (Gerace *et al.*, 1982). The deduced protein primary structure (Wozniak *et al.*, 1989), biochemical studies (Greber and Gerace, 1990) and immunological studies (Courvalin *et al.*, 1990a; Nickowitz and Worman, 1993) demonstrated that gp210 has a 58 amino acids carboxyl-terminus exposed

at the pore, a 20 amino acids transmembrane domain spanning at the nuclear pore membrane, and a 1783 amino acids glycosylated domain residing in the perinuclear space. The other characterized vertebrate pore membrane protein is Pom121p (Hallberg *et al.*, 1993). In contrast to gp210, Pom121p has the "FXFG" motifs which are present in most non-membranous nucleoporins. The term nucleoporin was first introduced to describe proteins of NPCs that are not anchored in pore membranes (Davis and Blobel, 1986), but it has been used to refer all NPC proteins recently. Rat p62 was the first nucleoporin to be identified (Davis and Blobel, 1986) and its encoding DNA was later isolated (Starr *et al.*, 1990). So far, at least sixteen nucleoporins have been molecularly cloned and characterized from yeast and vertebrates (Rout and Wente, 1994; Aitchison *et al.*, 1995). Most of the nucleoporins contain the characteristic repetitive peptide motifs including "FXFG" or "GLFG" or both. These repetitive sequences can form significant hydrophobic domains that may be involved in protein-protein interactions (Rout and Wente, 1993; Dingwall, 1993). While the integral proteins of pore membrane are more likely to be involved in the structural organization of the NPC, many of the non-membranous nucleoporins are involved in bi-directional nucleo-cytoplasmic material transport (Rout and Wente, 1994).

2. NPCs and Nucleo-Cytoplasmic Traffic

Ions, small molecules, and small proteins can diffuse freely through the 10 nm diameter aqueous channels of the NPC. Transport of proteins larger than 20-40 kDa depends on nuclear localization sequences (NLSs) (Kalderon *et al.*, 1984) for import and nuclear export sequences (NESs) (Wen *et al.*, 1995) for export. Export of RNAs requires certain structures such as the CAP of small nuclear RNA (Dargemont and Kuhn, 1992).

The nucleo-cytoplasmic traffic requires both NPC components and soluble cytoplasmic or nucleoplasmic factors. For nuclear protein import, cytosolic factors first recognize and bind to NLS-bearing proteins as complexes. These complexes are subsequently docked on the nuclear pore and translocated into the nucleus (Silver, 1991).

The lectin wheat germ agglutinin (WGA), which binds the N-actylglucosamine residues of NPC proteins, can block nuclear protein transport (Finlay *et al.*, 1987). Antibodies against p62 and gp210 can inhibit transport *in vitro* and *in vivo* (Featherstone *et al.*, 1988; Cordes, *et al.*, 1991; Panté *et al.*, 1993). Most yeast nucleoporin (Nup) gene mutants are lethal and their nuclei have defects in nuclear transport *in vitro* (Farbe and Hurt, 1994). P62 was found in a complex with two human cytosolic import factors NTF2 and Ran/TC4 (Moore and Blobel, 1994). A protein named RanBP with characteristics of nucleoporins has recently been identified to be able to interact with Ran/TC4, a GTP-binding protein involved in nuclear import (Moore and Blobel, 1993; 1994; Wu *et al.*, 1995; Yokoyama *et al.*, 1995). A nucleoporin-like polypeptide, named hRIP, can interact with the human immunodeficiency virus protein Rev which binds and facilitates the nuclear export of unspliced and partly spliced viral RNAs (Fritz *et al.*, 1995). These studies demonstrate that nucleoporins are directly or indirectly involved in nuclear import and export of proteins and RNAs.

3. Chromatin Binding of NPCs and Gene gating

The distribution of NPCs at the nuclear envelope is not random. NPCs appear to localize as clusters that correlate with the distribution of chromatin attached to specific membrane areas (Franke *et al.*, 1981; Nicolini *et al.*, 1984). Blobel (1985) has proposed in the "gene gating" hypothesis that distinct DNA-binding subunits of NPCs binds to specific DNA sequences that are repeated in all transcription-capable genes, thereby physically locating, or "gating", them to the NPCs. This may facilitate the specific export of transcripts from the gated genes. Conversely, the non-transcribed heterochromatin is located adjacent to the lamina and the inner nuclear membrane at the nuclear envelope. The isolation of the cDNA for a rat nucleoporin Nup153, which is localized exclusively on the nucleoplasmic side of the NPC, revealed that this protein has four zinc finger motifs characteristic of DNA-binding proteins (Sukegawa and Blobel, 1993). Nup153 may

indeed interact with DNA in cells as bacterially expressed Nup153 binds to DNA in a zinc-dependent fashion (Sukegawa and Blobel, 1993). Thus Nup153 is a candidate protein with a function consistent with the "gene gating" hypothesis, although it is not known whether it recognizes any specific DNA sequences (Dingwall, 1993; Sukegawa and Blobel, 1993).

B. The Nuclear Lamina

1. The Structure and Expression of Lamins

Lamins are the protein components of the nuclear lamina. Lamins A, B, and C were first identified as the three major lamin proteins expressed in rat liver cells (Gerace *et al.*, 1978). Protein sequence deduced for human lamins A and C cDNA clones first demonstrated that lamins belong to the intermediate filaments protein family (Fisher *et al.*, 1986; McKeon *et al.*, 1986). At the same time, it was shown that purified lamins can polymerize to form 10 nm filaments (Aebi *et al.*, 1986). The cDNAs encoding lamins have since been cloned from various organisms from insects to mammals demonstrating that all lamins have highly conserved primary and secondary structures (Nigg, 1992). Vertebrate lamins have been classified into two types: the neutral A-type lamins and the weakly acidic B-type lamin (Gerace and Blobel, 1980; Krohne *et al.*, 1987). Lamins have not been molecularly characterized in some lower eukaryotes (eg. yeast and plants) (Nigg, 1992), although proteins recognized by anti-lamin antibodies that can interact with vertebrate lamina components have been found in yeast (Georgatos *et al.*, 1990).

As is the case for all intermediate filament proteins, lamins consist of a central α-helical rod domain flanked by an amino-terminal head domain and a carboxyl-terminal tail domain (Stewart, 1990). *In vitro* lamin assembly studies indicate that the central rod domain is responsible for the formation of lamin dimers and the head and tail domains may be involved in the formation of lamin polymers (Heitlinger *et al.*, 1991). The lamins are

different from cytoplasmic intermediate filament proteins in that they have nuclear localization signals and (in most lamins) a motif known as the "CaaX" box ("C" as cysteine, "a" as aliphatic, "X" as any amino acid) at the carboxyl-terminal. The CaaX motif is the signal for isoprenylation at the cysteine residue (Nigg, 1992). Two major B-type lamin isoforms (lamins B1 and B2) are expressed in vertebrate somatic cells (Vorburger *et al.*, 1989; Weber *et al.*, 1990). Both lamin B1 and lamin B2 have permanently isoprenylated carboxyl-terminal tails, which may increase the affinity of these proteins for the membrane (Kitten and Nigg, 1991). Lamin A and lamin C are the products of the same gene and arise by alternative splicing (Lin and Worman, 1993). The amino-terminal 566 amino acids of lamins A and C are identical. At their carboxyl-termini, lamin A has unique 98 amino acids and lamin C has unique 6 amino acids. After incorporation into the lamina, the isoprenylated carboxyl-terminus of lamin A is trimmed off by an unknown proteinase (Weber *et al.*, 1989; Beck *et al.*, 1990). In contrast, lamin C does not contain a CaaX box and is never isopreneylated (Fisher *et al.*, 1986). Whether there are differences between the functions of lamin A and lamin C is not known.

The expression of different lamins are not uniform in different tissues or developmental stages of organisms. In the cells of early mouse embryos, only B-type lamins are expressed (Stewart and Burke, 1987). Lamins A, B and C are expressed in different ratios in different rat tissues (Worman *et al.*, 1988a). In some tumor cells, lamins A and C are absent (Guilly *et al.*, 1987; Stewart and Burke, 1987; Worman *et al.*, 1988a). In human lung cancer cell lines, the expression of lamins A and C are absent or greatly decreased while the expression of B-type lamins remains similar (Broers *et al.*, 1993; Kaufmann *et al.*, 1994). A specific B-type lamin isoform has been found to be expressed in rat germ cells (Furukawa *et al.*, 1994). The differential expression of lamins is not limited to mammals. A newly discovered *Drosophila* lamin C is found to be expressed only in late stages of development while the other *Drosophila* lamin, Dmo, is constitutively

expressed (Riemer *et al.*, 1995). These observations suggest that the differential expression of lamins, especially the expression of the A-type lamins, may be involved in the regulation of differentiation and development of higher eukaryotes.

2. Interaction of the Nuclear Lamina with Chromatin

The physical localization of the lamina suggests it as a plausible site for attachment of chromatin to the nuclear envelope. The structural association between lamins and chromatin has been observed by three dimensional fluorescence microscopy. In *Drosophila* embryo cells, a fraction of peripheral chromatin (15-20%) appears to have physical contact with the lamina (Paddy *et al.*, 1990). Localization of lamin B highly correlates with the chromatin distribution under the nuclear envelope in CHO cells (Belmont *et al.*, 1993). The first biochemical evidence to support the lamin-chromatin interaction was the demonstration that when nuclei were treated with Triton X-100 to solubilize nuclear membranes, the lamina remained closely associated with peripheral heterochromatin (Aaronson and Blobel, 1974). Subsequently, it was shown that purified rat lamins A and C can coat the surfaces of mitotic chromosomes *in vitro* (Glass and Gerace, 1990) and lamin A is able to bind to polynucleosomes (Yuan *et al.*, 1991). The α–helical domain of lamins A and C seems to be sufficient for their chromosome binding (Glass *et al.*, 1993). The tails of lamins A, B and C have also been shown to bind to chromatin fragments plated on microtiter wells, probably through interactions with core histones (Taniura *et al.*, 1995). Trypsin treatment abolishes the interactions between lamins and chromatin (Glass and Gerace, 1990; Yuan *et al.*, 1991), suggesting that lamins may bind to the protein components of the chromatin. Lamins can also directly interact with DNA (Luderus *et al.*, 1992; Shoeman and Traub, 1990). DNA sequences named matrix attachment regions (MARs), which are involved in the attachment site of the 80-90 kilobases (kb) chromatin "loops" at the nuclear matrix, can specifically bind to lamin B1 and lamin A polymers *in vitro* (Luderus *et al.*, 1992; 1995). These findings suggest that

the nuclear lamina could be the attachment sites for chromatin loops (Luderus *et al.*, 1992; 1995). It is therefore possible that the lamina interacts with both the DNA and protein components of the chromatin.

3. Lamina and Nuclear Envelope Breakdown during Mitosis

It has been widely accepted that the depolymerization of the nuclear lamina plays an important role in nuclear envelope breakdown during mitosis (Nigg, 1992). Lamins are depolymerized at the onset of the mitosis. Subcellular fractionation studies have shown lamins A and C in mammalian cells are dispersed throughout the cytoplasm in mitosis (Gerace and Blobel, 1980). In contrast, about 50% B-type lamins are remained associated with vesicles, presumably derived from the fragmented nuclear membranes (Gerace and Blobel, 1980; Burke and Gerace, 1986). Studies have shown that lamins are phosphorylated at a higher level during mitosis than during interphase (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985; Smith and Fisher, 1989), suggesting that hyperphosphorylation is responsible for the mitotic depolymerization of the lamina and the disassembly of the nuclear envelope. This hypothesis is strongly supported by the demonstration that $p34^{cdc2}$, the protein kinase that promotes mitosis, is responsible for the mitotic phosphorylation of lamins. Lamin B2 was shown to be phosphorylated by $p34^{cdc2}$ kinases both *in vitro* and *in vivo* and incubation of isolated nuclei with purified $p34^{cdc2}$ kinase results in lamina disassembly (Peter *et al.*, 1990). In addition, $p34^{cdc2}$ kinases can phosphorylate and cause lamin polymers to disassemble *in vitro* (Peter *et al.*, 1990; Dessev *et al.*, 1991; Goss *et al.*, 1994). Phosphorylation by $p34^{cdc2}$ kinases may affect the higher order organization of the nuclear lamina as *in vitro* phosphorylation of chicken lamin B2 by $p34^{cdc2}$ kinase can interfere with the head-to-tail polymerization of the lamins but not formation of the dimer itself (Heitlinger *et al.*, 1991). The mitotic phosphorylation sites on lamin proteins are the serine and threonine residues flanking the central rod domains, which are extremely well conserved

among different members of the lamin family (Peter *et al.*, 1990; Ward and Kirschner, 1990). Phosphorylation of these sites during mitosis may weaken the interactions between dimers of higher order structures (Ward and Kirschner, 1990). When these sites are mutated to amino acid residues that can not be phosphorylated, both lamin polymers form *in vitro* and the lamina formed *in vivo* become resistant to disassembly (Peter *et al.*, 1990; Heald and McKeon, 1991). Taken together, these results demonstrate that lamin phosphorylation by $p34^{cdc2}$ is essential for lamina disassembly during mitosis.

4. Lamins and Nuclear Envelope Reassembly

At the end of mitosis, the nuclear envelope reforms around each set of daughter chromosomes to generate two new nuclei. This process utilizes the fragments that derived from the original mother nucleus and does not depend on new protein synthesis or cytoplasmic stores of proteins. Nuclear envelope reassembly can be divided into three distinct stages: binding of nuclear membrane vesicles to the chromosome surfaces, vesicle fusion and assembly of pore complexes, and further fusion and import of proteins leading to nuclear envelope growth (Wiese and Wilson, 1993). Most of the knowledge about nuclear envelope reassembly has been obtained by studying *in vitro* nuclear envelope assembly. In *in vitro* nuclear envelope reassembly assays, extracts and membrane components prepared from synchronously dividing Chinese hamster ovary (CHO) cells (Burke and Gerace, 1986), fertilized and activated *Xenopus* eggs (Lohka and Masui, 1983; Newport, 1987), or *Drosophila* embryos (Crevell and Cotterill, 1991) are incubated with sperm chromatin, mitotic chromosomes or DNA. After incubation, a nucleus-like structure surrounding the chromatin can be observed under microscope. This reorganized structure has certain nuclear functions including transcription and DNA replication.

It has been proposed that the nuclear lamins play a pivotal role in the process of nuclear envelope reassembly. Gerace and Blobel (1980) proposed that during nuclear reformation, lamins A and C bind to chromatin first and then mediate the binding of B-type

lamin-containing nuclear membrane vesicles to the chromatin to generate a sealed nuclear envelope. In support of this model, it has been shown that lamins A and C associate directly with the surface of condensed chromosomes in CHO cell, and that depletion of lamins A and C inhibits nuclear envelope assembly (Burke and Gerace, 1986; Glass and Gerace, 1990). Evidence favoring this model has also been obtained in studies using the *Drosophila* embryo system (Smith and Fisher, 1989; Ulitzur *et al.*, 1992). Anti-*Drosophila* lamin antibodies can inhibit the attachment of nuclear membrane vesicles to the chromatin surface and nuclear envelope formation does not occur (Smith and Fisher, 1989; Ulitzur *et al.*, 1992). However, studies in the *Xenopus* system demonstrated that lamins were not essential in the nuclear envelope reassembly and led to another hypothesis of nuclear envelope reassembly (discussed below).

C. Integral Proteins of the Nuclear Inner Membrane

1. The Structure of Integral Proteins of the Nuclear Inner Membrane

The inner nuclear membrane is connected to the outer nuclear membrane by the nuclear pore membrane. Several integral membrane proteins localized to the inner nuclear membrane have been identified. The avian lamin B receptor (LBR) was the first inner nuclear membrane protein to have been identified (Worman *et al.*, 1988b). LBR is resistant to extraction with urea and alkali treatment indicating that it is an integral membrane protein. Indirect immunofluorescence using antibodies raised against LBR gives a nuclear rim pattern, demonstrating that it is a nuclear envelope protein (Worman *et al.*, 1988b). The cDNA for chicken LBR was subsequently cloned and the primary sequence of LBR was deduced (Worman *et al.*, 1990). Chicken LBR has 637 amino acids and a basic 204 amino acids amino-terminal domain that contains consensus sites for phosphorylation by protein kinase A and $p34^{cdc2}$ kinase and a stretch with putative nucleic acid binding sites. Following the amino-terminal domain are eight hydrophobic segments that can serve as transmembrane domains that span the inner nuclear membrane (Worman *et al.*, 1990).

Both the amino-terminal domain (Soullam and Worman, 1993; 1995) and the first transmembrane domain (Smith and Blobel, 1993; Soullam and Worman, 1995) of LBR can target and localize LBR to the inner nuclear membrane, likely by different mechanisms (Soullam and Worman, 1995).

Four rat proteins with molecular masses of 75 kDa, 68 kDa, 55 kDa and 53 kDa have also been localized to the inner nuclear membrane by immunoelectron microscopy and named LAPs 1A, 1B, 1C and LAP2 respectively for "lamina-associated polypeptides" (Senior and Gerace, 1988; Foisner and Gerace, 1993). Cloning of the cDNA for LAP2 (Furukawa *et al.*, 1995) indicates that it encodes a protein with 452 amino acids that is identical to a protein previously identified as β-thymopoetin, a presumed precursor of a thymocyte growth factor (Harris *et al.*, 1994). LAP2 has a large nucleoplasmic amino-terminal hydrophilic domain (residues 1-409) and a single putative transmembrane segment at residues 410-433 (Furukawa *et al.*, 1995). LAP1C is a protein of 506 amino acids with similar structure feature as LAP2, a hydrophilic nucleoplasmic domain (amino acids 1-310) and a single membrane-spanning region between residues 311-333 (Martin *et al.*, 1995). An integral nuclear membrane protein named "otefin" has also been identified in fruit fly (Harel *et al.*, 1989). Otefin has a nucleoplasmic domain with 452 amino acids and one potential membrane-spanning domain at the carboxyl-terminus (Padan *et al.*, 1990); the function of otefin is not known.

The integral membrane proteins that have been characterized to date show no sequence homology (except that of LAP1 A, B, C that may be derived from the same gene by differential splicing, Senior and Gerace, 1988). The common features they have are the presence of one or several transmembrane segments and the $p34^{cdc2}$ phosphorylation consensus sites in the nucleoplasmic domain. These integral membrane proteins are likely involved in three functions: association with lamins, association with chromatin and nuclear envelope reassembly at the end of mitosis.

2. Anchoring of the Nuclear Lamina

The nuclear lamina is tightly associated with the inner nuclear membrane although the lamins do not have transmembrane domains. During mitosis, while lamins A and C become soluble and randomly distributed in the cytosol, about 50% of B-type lamins remain attached to nuclear vesicles during mitosis (Gerace and Blobel, 1980; Burke and Gerace, 1986). B-type lamins are also more resistant to extraction than lamins A and C (Gerace *et al.*, 1984). These observations suggested that B-type lamins may interact with the inner nuclear membrane via an unique mechanism, for example, by attachment to a transmembrane receptor that binds to B-type lamins (Gerace *et al.*, 1984). The isoprenylation at the CaaX-cysteine has also been taken to explain the different fates of A- and B-type lamins during cell division (Kitten and Nigg, 1991), however, the CaaX modification seems to be necessary but not sufficient for a stable association of lamins with the membrane. For example, *Xenopus* lamin III (a B-type lamin), which is permanently isoprenylated and carboxyl methylated in oocytes, is soluble during mitosis (Firmbach-Kraft and Stick, 1993).

The first candidate for a B-type lamin binding protein identified was LBR. It has been shown that rat liver ^{125}I-lamin B but not ^{125}I-lamin A can bind to urea-extracted turkey erythrocyte nuclear envelopes in a saturatable manner. Lamin B also binds to LBR in a ligand blot assay (Worman *et al.*, 1988b). We further demonstrated that lamin B1 can bind to a glutathione S-transferase (GST)-LBR amino-terminal domain fusion protein (gtLBR-AT) (Ye and Worman, 1994). Indirect evidence has also shown that LBR interacts with the nuclear lamina in a cell cycle-dependent fashion (Bailer *et al.*, 1991). Autoantibodies against LBR are also anti-idiotypic to certain antibodies directed against B-type lamins (Lassoued *et al.*, 1991) and the autoantibodies recognizing the first 60 amino acids of LBR (Lin *et al.*, 1996), suggesting that they recognize a part of LBR involved in the LBR-B-type lamin interaction. An immunoprecipitate complex from chicken cell

nuclear extracts contains LBR, B-type lamins, a kinase which can modify LBR, and three other polypeptides with molecular sizes of 150 kDa, 34 kDa, and 18 kDa respectively (Simos and Georgatos, 1992; Meier and Georgatos, 1994). Partial sequence of the 34 kDa protein demonstrated it was homologous to the splicing factor 2 (SF2)-associated protein p32 (Simos and Georgatos, 1994). The biological function of this interaction has not been demonstrated. The identities of the 150 kDa, 18 kDa polypeptides and the associated kinase have not been demonstrated. When chicken LBR and human lamin B1 are co-expressed in yeast and detected by immunoelcetron microscopy, co-localization of lamin B and LBR were observed in nuclear envelope and membrane stacks (Smith and Blobel, 1994). This observation demonstrated that LBR and B-type lamin interaction can occur *in vivo*. LAPs can also bind to lamins. LAPs 1A and 1B bind to both lamins A, C and lamin B1, while LAP2 associates only with lamin B1 (Foisner and Gerace, 1993). These findings strongly support the hypothesis that B-type lamins associate with the nuclear envelope membrane by interacting with specific integral proteins at the nuclear inner membrane. In addition, other integral membrane proteins such as LAPs 1A, 1B and 1C that have general lamin binding affinity may also contribute to the attachment of the lamina to the nuclear inner membrane.

3. Interactions with Chromatin

Morphological and biochemical studies have shown that peripheral heterochromatin is associated with the inner nuclear membrane. It had been thought that the inner nuclear membrane may not be accessible to chromatin since the lamina, which may be 20 nm thick in some cells, could be a barrier for this interaction. However, more recent three-dimensional optical sectioning light microscopy studies have demonstrated that the lamina is actually a highly discontinuous network with spacing of about 0.5 μm between network components (Paddy *et al.*, 1990; Belmont *et al.*, 1993). In the spacing regions, inner nuclear membrane proteins can potentially interact with chromatin.

The primary structure of LBR showed that it has potential DNA binding motifs, leading to the hypothesis that LBR may interact with chromatin (Worman *et al.*, 1990). LBR-containing vesicles are targeted to the decondensing chromatin during mitosis (Chardhary and Courvalin, 1993). We have demonstrated that the amino-terminal domain of LBR can indeed bind to double-stranded DNA *in vitro* (Ye and Worman, 1994; see Chapter 3) and can interact with heterochromatin associated proteins *in vitro* and *in vivo* (see Chapter 5). These findings demonstrated that LBR can bind to chromatin, most likely heterochromatin, by both protein-DNA ad protein-protein interactions. Whether LBR recognizes specific DNA sequences in not known. Purified LAP2 is able to interact directly with mitotic chromosomes, while LAP1 cannot. LAP2-chromatin interactions also appear to be regulated by mitotic phosphorylation (Foisner and Gerace, 1993). It has not been demonstrated whether LAP2 interacts with chromatin proteins, or with DNA, or whether it can interact with interphase chromatin.

The interactions of integral membrane proteins with chromatin may have two biological functions. In interphase, they may be involved in the higher order organization of genome. At the end of mitosis, they may be responsible for the targeting of nuclear membrane vesicles to the chromatin for the reassembly of the nuclear envelope.

5. Inner Nuclear Membrane Proteins and Nuclear Envelope Disassembly

Increasing evidence implicates phosphorylation of proteins in the disassembly of the nuclear envelope during mitosis, the best characterized being the phosphorylation of lamins (Nigg, 1992). However, lamina disassembly seems to be necessary but not sufficient for nuclear envelope breakdown. The nuclear lamina is absent or only poorly developed in spermatocytes and spermatids (Stick and Schwarz, 1982) and disappears during meiotic prophase in chicken oocytes (Stick and Schwarz, 1983), however, the nuclear membranes and NPCs remain intact in these cells. Incubation of nuclei with mitotic cell extracts resulted in the depolymerization of the lamina but the nuclear membranes

remained unaffected (Newport and Spann, 1987). It has been proposed that phosphorylation-dephosphorylation of unidentified nuclear membrane components in the nuclear envelope may induce nuclear envelope breakdown and reassembly (Pfaller *et al.*, 1991). In agreement with this hypothesis, it has been shown that dephosphorylation of LBR reduces its lamin B binding ability (Appelbaum *et al.*, 1990) and that the phosphorylation of LBR by $p34^{cdc2}$ kinase is mitotically regulated (Courvalin *et al.*, 1992). A potential model is that both the hyperphosphorylation of lamins and dephosphorylation-phosphorylation of LBR at the onset of mitosis account for the breakdown of the nuclear envelope (Courvalin *et al.*, 1992). The binding affinity of LAP2 to lamin B1 and chromosomes can also be regulated by mitosis-specific phosphorylation (Foisner and Gerace, 1993). In these aspects, LAP2 and LBR appear to have redundant functions in mitosis.

6. Inner Nuclear Membrane Proteins and Nuclear Envelope Reassembly

Although there is evidence that lamins are involved in nuclear envelope reassembly, there are many findings that argue against the role of the lamins A and C in nuclear envelope reassembly as proposed (discussed previously). First, in some undifferentiated cells, lamin A and lamin C are not expressed and B-type lamins are the only major lamin proteins in the nuclear envelope (Guilly *et al.*, 1987; Stewart *et al.*, 1987; Lebel *et al.*, 1987; Worman *et al.*, 1988a, Rober *et al.*, 1989). Second, the nuclei present in cells during early *Xenopus* embryogenesis contain only one lamin protein (LIII), which is not bound to membrane vesicles during mitosis (Benavente *et al*, 1985; Stick and Hausen, 1985). Depletion of LIII from cytosolic fractions of *Xenopus* egg extracts does not block nuclear envelope formation around the chromatin, although the resulting nuclei appear small and fragile (Newport *et al.*, 1990; Meier *et al.*, 1991). Newport and colleagues (Wilson and Newport, 1988; Newport *et al.*, 1990) have proposed that the targeting of nuclear membrane vesicles to chromatin is independent of lamins and is mediated by an

integral membrane protein component(s) in the vesicles. Lamins may therefore be involved in the decondensation of the chromatin and in further growth of the nuclear envelope but not in the vesicle targeting.

In support of a lamin-independent mechanism of nuclear envelope reassembly, it has been shown that the binding of nuclear membrane vesicles prepared from *Xenopus* eggs to chromosomes is inhibited by trypsin treatment of the vesicles, suggesting that a protein component on the vesicle membranes is responsible for chromatin binding (Wilson and Newport, 1988). The protease-sensitive component(s) is resistant to treatments that disrupt protein-protein interactions, such as high salt, EDTA, or low ionic strength suggesting that it is an integral membrane protein, or proteins, tightly associated with the membranes (Wilson and Newport, 1988). The nuclear membrane vesicles that bind to chromosomes are a specific group of vesicles with diameters of 70 nm that bind to chromatin sites and sites spaced approximately 100 kb of DNA apart (Newport and Dunphy, 1992). When sperm chromatin was digested with trypsin, it was not able to bind membrane vesicles, suggesting that the interaction between decondensed chromatin and membrane vesicles requires both chromatin-bound and membrane-bound proteins (Newport and Dunphy, 1992). Several other groups have also demonstrated that nuclear membrane vesicles can bind to chromatin in the absence of lamins and subsequently fuse to form nuclear envelope-like structures (Pfaller *et al.*, 1991; Vigers and Lohka, 1991; Boman *et al.*, 1992).

Neither the lamin-dependent or lamin-independent model for the targeting of nuclear membrane vesicles to chromatin are definitive. The two different models are derived from different *in vitro* reassembly systems and the chromatin-targeting in the different systems may not be identical. The *in vitro* nuclear reassembly assays also have technical limits. For example, lamin depletion may not be complete and, in the case of *Xenopus* egg extracts, a new vesicle bound lamin II has been found since Newport's experiments,

suggesting that they might not have completely depleted the lamins (Lourim and Krohne, 1993). Second, the *in vitro* reassembly may not be identical to the nuclear envelope reassembly *in vivo*. In addition, the *in vitro* nuclear reassembly assays cannot define the order of steps in the pathway of nuclear envelope assembly. It is thus not known which components, the inner nuclear membrane proteins (LBR and LAP2) or the lamins or other components, initiate the reassembly of the nuclear envelope.

To overcome these difficulties, alternative approaches have been used. One example has been carried out by studying the behavior of LBR-containing vesicles during mitosis by indirect immunofluorescence microscopy and biochemical analysis. The immunofluorescence staining patterns of mitotic cells labeled with anti-LBR antibodies shows that LBR appears around the edges of chromatin as early as anaphase. The binding of LBR-containing membranes to chromatin further extends during telophase until a completed nuclear envelope is formed at the end of mitosis (Chardhary and Courvalin, 1993). When the time course of reassembly of B-type lamins and lamins A and C were examined during mitosis, they remained in the cytosol until early telophase. A trace amount of lamins starts to appear around nuclear envelope at late telophase. This observation strongly suggests that LBR-containing vesicles are targeted early to chromatin to reconstruct the nuclear envelope, earlier than lamins A and C and B-type lamins. Since LBR is an integral membrane protein of nuclear envelope, it is a likely candidate for the protein proposed by Newport that is responsible for the initiation of nuclear envelope reassembly during mitosis (Chardhary and Courvalin, 1993).

Chapter 2. Materials and Methods

Bacterial and Yeast Strains and Mammalian Cells

Escherichia coli strain DH5α (F^-, recA1, endA1, hsdR17, supE44, thi1, gyrA, relA1) was obtained from Gibco BRL Life Technologies, Inc and used as the bacterial transformation recipient for all plasmid constructs and expression of GST fusion proteins. *Saccharomyces cerevisiae* SFY526 (MATα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3::Gal1-LacZ) was a gift from Dr. S. Field (State University of New York, Stony Brook, New York). *S. cerevisiae* Y190 (MATα, gal4, gal80, his3, trp1-901, ade2-101, ura3-52, leu2-3,-112, URA::Gal1-LacZ, LYS2::GAL(UAS)-HIS3 cyh^r) was a gift from Dr. S.J. Elledge (Baylor College of Medicine, Houston, Texas). SFY526 was used as a recipient for all yeast transformations in studying lamin-lamin interactions (Chapter 4) and Y190 was used to performed two hybrid screen (Chapter 5) and the followed protein-protein interaction tests. Human cell lines used included Hela S3, 293T, NJBC and HepG2 cells.

Library Screening

A human Hela S3 cell lamda uni-Zap XR cDNA library (Stratagene) was used to screen human LBR cDNA using ^{32}P labeled chicken LBR cDNA DJ-5 (Worman *et al.*, 1990) as a probe by standard methods (Sambrook *et al.*, 1989). Hybridization was carried out at a specificity activity between $4x10^5$ and $1x10^6$ cpm/ml at 42°C in hybridization solution (6xSSC, 5xDenhardt's solution, and 0.1% SDS, 18.4 mM Na_2HPO_4, 4mM NaH_2PO_4, 50% formamide, and 0.1 mg/ml salmon sperm DNA). Nitrocellulose filters (Schleicher and Schuell) were finally washed at 55°C in 1xSSC and 0.1% SDS and exposed to XAR-5 film (Jersey Lab and Glove Supply) at -80°C with an intensifying screen. The plasmids were isolated according to the library manufacturer's instructions (Stratagene).

Northern Blotting

Northern blots of human poly(A+) RNA used to examine the expression of human LBR were provided by Dr. S. Chen-Kiang's laboratory (Mount Sinai School of Medicine, New York, NY). Hybridization was performed using ^{32}P-labeled human LBR cDNA as a probe at the same condition as cDNA library screening.

DNA Sequencing and Analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase Kit Version 2.0 (U.S. Biochemical Corp.) according to the manufacturer's instructions. Custom oligonucleotide primers were obtained from Genset. DNA and protein sequence analysis was performed using the computer facilities of the Department of Biomathematics of the Mount Sinai School of Medicine (New York, NY) and version 7.B of the GCG software package (Genetics Computer Group, Inc.) (Devereux *et al.*, 1984). Database searches were accomplished by using the BLAST network service at the National Center for Biotechnology Information (Atschul *et al.*, 1990).

Plasmid Constructions

(a) Plasmids that Express GST-Human LBR Amino-Terminal Domain Fusion Proteins (Chapter 3)

Different portions of the amino-terminal domain of human LBR were expressed as GST fusion proteins using the prokaryotic expression vector pGEX-2T (Pharmacia LKB Biotechnology Inc.). Polymerase chain reaction (PCR) primers were designed to amplify cDNA fragments encoding the corresponding portions of amino-terminal domain polypeptides. Restriction sites were engineered into the 5'-ends of the PCR primers to facilitate cloning of the amplified products into the BamH1 and EcoRI sites of pGEX-2T.

PCR was carried out using the human LBR cDNA QY1 as a template. The PCR products were digested with the BamH1 and EcoR1 and ligated into pGEX-2T that was also digested with BamH1 and EcoR1. The resultant plasmid was introduced into *E. coli* strain DH5α by standard transformation methods (Sambrook *et al.*, 1989). The recombinant plasmids were determined by both restriction analysis and DNA sequencing.

(b) Plasmids for Studying Lamin-Lamin Interactions in the Yeast Two-Hybrid System (Chapter 4)

To analyze interactions between different human lamins in the yeast two-hybrid system, complementary DNAs were cloned into plasmids pGBT9 and pGAD424 (provided by Dr. S. Fields). For cloning of lamin B1 into plasmids of pGBT9 and pGAD424, a full length cDNA of human lamin B1 in pBluescript (Pollark *et al.*, 1990) was provided by Drs. K. M. Pollard and E. M.Tan (Scripps Institute, La Jolla, CA). This plasmid was digested with StyI, treated with the Klenow fragment of DNA polymerase, then digested with BamH1. The fragment generated was cloned into pGBT9 and pGAD424 which were digested with EcoR1, treated with Klenow, and then digested with BamH1. These plasmids were called pGBT9-LmB and pGAD424-LmB, respectively. The fusion proteins encoded by each contained the respective domains of GAL4 fused in-frame to full length human lamin B1. To construct the plasmids encoding the GAL4 domains fused to the lamin B1 head domain, pGBT9-LmB and pGAD424-LmB were digested with SacI, which cuts just after the sequence encoding the head domain, and PstI, which cuts at the 3' end of the lamin B1 cDNA insert. The resulting linear plasmid was separated from the restriction endonuclease fragments, treated with T4 DNA polymerase, and ligated with T4 DNA ligase. The resulting constructs respectively encoded the DNA binding and activation domains of GAL4 fused to the head domain of lamin B1.

All other constructs were made using the PCR with the appropriate cDNA as the template to amplify specific sequences encoding various domains of lamin B1, the cDNA

clone described above was used as the template for PCR. A full-length human prelamin A cDNA was provided by Dr. N. Chauhary (Aronex Pharmaceutical Corporation, The Woodlands, TX) for use as a template to amplify sequence encoding prelamin A, lamin A, and lamin C. Restriction sites for BamH1 and SalI engineered for cloning of the cDNA fragments into BamH1 and SalI sites of pGBT9 and pGAD424 in-frame. For the carboxyl-terminal domain of lamin C, nucleotides reverse complementary to those encoding the last six amino acids that are not present in the prelamin A cDNA were engineered into the antisense primer. The amplified cDNAs were purified and digested with BamH1 and SalI for cloning into pGBT9 and pGAD424 that were also digested with BamH1 and SalI.

(c) Plasmids for Studying LBR-HP1 Interactions (Chapter 5)

Plasmid pGBT9-LBRAT was the bait plasmid used in the yeast two-hybrid library screening for LBR-interacting proteins. Custom oligonucleotide primers (Geneset) and plasmid QY1 DNA encoding the amino-terminal domain of human LBR from amino acid 1 to 208, with an EcoRI restriction site at its 5' end and a BamHI site at its 3' end, was amplified by the PCR. The amplified DNA was cloned into the EcoRI and BamHI sites of the GAL4 DNA binding domain fusion vector pGBT9 to produce pGBT9-LBRAT.

pBFT4-ATBP115 and pBFT4-ATBP8132 used for *in vitro* transcription and translation of HP1 proteins were constructed by digesting ATBP115 and ATBP8132 with PstI and XhoI and cloning the inserts into pBFT4 (supplied by Dr. J. Licht, Mount Sinai School of Medicine, New York, NY) at the same sites. pBFT4 constructs express $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$ lacking its first 17 amino acids with FLAG octapeptide (Asp Tyr Lys Asp Asp Asp Asp Lys) epitope at their amino-termini.

pSVK3-FLAG-ATBP115 and pSVK3-FLAG-ATBP8132 used to transfect Hela S3 cells were constructed by digesting pBFT4-ATBP115 and pBFT4-ATBP8132 with SpeI and XhoI and cloning the inserts into pSVK3 digested with XbaI and XhoI.

pQE31-ATBP115 and pQE31-ATBP8132 that express 6xHis-HP1 fusion proteins were constructed digesting pGADGH-ATBP115 and pGADGH-ATBP8132 by digestion with BamH1 and XhoI and cloning the inserts into pQE31 (Qiagen) at BamHI and SalI sites.

pACT2-HP1$^{Hs\alpha}$ and pACT2-HP1$^{Hs\gamma}$ constructs express different domains of HP1 proteins fused to GAL4 activation domain were constructed by cloning PCR amplified cDNA for these domains into pACT2 at EcoR1 and XhoI sites.

Expression and Purification of Recombinant Proteins in *E. coli*

(a) GST Fusion Proteins

For synthesis and purification of GST-human LBR amino-terminal domain fusion proteins, the selected plasmids were transformed into *E. coli* DH5α. A single colony was inoculated and grown in 25 ml LB medium supplemented with 100 μg/ml ampicillin at 37°C overnight. The overnight culture was inoculated into 250 ml LB (ampicillin 100 μg/ml) to grow for 90 minutes and then isopropyl-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce the expression of fusion proteins. After 3 hours, cells were pelleted and lysed in 10 ml lysis buffer (1xPBS, 1% Triton X-100, 1 mM pMSF). The lysate was briefly sonicated and centrifugated at 15,000 rpm for 10 minutes to collect the supernatant. GST fusion proteins were purified by Glutathione-Sepharose (Pharmacia) as described (Smith and Johnson, 1988).

(b) 6xHis-Fusion Proteins

Expression and purification of 6xHis-HP1 fusion proteins were carried out according to the manufacturer's instructions (Qiagen). Briefly, bacterial transformed with pQE31-HP1 constructs was grown in LB supplemented with ampicilin (100 μg/ml) and kanamycin (25 μg/ml) and induced with 2 mM IPTG for 3 hours. Cells were collected and

resuspended in lysis buffer (8M Urea, 0.1M Na-phosphate, 0.01M Tris-HCl [pH 7.0]). Ni-NTA beads were added to the supernatant of the bacterial lysate. After extensive wash with washing buffer (8M Urea, 0.1 M Na-phosphate, 0.01M Tris-HCl [pH 6.3]), proteins bound on the beads were eluted with washing buffer with 100 mM EDTA.

Protein Electrophoresis and Immunoblotting

For protein analysis, the samples were boiled in electrophoresis sample buffer and separated on SDS-polyacrylamide gels according to Laemmli (1970). The proteins on gels were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) using a semi-dry protein transfer apparatus (Bio-Rad) at 15 V for 1 hour. Before application of antibody, the nitrocellulose membranes were saturated with 5% nonfat dry milk in 1xTBST for 30 minutes at room temperature. The blots were then incubated with antibodies at appropriate dillution for 2 hours at room temperature and then washed three times for 15 minutes with 5% nonfat dry milk in 1xTBST to remove unbound antibodies. The blots were subsequently incubated with ^{125}I-labeled protein-A (DuPont NEN) at a dillution of 1:1000 for 1 hour and finally rinsed 5 times for 30 minutes with 5% nonfat dry milk in 1xTBST. To obtain autoradiograms, immunoblots were exposed to XAR film (Jersey Lab and Glove Supply) at -80°C with intensifying screens.

Affinity Purification of Antibodies

Antibodies from human serum were affinity-purified against human LBR fusion proteins using the method of Smith and Fisher (1984).

In Vitro Protein Binding Assays

(a) B-Type Lamin Binding Assay

Lamins were extracted with 8 M urea from nuclear envelopes prepared from rat liver according to the methods described (Dwyer and Blobel, 1976, Worman *et al.*, 1988a, 1988b). Lamin extracts were dialyzed against PBS, cleared by centrifugation and the suspension was incubated at room temperature for 60 minutes with 1 μg of GST-human LBR amino-terminal domain fusion protein, or control GST fusion protein, attached to glutathione-Sepharose beads (Pharmacia). The beads were washed three times with PBS and the proteins that remained bound to the glutathione-Sepharose beads were eluted with SDS sample buffer. The proteins in the unbound and wash fractions, and a portion of the lamin fraction before incubation with fusion proteins, were precipitated with 15% trichloroacetic acid (TCA). The proteins eluted from the glutathione beads and the proteins collected from the unbound fractions and washing fractions were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-lamin B1 antibodies (Cance *et al.*, 1992).

(b) Binding Assays Using *In Vitro* Translated Proteins

In each binding experiment, 10-20 μl of *in vitro* translated lysates of HP1 protein was added to 200 μl of binding buffer (150 mM NaCl, 20 mM Na-Hepes [pH 7.4], 10% glycerol, 0.05% NP-40) with 20 μl of glutathione-Sepharose coupled to equal amounts GST or GST-LBR amino-terminal domain fusion proteins. Suspensions were incubated at 4°C with rotation for 2 hours. After incubation, the Sepharose was washed 5 times with binding buffer and proteins that remained bound were eluted with protein sample buffer and analyzed by autoradiography of 12.5% SDS-polyacrylamide gels (Laemmli, 1970).

Binding assays using different salt or detergent concentrations were performed as described above except that the binding buffers contained the salt or detergent concentrations indicated. After incubation, the Sepharose was washed 5 times with binding buffers of the indicated salt or detergent concentration. The Sepharose was then

washed once with 150 mM NaCl, 20 mM Na-Hepes (pH 7.4), 10% glycerol, 0.05% NP-40 prior to eluting the bound proteins with protein sample buffer.

(c) Binding of Proteins from Cell Lysates to GST Fusion Proteins

Hela cell extracts were prepared as described below (see Immunoprecipitation) and 400 µl of cell extracts were incubated with 5 µg of the GST-LBR amino-terminal domain fusion protein or a GST fusion protein containing only the first 100 amino acids of LBR (Ye and Worman, 1994) or GST alone attached to glutathione-Sepharose beads (20 µl). Incubation was for 2 hours at 4°C with rotation. After incubation, the Sepharose was washed 5 times with 150 mM NaCl, 20 mM Na-Hepes (pH 7.4), 10% glycerol, 0.05% NP-40. The proteins that remained bound to the Sepharose were eluted with protein sample buffer, separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose sheets for immunoblotting as described (Courvalin *et al.*, 1990). Autoantibodies from a patient with scleroderma that recognized $HP1^{Hs\alpha}$ and related polypeptides (Saunders *et al.*, 1993; provided by Dr. W. C. Earnshaw, Johns Hophins University School of Medicine, Baltimore, MD) were used to detect the proteins on the immunoblots.

Protein-DNA Interaction Assays

(a) DNA Agarose Gel Shift Assay

DNA agarose gel shift assays were performed essentially as described (Sapp *et al.*, 1986; Wong *et al.*, 1992). 1µg of double-stranded M13mp19 DNA (Pharmacia) was incubated with various amounts of purified fusion proteins in binding solution (50 mM NaCl, 20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 5% glycerol). After a 30 minutes incubation on ice, the samples were loaded and electrophoresed on a 1% agarose gel, and the DNA was then visualized by staining with ethidium bromide. When heat-denatured

protein was used, the protein was heated at 95°C for 5 minutes prior to incubation with DNA. When proteinase treated protein was used, samples were incubated with 100 µg/ml proteinase K for 10 minutes at 37°C after incubation with DNA but prior to agarose gel electrophoresis.

(b) Nitrocellulose DNA Binding Assay

The assay for examining the binding of ^{32}P-labeled lambda DNAs to proteins immobilized on nitrocellulose ("Southwestern" blotting) was modified from two previously published procedures (Bowen *et al.*, 1980; Wang *et al.*, 1990). Proteins were separated by electrophoresis on SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose. The nitrocellulose filters were first blocked for 30 minutes in 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 20 mg/ml BSA, and 5% glycerol (Buffer D) at room temperature and then incubated with ^{32}P labeled HindIII-digested bacteriophage lambda DNA (New Eengland Biolabs) with a specific activity of $1x10^6$ cpm/ml in Buffer D for 1 hour. Nitrocellulose sheets were washed three times with large volume of Buffer D at room temperature and exposed to XAR-film.

Immunofluorescence Microscopy

For immunolocalization of proteins in cells, transfected cells or untransfected cells were grown to about 60% confluence on 35 mm-diameter petri dishes (NJBC and HepG2 cells were spun to slides using cytospin), washed three times with PBS, fixed with cold methanol at -20°C for 6 minutes, and then washed three times with solution A (1xPBS, 0.1% Triton X-100). Cells were then blocked with solution B (1xPBS, 2% BSA, 0.1% Triton X-100) for 30 minutes and then with the desired antibody diluted in solution B for 1 hour at room temperature. Cells were then washed five times with solution A, incubated for 1 hour with the desired secondary antibody in solution B plus 2 µg/ml 4,6-diamidino-2-phenylindole (DAPI), and then washed five times with solution A. Coverslips were

mounted with a 2% wt/vol solution of 1,4-diazabicyclo-(2.2.2)-ooctane (DABCO) in 85% glycerol/15% 0.5 M Tris-HCl (pH 8.6). Immunofluorescence microscopy was performed on either a Axiphot microscope (Carl Zeiss Inc.) or a laser scanning confocal microscope (TCS 4D; Leica Lasertechnik). To analyze fluorescent images on laser scanning confocal microscopy of the cells, a series of optical sections was collected at a spacing of 0.36-0.56 μm in the Z-axis using both green and red sources. These z-series were transferred to a Macintosh computer for editing and processing using Photoshop 3.0 Program. Image contrast and gain were adjusted to avoid signal saturation or reducing background. Fluorescence images were printed with a Tektronix Phaser II printer.

To detect human LBR in human cells, serum from patients with primary biliary cirrhosis (PBC) containing anti-LBR autoantibodies (Courvalin *et al.*, 1990; Nickowitz *et al.*, 1994) were used at 1:500 dilution and rabbit anti human LBR polycolonal antibodies were used at 1:500 dilution. Anti-FLAG M2 monoclonal antibody (Eastman Kodak Company) that recognizes the FLAG sequences used at 1:200 dilution in immunofluorescence to detect expression of FLAG-HP1 fusion proteins in Hela cells. Fluorescence-conjugated anti-rabbit and anti-mouse immunoglobulin antibodies used as secondary antibodies for immunofluorescence microscopy were obtained from Jackson Immuno Research Labs., Inc. and were used at a dilution of 1:100.

Yeast Two-Hybrid System

(a) Yeast Transformation

S. cerevisiae were transformed with plasmid DNA using the lithium acetate procedure (Ito *et al.*, 1983).

(b) Filter Assay and Liquid Assay of β-Galactosidase Activity

For the qualitative detection of β-galactosidase activity, yeast transformants were patched to solid synthetic complete (SC) medium with appropriate selection, incubated for 2-3 days at 30°C, and then transferred to nitrocellulose filters. Nitrocellulose filters were immersed in liquid nitrogen for 10 seconds or freezed in -80°C for 30 minutes and then transferred to filters of blotting paper that had been treated with 5-bromoindoyl-b-D-galactopyranoside (X-gal) in 100 mM Na-phosphate (pH 7.0), 10 mM KCl, and 1 mM $MgSO_4$. Filters were then incubated at 30°C for between 1 and 16 hours until the blue color was developed.

To quantitate β-galactosidase activity, yeast were grown in 2.5 ml culture with appropriate selection media to mid log phase. Cells were then collected and enzyme activity was measured as described (Guarente, 1983) using O-nitrophenyl-β-D-galactoside as the substrate. In each enzyme activity assay, 0.2 ml of yeast culture was used and the reaction was performed at 30°C until the yellow color developed. β-Galactosidase-specific activities were calculated as described and are given in standard units (Guarente, 1983). The reported specific activities are the means ± standard errors for three independent transformants.

(c) Yeast Two-Hybrid Screening

Yeast strain Y190 was cotransformed with pGBT9-LBRAT and DNA of a human Hela cell cDNA library in the GAL4 activation domain fusion vector pGADGH to give an approximately 10^6 recombinant transformants (Clonetech Lifetechnology Inc.). The transformants were spread on SC solid plates without histidine, leucine and tryptophan plus 25 mM 3-aminotriaole (3-AT). Colonies grown on the plates within 3 to 6 days were streaked onto the same selection plates and tested for β-galactosidase activities. The pGADGH-derived plasmids were rescued from ten yeast cotransformants with positive β-galactosidase activity and used to again cotransform yeast strain Y190 with pGBT9-LBRAT. Only two of these (ATBP115 and ATBP8132) remained positive. The ß-

galactosidase activities of these plasmid clones, when cotransformed with pGBT9-LBRAT and pGBT9, were again measured using the filter and the liquid assays.

In Vitro Transcription and Translation

The constructs pBFT4-ATBP115 and pBFT4-ATBP8132 were used in a coupled transcription and translation assay using the TNT coupled Reticulocyte Lysate System (Promega Corp.). The transcript was generated with T7 RNA polymerase from the circular plasmid template and translated in the presence of (^{35}S) Methionine (Amersham) in a combined reaction.

Cell Culture and Transfection

Hela cells and 293T cells were grown in DME media containing 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM glutamine. Media and other reagents for cell culture were obtained from GIBCO BRL. Cells were grown to 60% confluency on 10 mm-diameter dishes and transfected with plasmid DNA by phosphate precipitation method (Sambrook *et al.*, 1989). About 2 µg plasmid DNA was used for each dish. The precipitate was left on the dishes for 16 hours (overnight) and then rinsed twice with PBS and supplemented with fresh media and maintained in culture for 12 to 72 hours prior to preparation for immunofluorescence microscopy.

Immunoprecipitation

Four 100 mm-diameter petri dishes of Hela S3 cells grown to full confluency were washed 3 times with PBS and the cells harvested by scrapping with a rubber policeman. Cells were collected by centrifugation at 500xg and resuspended in 2 ml 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM pMSF, incubated on ice for 15 minutes and broken in a Dounce homogenizer (30 strokes with the B pestle). Broken cells were centrifugated at 6,000xg for 20 minutes at 4°C and the pellet resuspended in 800 µl of 50

mM Tris-HCl (pH 8.0), 5 mM EDTA, 2% BSA, 0.5% NP-40, 1 mM DTT, 0.2 mM pMSF and then disrupted with a tip sonicator. The suspension was centrifuged at 15,000 rpm for 30 minutes at 4°C in a microcentrifuge (Eppendorf). 400 µl of the supernatant were incubated for 4 hours at 4°C on a rotating mixer with the desired antibody that was coupled to protein A-Sepharose by using the ImmunoPure IgG Orientation Kit (Pierce). Anti-LBR antibodies (Nickowitz *et al.*, 1994; also see Chapter 3) were autoantibodies from a patient with PBC previously shown to recognize the protein. Control antibodies were from another patient with PBC but without LBR autoantibodies (Nickowitz *et al.*, 1994).

Other Materials

Unless otherwise indicated, routine chemical reagents were obtained from Sigma Chemical Co. or Fisher Scientific. Enzymes for DNA cloning were obtained from New England Biolabs.

Chapter 3. Primary Structure Analysis and Lamin B and DNA Binding of Human LBR, an Integral Protein of the Nuclear Envelope Inner Membrane

Abstract

We have isolated cDNAs for human LBR and determined its primary structure. Human LBR is 68% identical to chicken LBR and has a basic nucleoplasmic amino-terminal domain of 208 amino acids followed by a hydrophobic domain with eight putative transmembrane segments. The amino-terminal domain contains a Ser-Arg-rich stretch and consensus sites for phosphorylation by protein kinase A and $p34^{cdc2}$ protein kinase. *In vitro* phosphorylation analysis demonstrated that Thr^{200} of human LBR can be phosphorylated by $p34^{cdc2}$ protein kinase. The amino-terminal domain can affinity purify autoantibodies from autoimmune sera and these autoantibodies label the nuclear envelope when examined by immunofluorescence microscopy. GST-LBR amino-terminal domain fusion protein can bind lamin B *in vitro*. GST-LBR amino-terminal domain can also bind to DNA *in vitro*. The stretch between amino acids 71 and 100, which contains the Ser-Arg-rich stretch, is necessary for DNA binding. LBR is conserved among vertebrate species and its nucleoplasmic domain can potentially mediate the interaction of both the nuclear lamina and the chromatin with the inner nuclear membrane.

Introduction

The nuclear envelope consists of the nuclear membranes, the nuclear pore complexes, and the nuclear lamina (Nigg, 1989). The nuclear membrane has three domains including the outer nuclear membrane, the inner nuclear membrane, and the nuclear pore membrane. Each nuclear membrane domain has different integral membrane proteins that are involved in different aspects of the functions of the nuclear envelope. The outer nuclear membrane is continuous with the rough endoplasmic reticulum (ER) and these two membranes have no obvious distinguishable biochemical differences (Amar-Costesec *et al.*, 1974). The inner and outer nuclear membrane are connected by the nuclear pore membranes, which also have integral membrane proteins that have been proposed to be involved in the construction of the nuclear pore complexes (Wozniak *et al.*, 1989; Hallberg, *et al.*, 1993). The inner nuclear membrane is associated with the nuclear lamina and heterochromatin. These associations are likely mediated by the integral inner nuclear membrane proteins with lamin binding or chromatin binding affinities (Worman *et al.*, 1988b, 1990; Senior and Gerace, 1988; Harel *et al.*, 1989; Courvalin *et al.*, 1990; Bailer *et al.*, 1991; Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993).

LBR, an integral protein of the inner nuclear membrane that can bind lamin B *in vitro*, was first characterized in avian cells (Worman *et al.*, 1988b). Chicken LBR has a hydrophilic amino-terminal domain of 204 amino acids facing the nucleoplasm and a carboxyl-terminal domain consisting of eight hydrophobic segments (Worman *et al.*, 1990). Previous studies have shown that LBR can be phosphorylated by the protein kinase $p34^{cdc2}$ *in vitro* and *in vivo* (Courvalin *et al.*, 1992) and also can be phosphorylated by protein kinase A and an unknown kinase called p58 kinase (Appelbaum *et al.*, 1990; Simons and Georgatos, 1992). In addition, the amino-terminal domain of chicken LBR is basic, has several Ser/Thr-Pro-X-X sequences and a Ser-Arg-rich region, features which are often present in nucleic acid associated proteins, suggesting that LBR may interact with DNA or chromatin (Worman *et al.*, 1990).

Because autoantibodies from patients with primary biliary cirrhosis (PBC) that cross-react with chicken LBR recognize a nuclear envelope protein in Hela cells, we suspected that a human LBR existed (Courvalin *et al.*, 1990; Chaudhary and Courvalin, 1993). However, the primary structure and ligand binding properties of this human protein had not been elucidated. It was also not known from the past studies whether the nucleoplasmic domain of LBR alone could bind to B-type lamins or whether it could bind to DNA as hypothesized according to its sequence (Worman *et al.*, 1990). For these reasons, we isolated the human homologue of chicken LBR, determined its primary structure and examined its interactions with lamin B1 and DNA.

Results

Cloning of cDNA for Human LBR

To clone the human LBR cDNA, full-length chicken LBR cDNA DJ-5 (Worman *et al.*, 1990) was used as probe to screen a human Hella cell cDNA library (Stratagene) using standard procedures (Sambrook *et al.*, 1989). Ten positive clones were obtained after the tertiary screening. Plasmids isolated from these clones had inserts with sizes ranging from 1.5 to 3.7 kb and all these inserts hybridized with the chicken LBR cDNA in Southern blot analysis. DNA sequencing analysis demonstrated that all these clones overlapped with each other and were homologous to chicken LBR cDNA. The longest clone (3732 base pairs), named QY-1, had one open reading frame for a polypeptide of 615 amino acids displaying 68% overall sequence identity with chicken LBR. We concluded that QY-1 encoded the full length human LBR. The DNA sequences, the deduced amino acid sequences and hydropathy analysis of human LBR are shown in Fig. 3-1.

a

```
                                                    CCGGGTTGCTGTGCG -61
ACTATTCTCCGGGAGCCGTTCGTGTCACCGCCGGAACCTGGCGCAGGTTAATTATAGAAA  -1
ATGCCAAGTAGGAAATTTGCCGATGGTGAAGTGGTAAGAGGTCGATGGCCTGGGAGTTCA  60
 M  P  S  R  K  F  A  D  G  E  V  V  R  G  R  W  P  G  S  S   20
CTTTATTATGAAGTAGAAATTCTGAGCCACGACAGCACCTCCCAGCTTTACACTGTGAAG 120
 L  Y  Y  E  V  E  I  L  S  H  D  S  T  S  Q  L  Y  T  V  K   40
TATAAAGATGGAACAGAGCTTGAATTGAAAGAGAATGATATTAAGCCTTTAACTTCCTTT 180
 Y  K  D  G  T  E  L  E  L  K  E  N  D  I  K  P  L  T  S  F   60
AGGCAAAGGAAAGGTGGCTCAACTTCCAGTTCCCCTTCCAGACGCCGAGGGAGTCGATCA 240
 R  Q  R  K  G  G  S  T  S  S  S  P  S  R  R  R  G  S  R  S   80
AGGTCACGCTCCCGATCCCCTGGTCGACCACCTAAAAGTGCCCGCCGATCTGCTTCTGCT 300
 R  S  R  S  R  S  P  G  R  P  P  K  S  A  R  R  S  A  S  A  100
TCCCACCAGGCCGACATTAAGGAAGCAAGGAGGGAAGTGGAAGTTAAATTGACTCCGCTG 360
 S  H  Q  A  D  I  K  E  A  R  R  E  V  E  V  K  L  T  P  L  120
ATTCTGAAGCCATTTGGAAATAGCATCAGCAGATATAATGGGGAGCCTGAGCATATTGAG 420
 I  L  K  P  F  G  N  S  I  S  R  Y  N  G  E  P  E  H  I  E  140
AGAAATGACGCACCTCATAAAAATACACAGGAAAAATTCAGTTTGTCACAAGAAAGCAGT 480
 R  N  D  A  P  H  K  N  T  Q  E  K  F  S  L  S  Q  E  S  S  160
TACATAGCAACACAGTATAGCCTTCGTCCAAGAAGAGAAGAAGTCAAATTAAAAGAAATA 540
 Y  I  A  T  Q  Y  S  L  R  P  R  R  E  E  V  K  L  K  E  I  180
GATTCTAAGGAAGAAAAATACGTTGCAAAAGAACTGGCAGTGAGAACCTTTGAAGTGACC 600
 D  S  K  E  E  K  Y  V  A  K  E  L  A  V  R  T  F  E  V  T  200
CCCATCCGGGCAAAGGACTTGGAGTTTGGAGGAGTACCTGGTGTGTTTCTCATCATGTTT 660
 P  I  R  A  K  D  L  E  F  G  G  V  P  G  V  F  L  I  M  F  220
GGCCTGCCTGTGTTCCTCTTCCTGTTGCTGTTGATGTGTAAACAGAAAGATCCCAGTCTT 720
 G  L  P  V  F  L  F  L  L  L  L  M  C  K  Q  K  D  P  S  L  240
CTGAATTTCCCTCCTCCTTTGCCAGCTTTGTATGAGTTATGGGAAACCAGAGTATTTGGG 780
 L  N  F  P  P  P  L  P  A  L  Y  E  L  W  E  T  R  V  F  G  260
GTCTACCTCCTGTGGTTTTTGATTCAAGTCCTGTTCTACCTACTGCCAATTGGAAAGGTT 840
 V  Y  L  L  W  F  L  I  Q  V  L  F  Y  L  L  P  I  G  K  V  280
GTAGAAGGAACGCCTCTTATTGATGGAAGAAGACTCAAGTATAGATTAAATGGATTCTAT 900
 V  E  G  T  P  L  I  D  G  R  R  L  K  Y  R  L  N  G  F  Y  300
GCTTTTATCCTGACATCTGCAGTCATCGGAACATCTCTCTTCCAGGGCGTAGAGTTTCAT 960
 A  F  I  L  T  S  A  V  I  G  T  S  L  F  Q  G  V  E  F  H  320
TACGTGTACAGTCATTTTCTTCAGTTTGCACTTGCGGCCACTGTTTTTTGTGTGGTCTTG 1020
 Y  V  Y  S  H  F  L  Q  F  A  L  A  A  T  V  F  C  V  V  L  340
AGTGTGTATCTCTACATGCGCTCTTTGAAAGCGCCCCGGAATGACCTGTCGCCTGCCAGC 1080
 S  V  Y  L  Y  M  R  S  L  K  A  P  R  N  D  L  S  P  A  S  360
TCTGGAAATGCTGTCTATGATTTCTTCATTGGCCGTGAATTAAACCCTCGAATTGGTACT 1140
 S  G  N  A  V  Y  D  F  F  I  G  R  E  L  N  P  R  I  G  T  380
TTTGATCTCAAATACTTTTGTGAATTGCGCCCCGGATTGATTGGATGGGTGGTTATTAAC 1200
 F  D  L  K  Y  F  C  E  L  R  P  G  L  I  G  W  V  V  I  N  400
TTGGTGATGCTTTTGGCTGAAATGAAAATACAGGACCGCGCTGTTCCATCCTTGGCCATG 1260
 L  V  M  L  L  A  E  M  K  I  Q  D  R  A  V  P  S  L  A  M  420
ATTTTAGTTAATAGTTTCCAGCTTCTCTATGTGGTGGATGCTCTCTGGAATGAGGAAGCG 1320
 I  L  V  N  S  F  Q  L  L  Y  V  V  D  A  L  W  N  E  E  A  440
TTGTTGACGACCATGGACATCATCCACGATGGATTTGGATTCATGCTGGCTTTTGGAGAC 1380
 L  L  T  T  M  D  I  I  H  D  G  F  G  F  M  L  A  F  G  D  460
TTGGTGTGGGTTCCCTTTATTTACAGCTTCCAAGCCTTTTATTTAGTCAGTCATCCAAAT 1440
 L  V  W  V  P  F  I  Y  S  F  Q  A  F  Y  L  V  S  H  P  N  480
GAAGTGTCTTGGCCAATGGCTTCTCTAATTATTGTTCTGAAACTTTGTGGTTATGTAATC 1500
 E  V  S  W  P  M  A  S  L  I  I  V  L  K  L  C  G  Y  V  I  500
TCCGAGGTGCAAATTCTCAGAAAAATGCATTCCGGAAAAATCCCAGTGATCCAAAGCTT  1560
 F  R  G  A  N  S  Q  K  N  A  F  R  K  N  P  S  D  P  K  L  520
GCACATTTAAAAACCATTCATACTTCAACGGGAAAAAATCTTCTAGTTTCTGGATGGTGG 1620
 A  H  L  K  T  I  H  T  S  T  G  K  N  L  L  V  S  G  W  W   54
```

(contunied)

```
GGCTTTGTTCGCCACCCCAATTACTTGGGTGATCTCATCATGGCCTTGGCGTGGTCCCTC  1680
 G  F  V  R  H  P  N  Y  L  G  D  L  I  M  A  L  A  W  S  L   560
CCATGTGGTTTTAACCACATTCTGCCTTATTTCTACATAATTTATTTCACCATGTTGCTT  1740
 P  C  G  F  N  H  I  L  P  Y  F  Y  I  I  Y  F  T  M  L  L   580
GTCCACCGAGAAGCTCGTGACGAGTACCACTGTAAGAAGAAATACGGCGTGGCTTGGGAA  1800
 V  H  R  E  A  R  D  E  Y  H  C  K  K  K  Y  G  V  A  W  E   600
AAGTACTGTCAGCGTGTGCCCTACCGTATATTTCCATACATCTACTAATGCTCTTCTGGC  1860
 K  Y  C  Q  R  V  P  Y  R  I  F  P  Y  I  Y  ***             615
TTTCTACAAAATACTCCTGCAATTCCAGCTGCCATTTGCAAAAACAGGAAAAAAATCCGA  1920
AACTTTCTTTTGTTGCACTGACAGGGTCTGTACTTTTTTTTTTCTTTTTGAGTCAGGACT  1980
ATGGAGCCGAGTAGTTGATCTTTTAATATAGCCGTGTTTACTTGTATTAACTTACAGTTA  2040
ACATAGGAAAAATACAAGTAAGGATGTGAGAATTTGCATTTTAATGGGAAATTTTCAACC  2100
CTTAATCTGAAAACAGAAGACAGTCTTAATATAAATGTACTGTGAAGAATGCTATTGATG  2160
TTTATGGTTTCTGATTACTTTTCAAATTTTGATGTTTTTTTGCCAGTTGGCTTTTCTTAA  2220
ATGAAAACACTGTTCCATTTAAAGTACATTTATGTTTTATTCAGTAAGAGAATAGAATTT  2280
TCATTTGTTTTTCTTTAAATCCTTTACTAATTATATAATTTGAAAGCAAAAAGAAGGGCC  2340
TATATTAAATGCTGAAAGTGAAAAGTGATGACATTATTAGCAGACACTGCTTAAAGGAGA  2400
CCATTTGTAGCAGTTGGCTTAACCTCAACTTCTAAAACTACATTGAAAATGTAAATACAT  2460
AGCTTAGTTTTTTGTAATATATGGTGACTTCAGATTTTTTTGTACAGTATTTTGAATGTG  2520
AGATGATTGTCAGGACTAACTGTCTTTTTAACAAAACATTTTCAGTATTTTA[AATAAA]AT 2580
TTTGTAAAGTAATGTGAATTAAAAATTTTGGAACAATTAGAATTCATTCACTATTGTATA  2640
GAAGATGCTGTTAAAACATAGGAAGGGTATTTTTCTTGATCCAAAGTTTGTGAATTTGGC  2700
TTTGCTACCTCAATTGCAGGTGTTTGTTTGCCTTTATAAACTGTTGCAAATAGAAAAAAA  2760
ATAGAATAAGTATATATTTTTGGAGTAACATCAATATTTAAACATTTTTACACAGATCGG  2820
TGTTTGAAAATTTGCCATTTCAGGCTAATATTTTTATATATTTTTGACTTTTTAAAAGTT  2880
CATCAGTGTTTTTGCTACTGTTAAGCTTATGCAGTTTATACTGTATTTTTTATGTATCCT  2940
TTATATTTACCAAACCTGACTCCCTGTAAAGGAGTGCTGTCTTAAAAACAACTGAAGGGG  3000
TTAAAGTCGTTTCTTTTAGTTTAATAGATGTGCATAAGGTAGCTTTAGCAATTAAATTCT  3060
AGTGAAGTTGATATAGTCTCATTTTTAATTGTCCTGTAATGGAACAGTAGCAAATTCACT  3120
AAACTTTTGTTTCAGAGTTAAATTGTTCTCAGTACTTTCAATGTAGGGGAATGT[AATAAA] 3180
CATAGTGTGTATGTTTGGGTTTTAATTACACATTTTATATATGAGCCATTTAGATATGCA  3240
GTGTTAATTCTATACTGCATTTGAAGTGTATGTAACTTAGCTTATGTTAATGCAGTCATG  3300
AAGTTGGTTTGCTCCAGCATCCGGTAGTCTTTAAACATTCTTTTAGTGAAATTGTCATTG  3360
TTTTATCAGTGCTAATGTGTGCAAGCAGTTTTTTTATTTTGCTTTTCTCCTGGCATCAGA  3420
AAGTGGTGGCGTTTTCTGTACTGGATTGCACCAAGGAAGCTTTTGGGGAGGAAGGAAGGA  3480
CATTAAATTCTTTCCCTGGTAATGAAAAGAGCCCTTTATCAATACAGTGCTGCAATTTCT  3540
GGATATCAGCTACACTTTGTTTTTAAGTTTGTTTTTGACATGTTTATTTGGCAAATTTTA  3600
TAATGAAGTTTTAAGTTGAA[AATAAA]ATGTAGCAACA                        3637
```

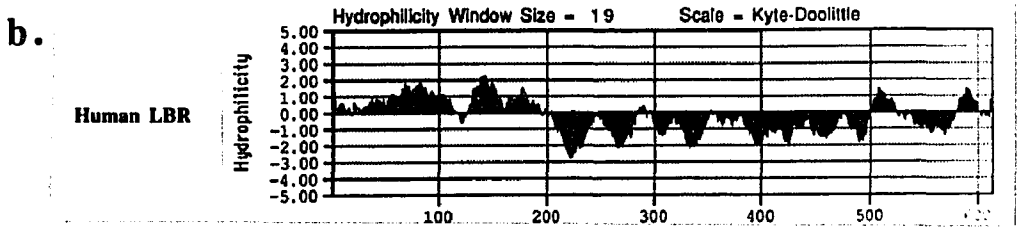


Fig. 3-1. DNA sequence, deduced amino acid sequence and hydropathy analysis of human LBR. **(a)** Nucleotide sequence of cDNA and deduced amino acid sequences of human LBR. The putative transmembrane domains are indicated by underlines below the amino acid sequences. Three sequences encoding AATAAA polyadenylation signals are shown in bold letters. Poly (A) tail was found 11 nucleotides downstream from the third AATAAA sequences. This sequence has been submitted to GenBank™/EMBL Data Bank with accession number L25931. **(b)** Kyte-Doolittle hydropathy plot of amino acid sequence of the human LBR. Analysis was performed using the MacVector program. The hydrophobic window was set at 19.

Human LBR has a 208 amino acid amino-terminal domain that is basic (pI 9.75), contains four Ser/Thr-Pro-X-X motifs (Ser^{71}, Ser^{86}, Thr^{118} and Thr^{200}) and a Ser-Arg-rich stretch (from amino acids Ser^{71} to Ala^{100}). Consensus sites for phosphorylation by $p34^{cdc2}$ kinase (Ser^{71}, Thr^{118}, Thr^{200}) and protein kinase A (Ser^{78}) are also present in the amino-terminal domain. Hydropathy analysis shows that there are eight hydrophobic segments in human LBR which may potentially function as transmembrane segments. All these features are conserved between chicken LBR and human LBR. Alignment of the amino acid sequences of human and chicken LBR is shown in Fig. 3-2 and a comparison of structural features of both proteins is summarized in Table 3-1.

Three yeast proteins, Sts1 of *Schizosaccharomyces pombe* (Shimanuki *et al.*, 1992), YGL022 (Chen *et al.*, 1991) and Erg24 (Lorenz and Parks, 1992) of *S. cerevisiae*, were found to have more than 25% sequence identity with the hydrophobic domain of LBR. These three yeast proteins do not contain amino-terminal domains homologous to those in human and chicken LBR are apparently not localized to the nuclear envelope. This finding and the genomic organization of the introns and exons of human LBR gene (Schuler *et al.*, 1994) suggest a hypothesis that the LBR gene may have evolved from an ancestral gene similar to these yeast genes by recombination of a gene for a soluble nuclear protein at the 5' end.

To examine the expression of LBR in human cells, full length QY-1 cDNA was used as probe to hybridize with poly(A+) mRNA isolated from human bladder carcinoma T24 cells, human B lymphocyte CESS cells and human T-lymphocyte NJBC-8 cells (Fig. 3-3). A single band of approximately 4.0 kb was detected in all three cell lines, indicating that human LBR is expressed in these cells. The RNA was of appropriate length to encode LBR.

```
hLBR   MPSRKFADGEVVRGRWPGSSLYYEVEILSHDSTSQLYTVKYKDGTELELKENDIKPLTSF   60
cLBR   --N--Y------M------V-----QVT-Y-DA-H----------A---S--RLQS--   60

                    71     78       86                          118
hLBR   RQRKGGSTSSSPSRRRGSRSRSRSRSPGRPPKSARRSASASHQADIKEARREVEVKLTPL  120
cLBR   K---SQ-S-------..----------A-GR---S-H-REHKEDKKKIIQ-TS-A-.   117
                71                84         95/96

hLBR   ILKPFGNSISRYNGEPEHIERNDAPHK.NTQEKFSLSQESSYIATQYSLRPRREEVKLKE  179
cLBR   .P--SE-NTR------DST----TSS-LLE-Q-LKPDV-MERVLD-----S----K-...   173

                              200                       TM 1
hLBR   IDSKEEKYVAKEL..AVRTFEVTPIRAKDLEFGGVPGVFLIMFGLPVFLFLLLLMCKQKD  237
cLBR   ...---I-AE-KIFE-IK-P-KPSSKT-E-----RF-T-ML--F--ATVLY-V-----D-   230
                         188
                                    TM 2
hLBR   PSLLNFPPPLPALYELWETRVFGVYLLWFLIQVLFYLLPIGKVVEGTPLIDGRRLKYRLN  297
cLBR   ---M--.------ES----K----F----FF-A---------L--SNP-K-Q--I-     289

               TM 3                          TM 4
hLBR   GFYPFILTSAVIGTSLFQGVEFHYVYSHFLQFALAATVFCVVLSVYLYMRSLKAPRNDLS  357
cLBR   ---A-L--A-A---L-YFQF-L--L-D--V---VS-AA-SMA--I---I------EE--A  349

                                              TM 5
hLBR   PAS.SGNAVYDFFIGRELNPRIGTFDLKYFCELRPGLIGWVVINLVMLLAEMKIQDRAVP  416
cLBR   -GGN--YL-----T-H-------S----------------A----------HNOSM-    409

            TM 6
hLBR   SLAMILVNSFQLLYVVDALWNEEALLTTMDIIHDGFGFMLAFGDLVWVPFIYSFQAFYLV  476
cLBR   --S----------------V------T-----------------V--L------       469

          TM 7
hLBR   SHPNEVSWPMASLIIVLKLCGYVIFRGANSQKNAFRKNPSDPKLAHLKTIHTSSGKNLLV  536
cLBR   G--IAI---V-AA-TI-NCI--Y---S------N--R--A----SY--V-P-AT--G---  529

                                        TM 8
hLBR   SGWWGFVRHPNYLGDLIMALAWSLPCGFNHILPYFYIIYFTMLLVHREARDEYHCKKKYG  596
cLBR   T--------------I--------------------V---IC----------H-------  589

hLBR   VAWEKYCQRVPYRIFPYIY                                           615
cLBR   L---R-------THISLHLLEHSTYLICKLKYTSHLCTWSVCYLGFKH              637
```

Fig. 3-2. Alignment of the amino acid sequences of human LBR and chicken LBR. The sequence of chicken LBR (cLBR) is vertically aligned below that of human LBR (hLBR). Amino acids are represented by single-letter codes. When an amino acid in chicken LBR is identical to the one in the corresponding position of human LBR, a dash is shown. Gaps in alignment are indicated by dots. The putative transmembrane segments (TM) are underlined. The position of the amino acids that are the consensus sites for protein kinases or DNA binding motifs (see Table 3-1) are also indicated by the numbers above (hLBR) or under (cLBR) the corresponding amino acid residue.

Table 3-1. Comparison of human LBR and chicken LBR

Structure Features	Human LBR	Chicken LBR
Amino-terminal domain		
Length (amino acids)	208	204
pI	9.75	9.89
Protein kinase A sites (Arg-Arg-X-Ser)	Ser^{78}	Ser^{95}, Ser^{96}
$p34^{cdc2}$ kinase sites (Ser/Thr-Pro-X-X)	Ser^{71}, Thr^{118}, Thr^{200}	Ser^{71}, Thr^{188}
Nucleic acid binding Motifs (Ser/Thr-Pro-X-X)	Ser^{71}, Ser^{86}, Thr^{118}, Thr^{200}	Ser^{71}, Ser^{84}, Thr^{190}
(Ser-Arg rich stretch)	Ser^{71} to Ala^{100}	Ser^{71} to Lys^{107}
Carboxyl-terminal domain		
Length (amino acids)	407	433
Putative transmembrane segments	Eight	Eight

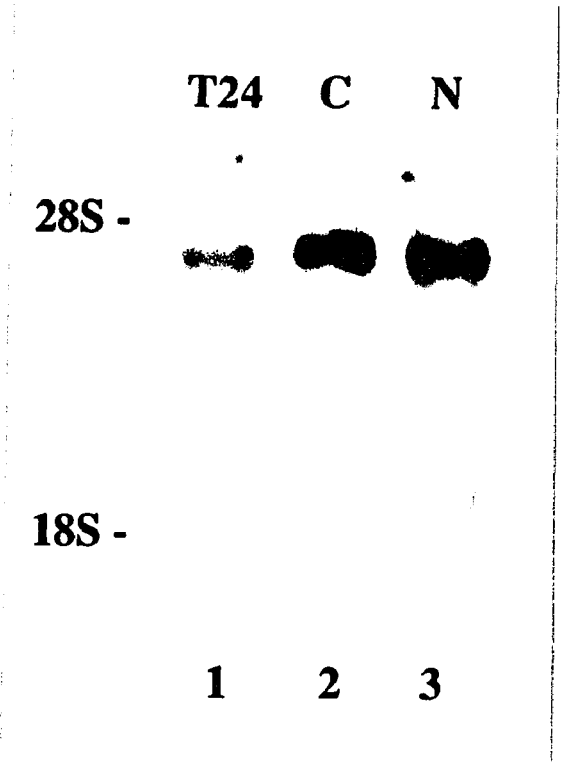


Fig. 3-3. Northern blot using ^{32}P-labeled human LBR cDNA probe QY1. RNA is from human bladder carcinoma T24 cells (lane 1), human B-lymphocyte CESS cells (lane 2), and human T-lymphocyte NJBC-8 cells (lane 3). Positions of the migration of rRNAs are indicated at left.

The Amino-Terminal Domain of Human LBR Is Recognized by Autoantibodies from Patients with PBC

Autoantibodies from patients with PBC that recognize purified amino-terminal domain of chicken LBR also recognize a mammalian nuclear envelope protein of similar size (Courvalin *et al.*, 1990). To determine whether these autoantibodies also recognize the protein encoded by the isolated human LBR cDNA, we expressed the amino-terminal domain of human LBR from Pro^2 to Phe^{216} as a GST fusion protein (gtLBR-AT) in *E. coli*. The purified gtLBR-AT has a molecular mass of 51 kDa estimated by SDS-polyacrylamide gel electrophoresis (Fig. 3-4, lane 2). Lower bands are the degradation polypeptides of gtLBR-AT. Sera from two patients with PBC previously shown to recognize chicken LBR (Courvalin *et al.*, 1990; Nickowitz *et al.*, 1994) were used in western blots to detect gtLBR-AT. Western blotting demonstrated that gtLBR-AT was recognized by these two sera (Fig. 3-4. lanes 3, 4), but not by a control serum (lane 5). The degradation products of gtLBR-AT were also recognized by these two autoantibodies. Thus the isolated cDNA QY-1 encodes the human homologue of chicken LBR identified in previous studies (Courvalin *et al.*, 1990; Chaudhary and Courvalin, 1993).

We further performed immunofluorescence microscopy to confirm that human LBR, encoded by QY-1 cDNA, is a nuclear envelope protein. When examined by immunofluorescence microscopy, serum from one patient with PBC (Nickowitz *et al.*, 1994) labeled both the mitochondria and the nuclear envelope of HepG2 cells and NJBC cells (Fig. 3-5, left panel). To determine if gtLBR-AT selectively reacted with the antibodies that recognize the nuclear envelope, antibodies in this sera were affinity purified against gtLBR-AT using previously described methods (Smith and Fisher, 1984). When used in immunofluorescence microscopy, the purified antibodies only labeled the nucleus of both cell types in a smoothy rim-like pattern. This result is consistent with the localization of LBR at the nuclear envelope (Fig. 3-5, right panel).

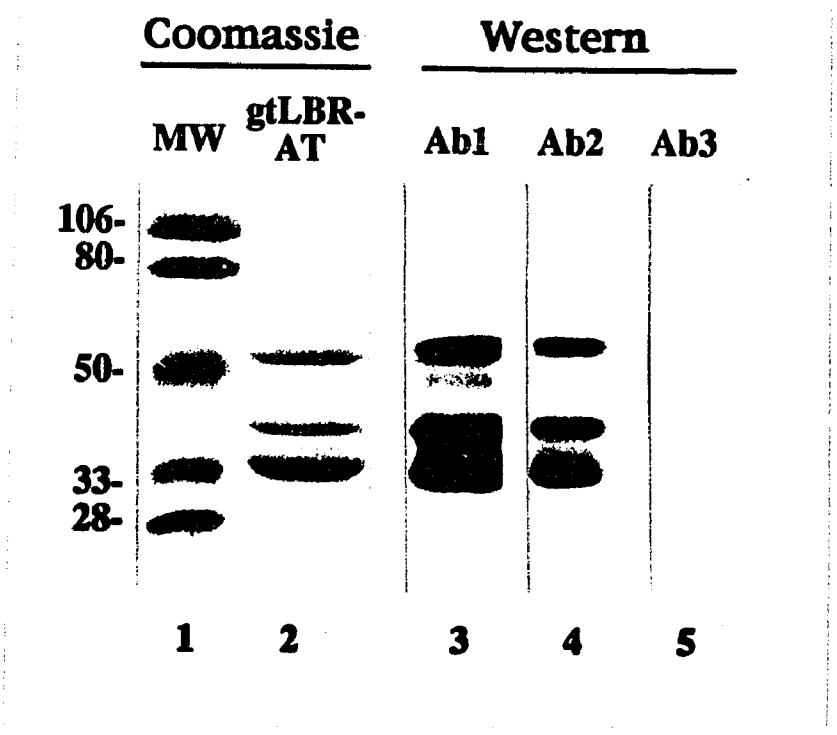


Fig. 3-4. Autoantibodies from two patients with PBC react with GST fusion protein containing amino-terminal domain of human LBR. At left is a Coomassie blue-stained polyacrylamide slab gel (Coomassie) showing purified GST-LBR fusion protein (Pro^2 to Phe^{216}) and molecular mass standards (indicated at left in kDa). The full-length fusion protein migrates with an apparent molecular mass of approximately 51 kDa. At right is an autoradiogram of the corresponding immunoblot (Western) using serum from two different patients with PBC (Ab1 and Ab2) with autoantibodies previously shown to recognize avian LBR and control serum from a normal individual (Ab3). In addition to full-length fusion protein, Ab1 and Ab2 also react with degradation products (lower two bands).

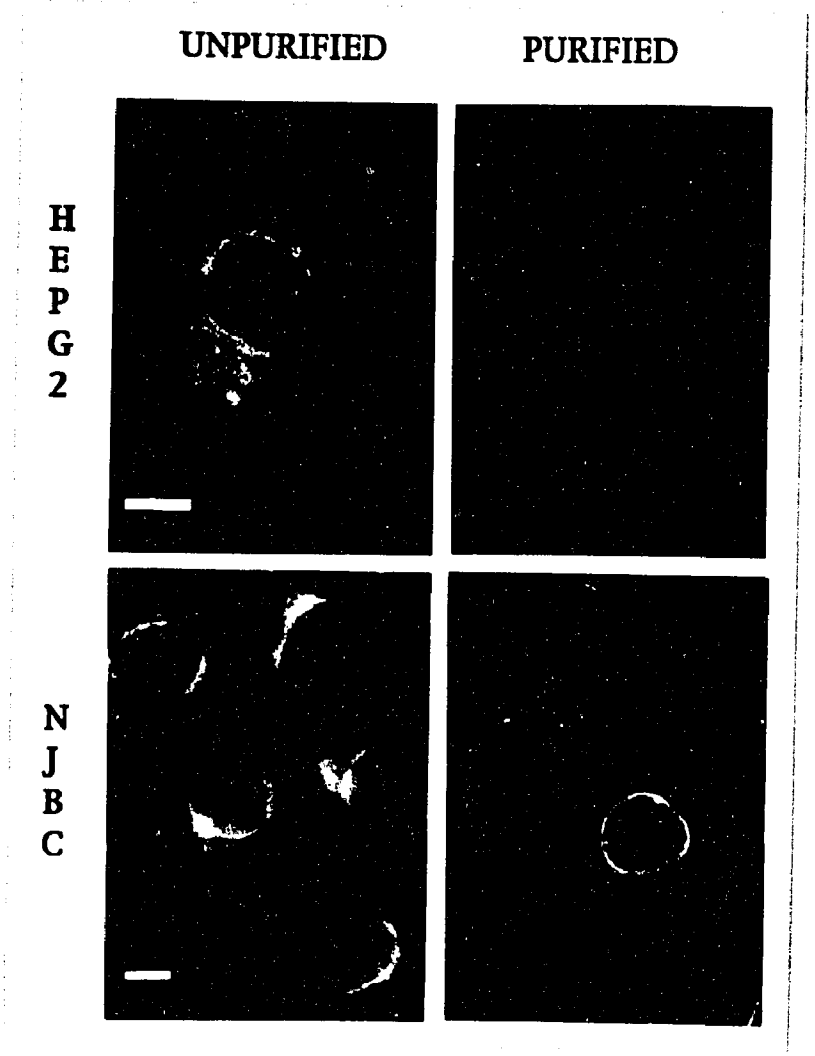


Fig. 3-5. Immunofluorescence micrographs showing nuclear envelope labeled by autoantibodies that recognize the amino-terminal domain of human LBR. At left are HepG2 cells (top panel) and NJBC-8 cells (bottom panel) labeled with whole serum (unpurified) from a patient with PBC that contains autoantibodies against both LBR and mitochondiral antigens. At right are HepG2 cells (top panel) and NJBC-8 cells (bottom panel) labeled with antibodies from the same serum used as at left that were affinity purified (purified) against gtLBR-AT. Bars indicate 5 μm.

The Amino-Terminal Domain of Human LBR Binds to B-type Lamins

Previous *in vitro* filter binding assays demonstrate that chicken LBR selectively interacts with B-type lamins (Worman *et al.*, 1988b). Most likely the amino-terminal domain of LBR is involved in the interaction with B-type lamins as this domain faces the nucleoplasm and the rest of this protein is composed primarily of transmembrane segments. To examine this possibility, we tested the interaction of the amino-terminal domain of human LBR with B-type lamins in a solution binding assay. Lamins were extracted with 8 M urea from nuclear envelope preparations of rat liver to give a solution enriched in lamins and trace amounts of other proteins according previous method (Worman *et al*, 1988a; 1988b). These lamin-enriched extracts were dialyzed against PBS to give a dilute solution of lamins. The diluted lamin solution was then incubated at room temperature for one hour with gtLBR-AT, or GST protein as control, attached to glutathione-Sepharose. The Sepharose and attached proteins were pelleted by centrifugation, washed three times with PBS and the proteins that remained bound were then eluted with SDS-sample buffer. To concentrate the proteins, the unbound and wash fractions were precipitated with 15% TCA. The unbound (F), wash (W) and bound (B) fractions were analyzed by western blotting using polycolonal anti-lamin B1 antibodies. A fraction of lamin B1 remained bound to the LBR fusion protein but not to the GST control protein attached to glutathione-Sepharose (Fig. 3-6). This result demonstrate that the amino-terminal domain of LBR can bind to B-type lamins *in vitro*. However, we cannot rule out the possibility that the transmembrane domains are involved in the B-type lamin binding *in vivo*, or if other minor components are involved in LBR's B-type lamin binding in this experiment.

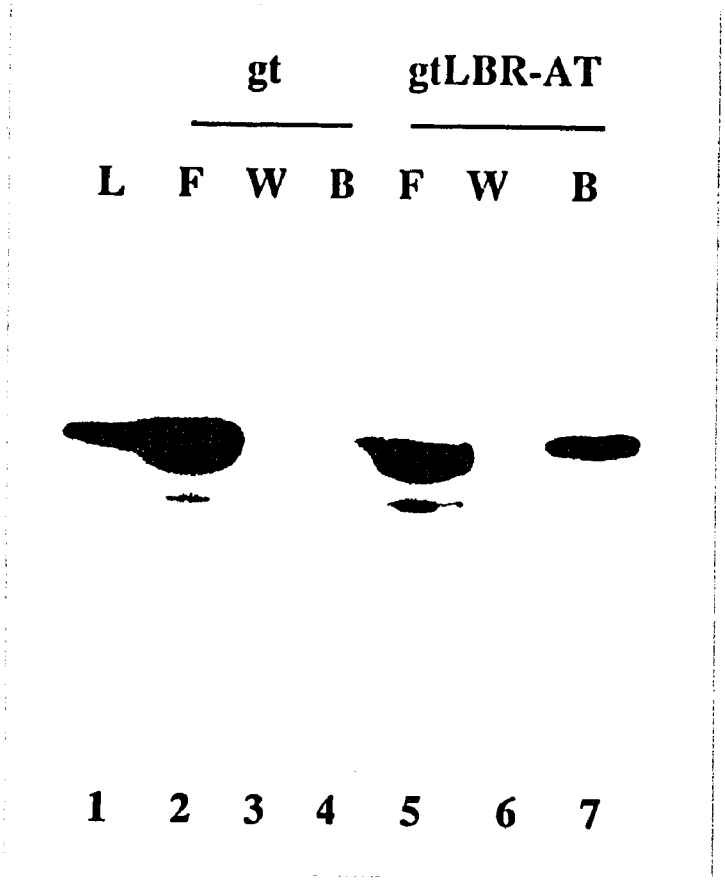


Fig. 3-6. LBR amino-terminal domain GST fusion protein binds to lamin B1. Autoradiogram of immunoblot showing bound and unbound lamin B1 when fractions were incubated with either GST (gt) or gtLBR-AT attached to glutathione-Sepharose beads. The blot was probed with anti-lamin B1 antibodies. Lane 1 (load) shows 10% of the load that was identical in each case. Lanes indicated by F (Flow through) contain proteins that did not bind to the beads. Lanes indicated by W (wash) contain proteins eluted from the beads by the PBS wash. Lanes indicated by B (bound) contain proteins eluted from the beads with SDS-sample buffer.

The Amino-Terminal Domain of Human LBR Binds to DNA

The amino-terminal domains of chicken and human LBR are basic and have several conserved Ser/Thr-Pro-X-X motifs and a Ser-Arg repeats. These features are common in many nucleic acid binding proteins (Suzuki, 1989). A chromatin or DNA binding function of LBR had been previously hypothesized (Worman *et al*, 1990; Chaudhary and Courvalin, 1993; Soullam and Worman, 1993). We therefore performed two different *in vitro* DNA binding assays to determine if the fusion protein gtLBR-AT can bind to DNA. DNA binding to the amino-terminal domain of LBR was first examined by a gel-shift assay utilizing double-stranded M13 DNA according to the methods described (Varshavsky, 1987; Wong *et al.*, 1992). GST fusion protein gtLBR-AT was incubated with DNA at 4°C for 30 minutes. The DNA-protein complexes was subjected to 1% agarose gel electrophoresis at a current of 20 mA for 3-4 hours. After electrophoresis, DNA was observed by ethium bromide staining. As shown in Fig. 3-7, increasing amounts of gtLBR-AT significantly retarded the migration of DNA while increasing amounts of GST control protein did not (Fig. 3-7, lanes 5-7). When the protein was denatured by heat treatment (Fig. 3-7, lane 8) or destroyed by proteinase K digestion (Fig. 3-7, lane 9), its ability to retard the migration of M13 double-stranded DNA was abolished. The inactivation by heat suggests that secondary and tertiary structures must be maintained for the amino-terminal of LBR to bind to DNA.

To further define the DNA binding within LBR's amino-terminal domain, three different GST fusion proteins were expressed. The first fusion protein contains the stretch of LBR from Pro^{2} to Ser^{71}, the second from Pro^{2} to Ala^{100} and the third from Ser^{97} to Phe^{216}. In the second DNA binding assay ("Southwestern" blotting), LBR amino-terminal fusion proteins of different lengths and control proteins were subjected to SDS-PAGE and transferred to nitrocellulose (Fig. 3-8). The nitrocellulose sheets were incubated with ^{32}P-labeled DNA. DNA was shown to associate with the LBR amino-terminal domain fusion protein and also with histone H1, a known DNA binding protein, but not with cytochrome

c, a basic control protein (pI 9.6). Of the three GST fusion proteins that contained regions of LBR from Pro^2 to Ser^{71}, Pro^2 to Ala^{100} and Ser^{97} to Phe ^{216}, only the fusion protein containing from Pro^2 to Ala^{100} associated with DNA (Fig. 3-8). This finding indicates that the stretch of LBR from amino acid Ser^{71} to amino acid Ala^{100} is necessary for DNA binding. This portion of LBR contains two Ser-Pro-X-X motifs and the Ser-Arg-rich stretch of the potential DNA binding motifs conserved in both human and avian LBR.

Thr^{200} of Human LBR Is a $p34^{cdc2}$ Protein Kinase Site *In Vitro*

Chicken LBR Thr^{188} is a cell cycle-dependent phosphorylation site for $p34^{cdc2}$ kinase (Courvalin *et al.*, 1992). The potential corresponding $p34^{cdc2}$ phosphorylation site in human LBR is Thr^{200}-Pro-Ile-Arg. To determine whether this site is actually a $p34^{cdc2}$ kinase phosphorylation site, an oligonucleotide was designed to amplify and express gtLBR-AT as a mutant protein in which Thr^{200} is replaced by an alanine that cannot be phosphorylated. The GST fusion proteins were phosphorylated by $p34^{cdc2}$ kinase *in vitro* and the labeled bands were excised and analyzed by two-dimensional phosphopeptide mapping as previously described (Courvalin *et al.*, 1992; *in vitro* phosphorylation and phosphopeptide mapping was performed by colleagues in Dr. J.-C. Courvalin's laboratory). The result is shown in Fig. 3-9. The wild type protein (gtLBR-AT^{T200}) has four major phosphopeptides while the mutant protein (gtLBR-AT^{A200}) lacks one of the phosphopeptides, likely due to the replacement of Thr^{200} with Ala^{200}. The same phosphopeptide was phosphorylated in mitosis in ^{32}P-labeled cells when examined by Immunoprecipitation (Dr. J.-C. Courvalin, personal communication). These results demonstrate that Thr^{200} of human LBR is a $p34^{cdc2}$ phosphorylation site, and like the corresponding site of chicken LBR, may be a cell cycle-dependent phosphorylation site. The cell cycle-dependent phosphorylation of LBR may regulate the affinity of LBR to B-type lamins and chromatin.

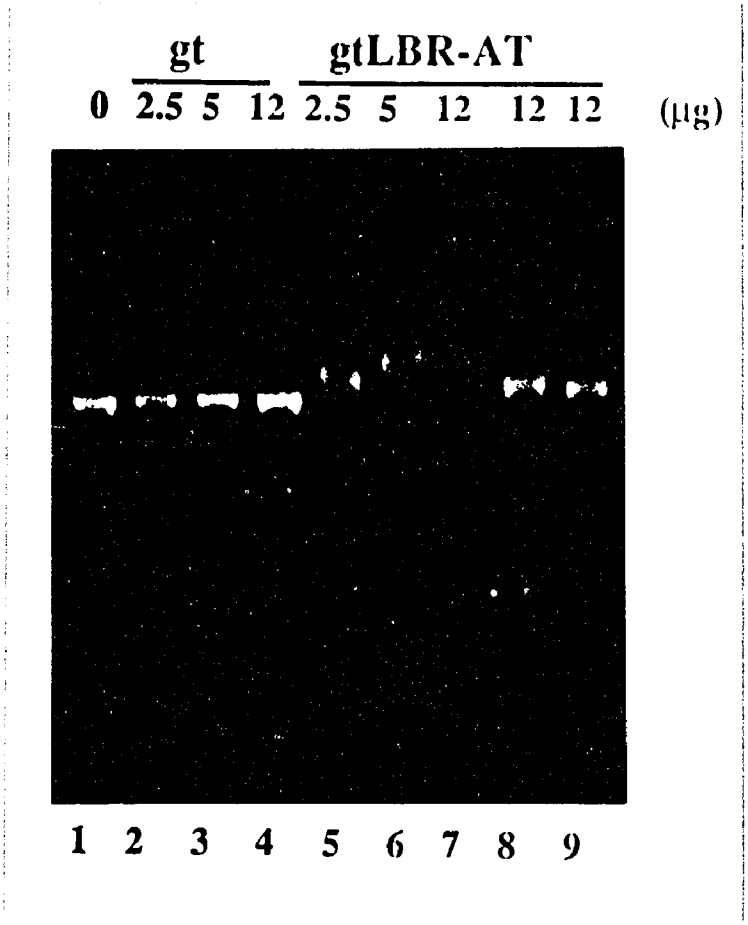


Fig. 3-7. Human LBR amino-terminal domain fusion protein retards migration of M13 double-stranded DNA subjected to electrophoresis in agarose gel. Photograph of ethidium bromide-stained agarose gel is shown. Lane 1 is control of DNA incubated with binding buffer. Lanes 2-4 show migration of DNA incubated with increasing amounts of GST (gt). Lanes 5-9 show migration of DNA incubated with increasing amounts gtLBR-AT. Lane 8 shows migration of DNA incubated with heat-denatured gtLBR-AT and lane 9 migration of DNA incubated with proteinase K-treated gtLBR-AT. Numbers above lanes 1-9 indicate micrograms of protein incubated with DNA

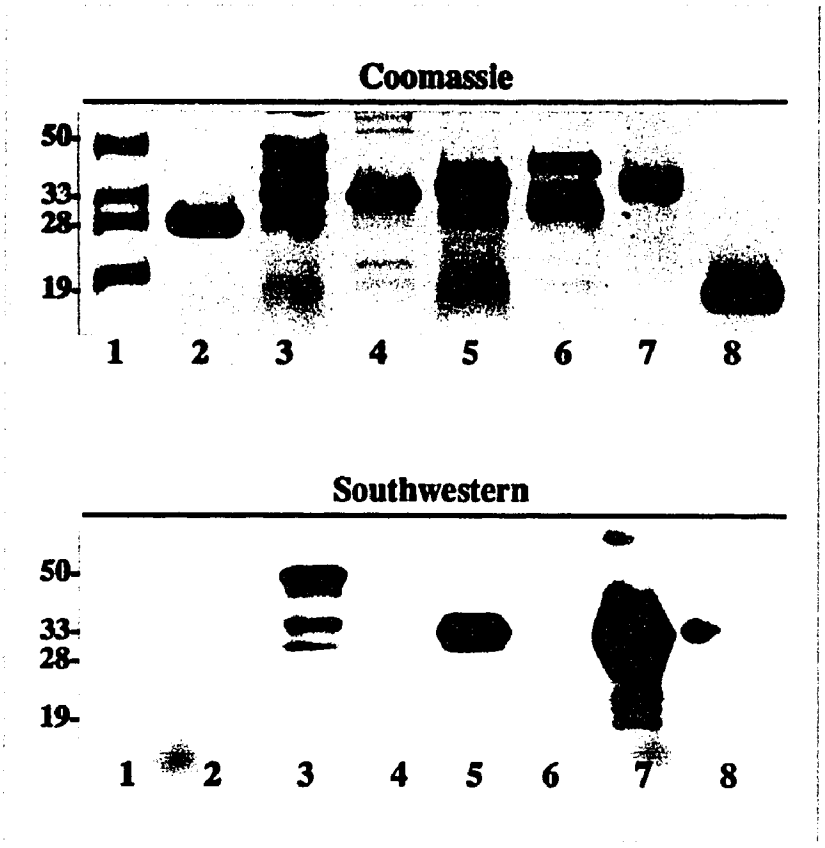


Fig. 3-8. ^{32}P-labeled bacteriophage lambda-DNA binds to the amino-terminal domain of human LBR immobilized on nitrocellulose. Coomassie blue-stained SDS-polyacrylamide slab gel (top) and matching "Southwestern" blot (bottom) probed with ^{32}P-labeled fragments of bacteriophage lamda DNA are shown. Lane 1 shows molecular mass standards (indicated at left in kDa). Lane 2 contains GST. Lane 3 contains GST-LBR fusion protein from Pro^{2} to Phe^{216} (gtLBR-AT). Lanes 4-6 contain LBR fusion proteins that contain amino acids from Pro^{2} to Ser^{71} (lane 4), Pro^{2} to Ala^{100} (lane 5), and Ser^{97} to Phe^{216} (lane 6) of LBR. Lane 7 contains histone H1, and lane 8 contains cytochrome c.

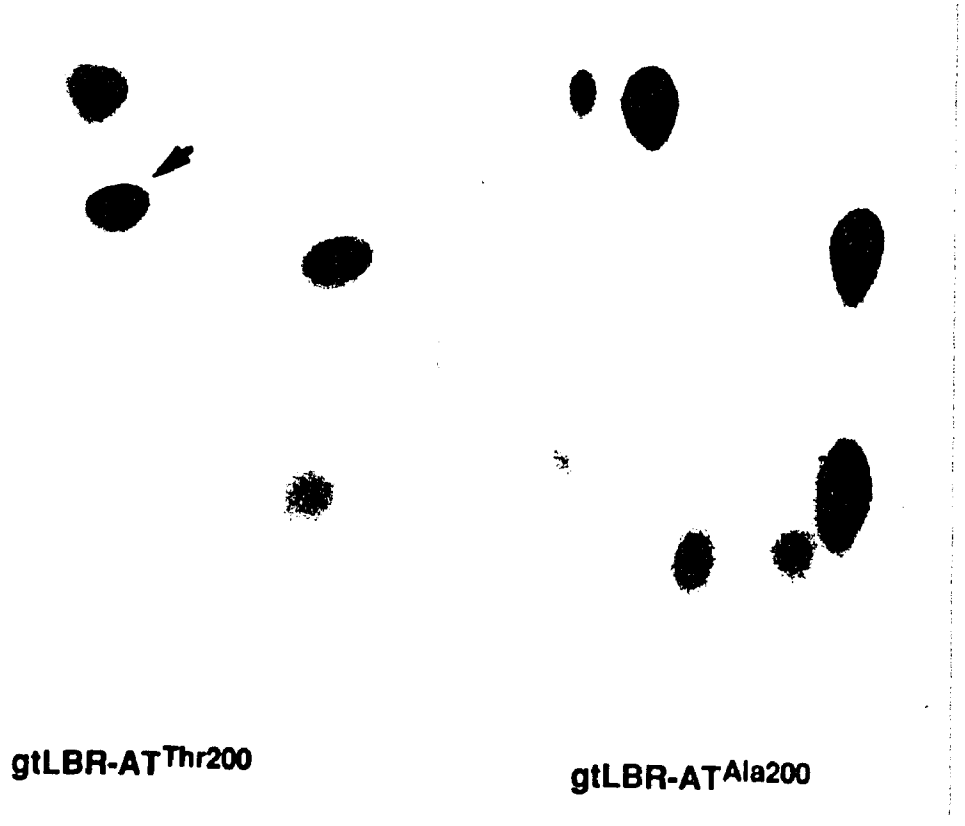


Fig. 3-9. Thr^{200} of the amino-terminal domain of human LBR is a phosphorylation site for p34^{cdc2}. Autoradiograms showing two-dimensional phosphopeptide maps of ^{32}P-labeled fusion proteins gtLBR-AT^{T200} (left panel) and gtLBR-AT^{A200} (right panel). Arrowhead indicates prominent ^{32}P-labeled phosphopeptides from gtLBR-AT^{T200} that is absent from fusion protein gtLBR-AT^{A200}. This same phosphopeptide is also phosphorylated during mitosis *in vivo* (Dr. J-C. Courvalin, personal communication). The *in vitro* phosphorylation and phosphopeptide mapping was performed by the laboratory of Dr. J-C. Courvalin.

Decreased Concentrations of Human LBR mRNA Induced by IL-6

When the expression of human LBR was examined on northern blots containing mRNA isolated from human cells treated with interleukin 6 (IL-6), we observed that human LBR mRNA was significantly reduced 2 hours after IL-6 treatment of NJBC cells and that no significant signal could be detected after 2 days of induction (Fig. 3-10). This observation suggests that the expression of LBR may be down-regulated by IL-6, although LBR at the nuclear envelope was not found to be reduced examined by immunofluorescence microscopy (data not shown). In fact, the 5' upstream region of the LBR gene contains several consensus sequences for binding of transcription factors including Sp1, Ap-2, and NF-kB (Schuler *et al.*, 1994), suggesting that the expression of LBR can be regulated by several common signaling pathways. While the biological implications of these findings are not yet known, it has been hypothesized that variations in the expression of proteins such as LBR, could be potentially alter nuclear morphology and genome organization in processes such as cell differentiation, development, and carcinogenesis (Schuler *et al.*, 1994). In light of this, it is possible that IL-6, a pleiotropic cytokine that is involved in inducing differentiation and regulating cell growth (Chen-Kiang *et al.*, 1993), regulates the expression of LBR to contribute to the remodeling of the nuclear envelope and the genome.

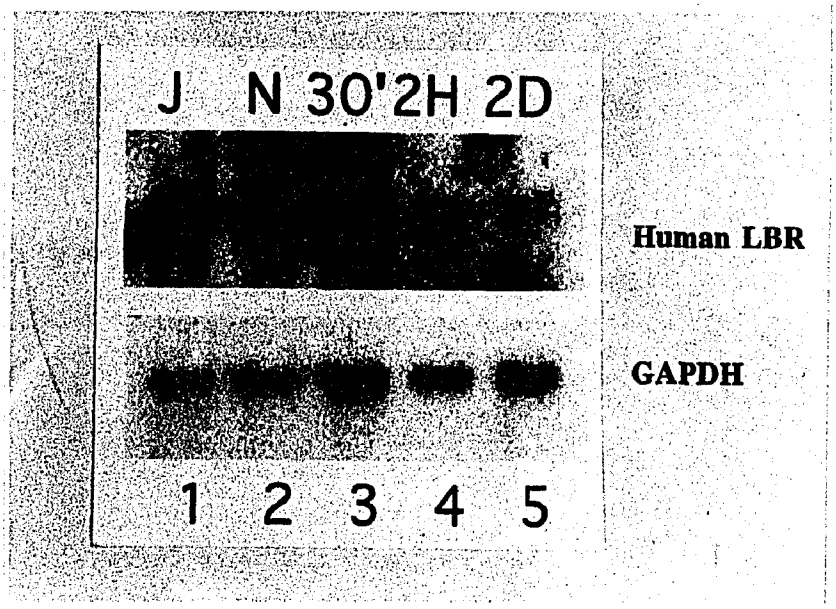


Fig. 3-10. Decreased concentrations of human LBR mRNA induced by IL-6. Northern blot analysis of poly(A+) mRNA isolated from human T-lymphocyte NJBC-8 cells treated with IL-6 using QY-1 cDNA as a probe (upper panel). The lower panel is the signals of GAPDH as reference of RNA loading of the same blot. Cell lines and the time course of IL-6 induction are shown at the top. RNA is from Jurket cells without IL-6 induction (lane 1), NJBC-8 cells without IL-6 treatment (lane 2) and with IL-6 treatment by 30 minutes (lane 3), 2 hours (lane 4) and 2 days (lane 5). The northern blot is provided by Dr. Chen-Kiang's laboratory.

Discussion

Certain autoantibodies from patients with PBC that had previously been shown to recognize avian LBR also recognized a mammalian protein at the nuclear envelope, suggesting the existence of a mammalian analog of chicken LBR (Courvalin *et al.*, 1990; Chaudhary and Courvalin, 1993). The primary structure and ligand binding properties of this protein had not been known. We have now cloned the cDNA for human LBR and demonstrated that it is homologous to avian LBR. The nucleoplasmic amino-terminal domain of human LBR can bind to B-type lamins *in vitro*. This is consistent with the proposed function as a B-type lamin receptor that anchors the lamina to the inner nuclear membrane in interphase (Worman *et al.*, 1988b). We also demonstrated that the amino-terminal domain of LBR can bind to DNA *in vitro*, providing evidence for the previous hypothesis that LBR may have a chromatin or DNA binding function (Worman *et al.*, 1990; Chaudhary and Courvalin, 1993; Soullam and Worman, 1993).

The DNA binding activity of LBR can be involved in several cellular functions. First, DNA binding, as well as B-type lamin binding, can contribute to the targeting and retention of the protein in the inner nuclear membrane after synthesis. This hypothesis has been supported by previous observations that the amino-terminal domain of LBR can target a type II integral membrane protein to accumulate at the nuclear envelope (Soullam and Worman, 1993). It has recently been proposed that multiple factors, including ligand binding and secondary and tertiary structures, are responsible for targeting integral membrane proteins like LBR to the inner nuclear membrane (Soullam and Worman, 1995).

Second, the association of DNA with LBR can be involved in attaching chromatin to the inner nuclear membrane in interphase. Heterochromatin is located adjacent to the inner nuclear membrane and the nuclear lamina in most cells (Fawcett, 1966). High resolution digital subtraction immunofluorescence microscopy has demonstrated that the lamina is a discontinuous structure covering only a portion of the nuclear envelope (Paddy

et al., 1990). Therefore, it is possible that chromatin may directly associate with the inner nuclear membrane at the points of the nuclear periphery where there is no lamina. Hence, in addition to functioning as a B-type lamin attachment site, LBR may also function in the attachment of chromatin to the inner nuclear membrane by binding with DNA or other chromatin proteins (see chapter 5).

It is also possible that the DNA binding activity of LBR is responsible for targeting mitotic membrane vesicles to decondensing chromosome at the end of mitosis. In *Xenopus* oocytes, a trypsin-sensitive integral membrane protein(s) is responsible for targeting mitotic membrane vesicles to chromatin to initiate nuclear envelope reassembly *in vitro* (Wilson and Newport, 1988). In mammalian cells, LBR-containing membranes are the first population of mitotic membranes to be targeted to decondensing chromosomes in anaphase and telophase (Chaudhary and Courvalin, 1993). The DNA binding activity of LBR can be responsible for the targeting of LBR-containing membranes to chromosomes during mitosis.

LBR is highly conserved between human and chicken. In the amino-terminal domain, the potential consensus sites for protein kinase A and $p34^{cdc2}$ protein kinase are conserved. The DNA binding motifs are also conserved. These findings suggest that these sequences may be important for LBR's functions. We have demonstrated that the conserved Ser-Arg rich stretch of LBR from amino acids 71-100 in human LBR is essential for its DNA binding ability. It is very likely that the same stretch in chicken LBR is also able to bind to DNA. When the GST fusion proteins of the amino-terminal domain of LBR (Pro^2 to Ser^{71}, Pro^2 to Ala^{100} and Ser^{97} to Phe^{216}) were used to perform *in vitro* lamin B binding assays, none of them associated with lamin B, suggesting that structural features of the entire amino-terminal domain may be necessary for the interaction of LBR with lamin B. The hydrophobic carboxyl-domain is also highly conserved between human and chicken LBR. However, whether these putative transmembrane segments have functions other than spanning at the nuclear membrane is not known. Other characterized integral

proteins of the nuclear pore membrane and inner nuclear membrane have only one or two transmembrane segments (Padan *et al.*, 1990; Furaukawa *et al.*, 1995; Martin *et al.*, 1995). It seems unlikely that LBR's eight transmembrane domains only contribute to the stabilization of the protein at the nuclear membrane. The first transmembrane segment of chicken LBR may serve as an inner membrane targeting signal for LBR (Smith and Blobel, 1993, Soullam and Worman, 1995). It is also possible that certain regions of the hydrophobic carboxyl-domain of LBR contribute to the association with B-type lamins at the nuclear membrane in cells. In addition, mutants of the yeast homologous gene *Sts1* are supersensitive to the protein kinase inhibitor stauroporine and also to divalent cations and other drugs (Shimanuki *et al.*, 1992). The homology between the Sts1 protein and LBR in transmembrane segments suggests the possibility that LBR's carboxyl-terminal domain may be involved in the transport of ions or organic compounds between the nuclear and perinuclear space.

Thr^{188} of chicken LBR is a cell cycle-dependent phosphorylation site for $p34^{cdc2}$ kinase (Courvalin *et al.*, 1992). The corresponding site (Thr^{200}) in human LBR is also a phosphorylation site for $p34^{cdc2}$ *in vitro* and *in vivo*. Phosphorylation of $Thr^{188/200}$ during mitosis might modify the structure of the amino-terminal domain and change its affinity to its binding ligands including B-type lamins, DNA and heterochromatin components (see chapter 5). In reverse, dephosphorylation of $Thr^{188/200}$ in interphase may increases the affinity of the amino-terminal domain of LBR to its ligands. Dephosphorylation of serine residues was shown to reduce LBR's binding to B-type lamins (Appelbaum, 1990). At least one serine residue that is phosphorylated in interphase is not phosphorylated in mitosis (Courvalin *et al.*, 1992). Thus it is likely that binding affinity of LBR to its ligands are regulated by a combinatorial mechanism of phosphorylation and dephosphorylation.

Acknowledgments

We thank Dr. Selina Chen-Kiang for providing northern blots and human cell lines, Dr. Jean-Claude Courvalin for analysis of the *in vitro* phosphorylation site on human LBR, Dr. Ekkehard Schler for help in DNA sequencing, and Xiaokui Zhang for help in preparing figures.

Chapter 4. Protein-Protein Interactions between Human Nuclear Lamins Expressed in Yeast

Abstract

The nuclear lamina is a meshwork of intermediate filaments that underlies the inner nuclear membrane. Lamins are the protein components of the nuclear lamina and belong to the intermediate filament protein family. An intermediate filament protein has three domains including the head domain, the rod domain and the tail domain. The rod domain can further be divided into three coiled-coil subdomains called coil 1a, coil 1b and coil 2 respectively. The nuclear lamina is assembled via protein-protein interactions between the nuclear lamins. We have used the yeast two-hybrid system to examine the interactions between the predominant human nuclear lamins including lamin A, lamin B1, lamin C and lamin A precursor prelamin A. All of these four lamin proteins were able to form homodimers as well as heterodimers when expressed as GAL4 fusion proteins in *Saccharomyces cerevisiae*. Analysis of the different structural domains of lamin B1 demonstrated that the head and tail domains are not involved in the formation of homodimers, all of the subdomains of the rod contribute to the formation of homodimerization, and the second half of coil 2 of the rod domain was necessary for the formation of the most stable homodimers. These results suggest that the lamin filaments can be homotypic as well as heterotypic. The results also demonstrate that the yeast two-hybrid system can be used to study the interactions between structural proteins and their domain.

Introduction

The nuclear lamina is a filamentous meshwork of 10 nm-diameter intermediate filaments (IF) that underlies the inner nuclear membrane (Aebi *et al.*, 1986). The cloning of cDNAs for human lamins A and C first clearly demonstrated that they are members of IF protein family (Fisher *et al.*, 1986; McKeon *et al.*, 1986). IF proteins can self assemble into complex 10 nm-diameter filaments. According to their primary structure or tissue-specific expression, the IF protein family is classified into six types. Nuclear lamins belong to type V; types I-IV and VI are various cytoplasmic IF proteins (Albers and Fuchs, 1992).

All IF proteins consist of three domains: a non-helical "head" domain at the amino-terminus, a non-helical "tail" domain at the carboxyl-terminus and a α-helical central rod domain in the middle. The central rod domain can be further divided into three coiled-coil regions termed coil 1a, coil 1b, and coil 2 that are separated by short linker sequences. The central rod domain is highly conserved among IF proteins. In contrast, the primary structures of the head and tail domains can vary significantly. The central rod domain contains hydrophobic amino acid residues that are arranged in a repeat pattern which creates a hydrophobic environment favoring protein-protein interactions (Albers and Fuchs, 1992; Stewart, 1993). The 10-nm diameter IFs are assembled through protein-protein interactions among IF proteins. The first level of organization is the formation of a dimer through interactions between two α-helical rod domains. IF dimers can either be homodimers or heterodimers. Basic and acidic keratins, the type I and II IF proteins, can form both homodimers and heterodimers *in vitro* (Coulombe and Fuchs, 1990), but only the heterodimers are involved in filament formation *in vivo* (Hatzteld and Weber, 1990). The type III IF proteins, such as vimentin, desmin, peripherin, and glial acidic fibrillary protein, normally form as homodimers. IF dimers further interact to form higher-ordered polymers in different fashions and that ultimately assemble into 10 nm-diameter filaments (Albers and Fuchs, 1992).

Lamins are different from cytoplasmic IF proteins in that they contain a nuclear localization sequences in their carboxyl-terminal tail domains and an extra 42 amino acids stretch in coil 1b (Dodemont *et al.*, 1990). The nuclear lamina of most terminally differentiated mammalian cells is comprised of lamins A, C, B1 and B2 (Nigg, 1992). Vertebrate lamins are designated as A- or B-type lamins according to their biochemical properties and primary structure. A-type lamins are neutral and B-type lamins have a weak acidic charge (Gerace and Blobel, 1980). While B-type lamins are constitutively expressed, lamins A and C are not present in many undifferentiated cells including some human cancers (Nigg, 1992). Mammalian cells potentially express three different isoforms of B-type lamins (Höger *et al.*, 1988; 1990; Pollard *et al.*, 1990; Furukawa and Hotta, 1993). Lamin B1 is the predominant B-type lamin isoform present in mammalian somatic cells (Höger *et al.*, 1988; 1990; Pollard *et al.*, 1990) and in humans it is expressed from a gene (Lin and Worman, 1995) on chromosome 5 (Wydner *et al.*, 1996). Lamin B2 arises from another gene and is expressed in most somatic cells in minor amounts (Vorburger *et al.*, 1989, Weber *et al.*, 1990). The third mammalian B-type lamin isoform, termed lamin B3, is expressed only in spermatocytes and may arise from the lamin B2 gene by alternative splicing (Furukawa and Hotta, 1993). Human lamins A and C are identical in the first 566 amino acids but differ in the carboxyl termini (Fisher *et al.*, 1986; McKeon *et al.*, 1986). Genomic cloning of genes for human lamins A and C has demonstrated that they are encoded by the same gene which in human is on chromosome 1 (Wydner *et al.*, 1996) and arise by alternative splicing (Lin *et al.*, 1993). A germ cell-specific lamin that is identical to lamin C but with a different amino-terminal domain may also arise from this gene (Furukawa *et al.*, 1994). Mature lamin A is derived from a precursor called prelamin A by the removal of its last 18 amino acids by an unknown proteolytic processing reaction (Weber *et al.*, 1989; Beck *et al.*, 1990). Both A-type lamins (except lamin C) and B-type lamins have conserved CaaX signal sequences at their carboxyl-termini for farnesylation. While the farnesyl modification on B-type lamins is permanent, the prenyl-group is present

only on prelamin A but not on mature lamin A (Nigg, 1992). The farnesylation modification has been shown to be required for targeting newly synthesized lamins to the nuclear membrane (Holtz *et al.*, 1989; Krohne *et al.*, 1989; Kitten and Nigg, 1991).

Because various combinations of lamins are expressed in different mammalian cells during different stages of development, it is possible that lamins, like cytoplasmic intermediate filament proteins, can be heteropolymeric or homopolymeric. The demonstration that intranuclear complexes associated with sites of DNA replication contain only B-type lamins but not lamins A and C further suggests that different associations between various lamin proteins with functional implications can occur within the same nucleus (Moir *et al.*, 1991). Despite these observations, little is known about the first order interactions between the different human nuclear lamins. It is also not known if prelamin A can interact with itself or with other lamin proteins. In addition, the roles of individual lamin domains in the formation of dimers have not been well defined. For these reasons, we have used the yeast two-hybrid system, which is designed to study interactions between two proteins in a cellular environment, to examine protein-protein interactions between the major human nuclear lamins and their domains.

Results

Homodimerization and Heterodimerization of the Major Human Nuclear Lamins

We used the yeast two-hybrid system (Fields and Song, 1989) to detect protein-protein interactions between the major human nuclear lamins expressed in yeast. In the two-hybrid system, the DNA binding domain of yeast transcription factor GAL4 is expressed from one plasmid (pGBT9) and the transcription activation domain from another (pGAD424). Constructs derived from these two plasmids are used to cotransform specific yeast strains in which the GAL4 gene has been deleted and contain a *lacZ* fusion gene that can be activated by a GAL4 binding site. Neither the DNA binding domain nor the activation domain alone can activate transcription. Transcription from the *lacZ* gene can be activated when the DNA binding domain and the activation domain come together as a result of an interaction between polypeptides fused to them. Activity of the *lacZ* gene can be detected by measuring β-galactosidase activity in the transformed yeast using qualitative filter assays or quantitative liquid assays. A positive β-galactosidase activity generally indicates an effective protein-protein interaction while a negative one suggests no interaction. To a certain degree, the activities of β-galactosidase also approximate the strength of the protein-protein interactions (Fields and Song, 1989).

The cDNAs encoding human prelamin A, lamin A, lamin B1, lamin C were cloned into the two-hybrid system plasmids pGBT9 and pGAD424 as in frame fusions with the yeast GAL4 DNA binding domain and activation domain respectively. The cDNA for a portion of neurofilament protein NF-L, a type IV IF protein, was constructed into both pGBT9 and pGAD424 for use as controls (Fig. 4-1A). All combinations of these constructs were used to cotransform yeast strain SFY526, and the transformants were for β-galactosidase activity by filter assays (Fig. 4-1B). The β-galactosidase activity was positive for all combinations of lamin fusion proteins and negative for all combinations of

transformation of lamin fusion proteins with NFL^{23-349} (partial NF-L protein, includes amino acids 23 to 349) fusion proteins. The cotransformants of pGBT9-NF-L^{23-349} and pGAD424-NF-L^{23-349} were detected as β-galactosidase positive, indicating that this fragment of NF-L can form a homodimer with itself. These results demonstrated that prelamin A, lamin A, lamin B1 and lamin C can form homodimers and heterodimers in all combinations.

The β-galactosidase activities of yeast transformants were also measured by the "liquid method" which semi-quantitatively reflects the strength of protein-protein interactions (Fields and Song, 1989). The β–galactosidase activities measured ranged from 2 units to about 17 units (Table 4-1). The highest β-galactosidase activity detected was for the cotransformants of pGBT9-PreA and pGAD424-LmB (16.8 ± 4.2 units) and the lowest was of pGBT9-LmC and pGAD424-PreA. Generally higher β-galactosidase activities were detected in co-transformants with pGAD424-LmB. The reason for the higher activities when lamin B1 was expressed in this context is unclear. No significant differences of β-galactosidase activities were observed among all other cotransformation combinations. Cotransfromation of the pair of plasmids expressing GAL4-NF-L^{23-349} fusions gave an enzyme activity of 2.8 units, which is as high as several heterotypic lamin-lamin interactions including PreA-LmA, LmB-PreA, LmB-LmC and LmC-PreA. The enzyme activities measured in the cotransformants of NF-L with all the lamins were the same as the negative control (cotransformants of pGBT9 and pGAD424), demonstrating that NF-L does not form heterodimers with lamins. Transformation with any of the GAL4-lamin fusion proteins alone did not give β-galactosidase activity above control (data not shown), indicating that lamins do not bind the GAL4 binding sequence in DNA or nonspecifically activate transcription.

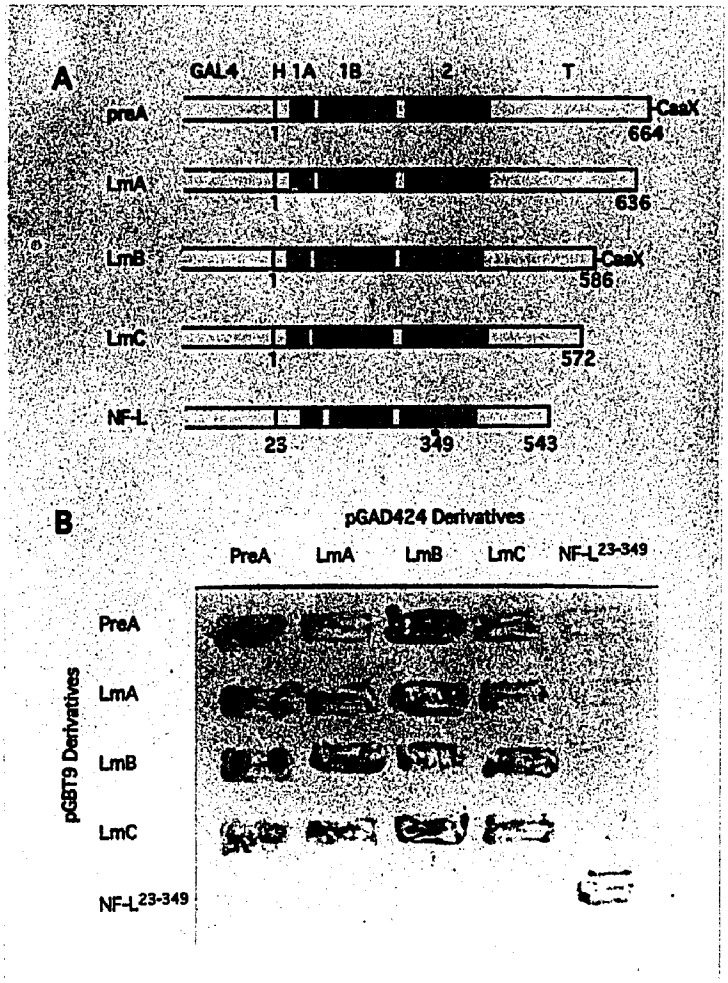


Fig. 4-1. Interactions between human nuclear lamins expressed in yeast detected using the yeast two-hybrid assay. **(A)** Schematic diagrams showing pre-lamin A (PreA), lamin A (LmA), lamin B1 (LmB), lamin C (LmC) and neurofilament filament protein NF-L fusion proteins. At left of each diagrammed polypeptide is the GAL4 fusion domain. The amino acid numbers of the proteins are indicated below each diagram. Coil 1a (left), coil 1b (middle), and coil 2 (right) are shaded in each diagram. CaaX indicates isoprenylation consensus sequences at the carboxyl termini of prelamin A and lamin B1. "*" indicates the position of amino acid 349 of NF-L. **(B)** Detection of β-galactosidase activity by a colorimetric filter assay in yeast cotransformed with the indicated plasmids. pGAD424 derivatives express the activation domain of GAL4 fused to the indicated lamin. pGAD424 derivatives (vertical column) and pGBT9 derivatives (horizontal row) are as indicated. The transformed yeast cultures were plated on solid media and transferred to filters as described in Materials and Methods. Yeast in which positive protein-protein interactions occurred are blue (gray in the black and white photo) as a result of the induction of β-galactosidase activity. White colonies are those in which β-galactosidase activity was not induced in the cotransformed yeast.

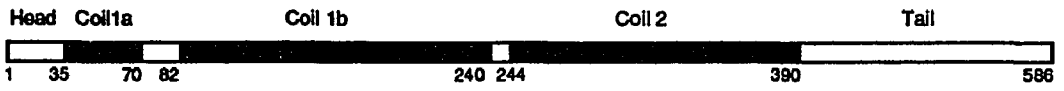
Table 4-1. β-Galactosidase activities in the two-hybrid assay for yeast cotransformed with plasmids that express GAL4 fusion proteins of human nuclear lamins

	pGAD424				
	Prelamin A	Lamin A	Lamin B	Lamin C	NF-L$^{23-349}$
pGBT9					
PreA	3.9 ± 1.1	2.3 ± 1.0	16.8 ± 4.2	5.1 ± 0.8	0.9 ± 0.1
LmA	4.9 ± 4.2	8.2 ± 1.6	6.3 ± 1.0	6.3 ± 1.1	0.9 ± 0.1
LmB	3.2 ± 0.1	3.5 ± 0.3	10.8 ± 0.1	2.8 ± 0.5	0.9 ± 0.1
LmC	1.9 ± 0.4	3.9 ± 0.5	10.8 ± 0.1	2.8 ± 0.5	1.0 ± 0.1
NF-L$^{23-349}$	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	2.8 ± 0.9

Note. pGAD424 derivatives (vertical columns) express the activation domain of GAL4 domain of GAL4 fused to the indicated lamin or NF-L. pGBT9 derivatives (horizontal rows) express the DNA binding domain of GAL4 fused to the indicated intermediate filament protein. Yeast were transformed with the pGAD424 derivatives and pGBT9 derivatives as indicated. Values are mean β–galactosidase activities in standard units (see Materials and Methods) ± standard error for n = 3 experiments. For yeast cotransformed with pGBT9 and pGAD424 (negative control), the activity was 1.0 ± 0.1)

Domains of Lamin B1 Involved in Homodimerization

To analyze the domains of lamin B1 involved in homodimerization, we cloned various cDNAs encoding domains of lamin B1 (Fig. 4-2, diagram on top) into both pGBT9 and pGAD424 vectors to be expressed as GAL4 fusion proteins. These plasmids were used to cotransform yeast SFY526 in pairs. The β-galactosidase activities of these transformants were tested by both filter and liquid assays (Fig. 4-2, table on bottom). The head domain (H), coil 1a (C1a), coil 1b (C1b), the head domain plus coil 1a (HC1a) and the tail domain (T) were negative, indicating that they were not able to form homodimers to induce β-galactosidase activity. Coil 2 alone (C1a), coil 2 plus the tail domain (C2T), and coil 1a plus coil 1b with or without the head domain (C1a1b or HC1a1b) all induced similar levels of β-galactosidase activity. Full-length lamin B1 and lamin B1 lacking the carboxyl-tail domain gave slightly higher levels of β-galactosidase activity than C1a, C2T, C1a1b and HC1a1b homodimerization. The highest measured level of β-galactosidase activity was obtained for the domain containing coil 1b and coil 2 of lamin B1, but deletion of the second half of coil 2 from this polypeptide abolished its ability to induce β-galactosidase activity in the transformed yeast. The second half of coil 2 therefore appears to be needed for the formation of the most stable lamin dimers. Coil 1b plus coil 2 of lamin B1 induced twice as much β-galactosidase activity as full-length lamin B1, suggesting that the head and tail domains of the protein may partially inhibit dimerization.



Lamin B Domain	Amino Acid	Beta-Gal Activity Liquid Assay	Filter Assay
H	1-38	1.3 ± 0.1	White
C1a	31-75	1.3 ± 0.1	White
C1b	78-243	1.2 ± 0.1	White
C2	241-394	3.6 ± 0.5	Blue
C2T	241-586	2.7 ± 0.8	Blue
C1a1b	31-243	3.3 ±1.0	Blue
C1b2	78-394	24.8 ±1.4	Blue
C1b2m	78-321	0.8 ± 0.1	White
HC1a	1-75	1.3 ± 0.1	White
HC1a1b	1-243	1.4 ± 0.1	Blue
HC1a1b2	1-394	7.2 ±1.9	Blue
T	390-586	1.2 ± 0.1	White
LmB	1-586	13.0 ± 4.5	Blue
pGBT9+pGAD424		1.2 ± 0.1	White
pCL1(Gal4)		118.0 ± 7.3	Blue

Fig. 4-2. Interactions between the various domains of human lamin B1 detected in the yeast two-hybrid assay. At top is a schematic diagram of lamin B1. The amino acid numbers of lamin B1 are indicated below the diagram. Coil 1a, coil 1b, and coil 2 are represented by black rectangles. Yeast's were cotransformed with pGBT9 and pGAD424 derivatives that respectively expressed the DNA binding domain and activation domain of GAL4 fused to the indicated domain of human lamin B1. The first and the last amino acid in lamin B1 is given for each domain. Also shown are results from experiments in which yeast were cotransformed with pGBT9 and pGAD424 (negative control) and plasmid pCL1 that expressed full-length GAL4 (positive control). The results of the quantitative liquid β-galactosidase activity assays (means ± standard errors for n = 3 experiments) are given for each combination as are the results of the colorimetric filter assays in which blue indicates a positive protein-protein interaction and white is negative. Abbreviations used: H, head domain; C1a, coil 1a; C1b, coil 1b; C2, coil 2; C2T, coil 2 plus tail domain; C1a1b, coil 1a plus coil 1b; C1b2, coil 1b plus coil 2; C1b2m, Coil 1b plus the first half of coil 2; HC1a, head domain plus coil 1a and 1b; HC1a1b, head domain plus coil 1a and 1b; HC1a1b2, the tail-less lamin B; T, tail domain; LmB, full-length lamin B1.

Discussion

We have demonstrated that human nuclear lamins can specifically form both homodimers and heterodimers using the yeast two-hybrid system. A functional nuclear lamina can be organized from solely homotypic B-type lamins since, in undifferentiated cells and some cancer lines, lamins A and C are not expressed (Guilly *et al.*, 1987; 1990; Lebel *et al.*, 1987; Stewart *et al.*, 1987; Worman *et al.*, 1988; Rober *et al*, 1989; Cance *et al.*, 1992). Normally, mammalian somatic cells express multiple lamins including lamin A, lamin C and two B-type lamin isoforms (Nigg 1992). Our results demonstrate that when lamins A, B1 and C are all expressed, the lamina can potentially be composed of heteropolymeric filaments containing various combinations of these proteins. However, we are not certain whether all these combinations of lamins are involved in the formation of higher-ordered lamin polymers in the cell, since in theory the yeast two-hybrid system detects the protein-protein interactions between two single polypeptides (Fields and Song, 1989). Even so, the different combinations of lamin homodimers or heterodimers can potentially be involved in forming individual lamin filaments or other substructures within the nucleus. For example, in some cells, there are intranuclear structures which appear to contain only B-type lamins, although the lamina is composed of lamins A, B, and C (Moir, 1994).

Homotypic and heterotypic interactions between mammalian lamins have been studied using *in vitro* binding assays (Krohne *et al.*, 1987; Georgatos *et al.*, 1988). *Xenopus* lamin L1(a B-type lamin) has been shown to bind strongly to mammalian lamins A and C in a blot binding assay. Binding to itself and B-type lamin was much weaker but mammalian lamin C could bind to itself and to lamins A, B-type lamin and L1 with the same affinities (Krohne *et al.*, 1987). Solid phase binding assays and column chromatography analysis showed that homotypic lamin A-A and C-C interactions were weaker than the heterotypic lamin A-B, and B-C interactions. The association of B-type lamin with itself was barely detected unless it was "superphosphorylated" *in vitro* by

protein kinase A (Georgatos *et al.*, 1988). However, bacterial-expressed chicken lamin B2 has been shown to be able to form intermediate filaments *in vitro* (Heitlinger *et al.*, 1991; 1992). In addition, lamin A alone, lamin C alone and mixtures of lamin A and lamin C can also assemble to form filaments or paracrystals (Aebi *et al.*, 1986; Ward and Kirschner, 1990; Gieffers and Krohne, 1991; Moir *et al.*, 1991; Heitlinger *et al.*, 1992). Our results in the yeast two-hybrid system are consistent with these previous *in vitro* reconstitution experiments and demonstrate that these lamin-lamin interactions can occur *in vivo*. The discrepancies among different *in vitro* binding assays may result from the differences in buffer conditions, protein concentrations, or post-translational modifications such as phosphorylation (Georgatos *et al.*, 1988).

In the yeast two-hybrid system, prelamin A can also form both homodimers and heterodimers suggesting that the 18 amino acids at the carboxyl-terminus of prelamin A do not function to interfere its interaction with itself or other lamins. In mammalian cells, the carboxyl-terminus of prelamin A is farnesylated and subsequently removed to form mature lamin A after proteolytic processing (Lutz *et al.*, 1992). Although we do not know if prelamin A is subjected to posttranslational modifications in yeast, isoprenylation is likely to occur since prenyltransferases and the consensus sequences for prenylation are conserved in yeast and mammals (Brown and Goldstein, 1993). However, it is unlikely that yeast have the protease that processes prelamin A to lamin A. The precise function of prelamin A in vertebrate cells is not known. It has been observed that mutant prelamin A protein that is resistant to the proteolytic processing cannot incorporate into the lamina in cells (Hennekes and Nigg, 1994). Our results suggest that this is probably not because prelamin A is not able to form dimers, or to associate with other type of lamins. It is more likely that unknown mechanism(s), for example, interactions of monomers or dimers of prelamin A with other proteins in the mammalian nucleus, are responsible for preventing the unprocessed protein from binding to other lamina or incorporating into the lamina.

We have shown that the second half of coil 2 appears to be important for the

formation of strong lamin B1 homodimers. Coil 2 of lamin B1 contains 145 amino acid residues. Coil 2 alone can form homodimers, and the β-galactosidase activity detected in the yeast two-hybrid assay is as strong as that of C1a1b, a longer coiled-coil polypeptide (210 amino acids). The cotransformants that express the 158 amino acid coil 1b have no detectable β-galactosidase activity, suggesting that it does not form homodimer. β-galactosidase activities of an order of magnitude greater were detected in the two-hybrid assay with a polypeptide containing both coil 1b and coil 2 (C1b2), but when the second half of coil 2 was deleted from this polypeptide (C1b2m), the ability to form homodimers was essentially abolished. It has been known that the homology between different IF proteins is particularly high at the start of coil 1a and near the end of coil 2. A consensus sequence "YRR/KL/MLEGE" is found at the end of coil 2 in all IF proteins (Albers and Fuchs, 1992). In lamins, the amino acid identity in coil 2 can be as high as 90% (Krohne *et al.*, 1987). It has been shown that when the last 10 to 30 amino acids in coil 2 of a keratin are deleted, dramatic alterations in cellular keratin networks are observed (Albers and Fuchs, 1987). However, deletion of the tail domain did not cause any visible perturbations on keratin network. In our assay, NF-L^{23-349} which has the second half of coil 2 deleted, can form homodimers with itself but can not form heterodimers with lamins. This interaction may result from the interactions between coil 1a plus coil 1b, because the homodimerization of C1a1b of lamin B1 also gave a significantly positive β-galactosidase activity.

NF-L^{23-549}, which contains full-length NF-L lacking the first 23 amino acids, does not form homodimers (data not shown). Another full-length neurofilament protein NF-M also does not form homodimers with itself in the yeast two hybrid system (data not shown). This is probably because mammalian neurofilament are coassembled from three subunits including NF-L, NF-M and NF-H (Albers and Fuchs, 1992). It is possible that the second half of coil 2 and the tail domain function to specify the formation of NF units as heterotrimers but not homodimers or heterodimers. Therefore, deletion of the second

half of coil 2 and the tail of NF-L enable this polypeptide to form homodimers in our assays.

The head and tail domains of lamins have been proposed to be involved in the second level of organization of the lamina because lamin dimers associate longitudinally to form polar head-to-tail polymers (Heitlinger *et al.*, 1991). In such a case, the head and tail domains may interact with each other to form the head-to-tail polymers. In the two-hybrid system, neither the head domain nor the tail domain can form homodimers, suggesting that they may not contribute to the formation of lamin dimers. Cotransformation of pGBT9-H and pGAD424-T or pGBT9-T and pGAD424-H into yeast also did not induce expression of β-galactosidase, indicating that head and tail domains did not interact in the two-hybrid system. In fact, in our assays, the head and tail domains may inhibit dimerization as full-length lamin B1 appears to interact less strongly with itself than the polypeptide that contains only coil 1b and coil 2. It is possible that the head and tail domains needed to be in the context of lamin dimers to interact with each other to form the head-to-tail polymers.

The interactions of different domains of vimentin (a type III IF protein) have recently been studied by the two-hybrid assay (Meng *et al.*, 1996). Coil 1b, the amino half, and coil 2b of the vimentin molecule have homotypic interactions with β-galactosidase activities of 18, 44, and 52% of the full-length vimentin homotypic interaction (Meng *et al.*, 1996). These three domains corresponds to the domains of C1b, HC1a1b, and C2 of lamin B1. The head domain and the tail domain of vimentin do not interact either homotypically or heterotypically (Meng *et al.*, 1996). These results are consistent with the interactions of the domains of lamin B1, suggesting that the functions of the IF protein domains in IF organization are conserved.

The contributions of different domains of lamin B1 in homodimerization inferred from the β-galactosidase activities measured in the two-hybrid assay are generally consistent with the known interactions between intermediate filament protein rod domains (Steinert and Roop, 1988; Albers and Fuchs, 1992; Stewart, 1993). However, many

factors such as the level of protein expression, the stability of the chimerical fusion proteins, conformational effects, and possible interactions of GAL4 fusion proteins with endogenous yeast proteins, may influence the transcription activation of *lacZ* gene in the two-hybrid assay. It is also possible that the lamin B1 domains dimerize in a random orientation in the yeast two-hybrid assay, and not necessarily in the parallel orientation known to occur between two full-length in intermediate filament subunits (Albers and Fuchs, 1992; Stewart, 1993). For these reasons, a definitive analysis of the quantitative aspects of the interactions between lamin B1 domains will require complementation by methods other than the two-hybrid system.

Despite the caveats mentioned above, our result nonetheless demonstrate that the yeast two-hybrid system can be utilized as a semiquantitative method to examine the interactions between structural proteins of higher eukaryotic cells. The studies on the interactions of vimentin domains have also confirmed this (Meng *et al.*, 1996). Although the amino-terminal domain of LBR can interact with B-type lamins *in vitro* (see Chapter 3), this interaction was not detected in the two-hybrid system. This may due to the strong homodimerization of lamin B1 so that a weaker interaction between LBR and lamin B was masked.

Acknowledgment

We thank Dr. S. Fields for plasmids and yeast strains for the two-hybrid system, Drs. N. Chaudhary, K.M. Pollard, and E. M. Tan for clones, and Drs. J. Hirsch and R.A. Lazzarini for advice and helpful discussions.

Chapter 5. Interaction between Heterochromatin Proteins and LBR, an Integral Protein of the Nuclear Envelope Inner Membrane

Abstract

The eukaryotic chromatin is comprised of euchromatin and heterochromatin. Heterochromatin is partially localized to the inner nuclear membrane. Integral proteins of the inner nuclear membrane that bind the nuclear lamina may also associate with the heterochromatin. We have used the nucleoplasmic domain of human inner nuclear membrane protein LBR to perform a yeast two-hybrid screen and isolated two human proteins homologous to a *Drosophila* heterochromatin HP1. We demonstrated that these two human proteins, termed $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$, interact with LBR *in vitro* and *in vivo*. *Drosophila* HP1 is involved in position-effect variegation of gene expression. Thus, LBR can mediate the association of the heterochromatin with the inner nuclear membrane and also play a role in packaging chromatin into transcriptionally inactive domains at the nuclear envelope.

Introduction

The nuclear envelope of eukaryotic cells encloses and organizes the chromatin in the nucleus into functional domains that influence gene expression (Blobel, 1985). The eukaryotic genome is composed of euchromatin and heterochromatin. Heterochromatin is the chromatin material that remains condensed throughout interphase and has little transcription activity. Euchromatin is highly transcribable and is only condensed in mitotic chromosomes and decondensed in interphase (Brown, 1966). During interphase, heterochromatin is characteristically localized at the nuclear periphery, at specific sites in the nuclear core and surrounding the nucleolus while the euchromatin is localized adjacent to the nuclear pore complex and in the interior of the nucleus (Franke *et al.*, 1981; Bouteille *et al.*, 1983; Mathog *et al.*, 1984; Blobel, 1985).

An early biological function linked to heterochromatin is a genetic phenomenon called "position effect variegation" (PEV) in *Drosophila melanogaster* (Muller, 1930). When the *white* gene required for pigmentation of fly eyes is translocated next to heterochromatin from the euchromatin in the X chromosome, a mosaic pattern of eye color is observed (Muller, 1930). It has been suggested that the spreading heterochromatin encompasses the regions of the *white* gene in some cells making it transcriptionally inactive (Tartof *et al.*, 1984). Since heterochromatin is present in all eukaryotic cells, the position effect repression has been proposed as a general mechanism for gene silencing or inactivation (Loo and Rine, 1994).

The condensed appearance of heterochromatin is thought to be due to a specialized nucleoprotein structure. The first nonhistone chromosomal protein associated with heterochromatin to be identified was heterochromatin protein 1 (HP1) in *Drosophila melanogaster* (James and Elgin, 1986). *Drosophila* HP1 is a 206 amino acid nuclear protein localized by immunofluorescence microscopy to heterochromatic regions of interphase and polytene chromosomes (James and Elgin, 1986; James *et al.*, 1989). *Drosophila* HP1 is encoded by *Sur(var)*205, a gene that can suppress PEV in a dosage-

dependent manner (Eissenberg *et al.*, 1990; 1992), suggesting that HP1 plays an important role in the formation and maintenance of heterochromatin. Homologs of *D. melanogaster* HP1 have been subsequently found in *Drosophila virilis* (Clark and Elgin, 1992), mouse (Singh *et al.*, 1991), human (Saunders *et al.*, 1992), mealy bug (Epstein *et al.*, 1992), and *Schizosaccharomyces pombe* (Lorentz *et al.*, 1994). HP1 proteins have two highly conserved regions. The first domain is a 37 amino acids region near the amino-terminus designated as the "chromodomain" that has 65% identity with *Drosophila* Polycomb protein (Pc) (Paro and Hogness, 1991). Pc is known to be involved in mediating repression of homeotic genes in *Drosophila* (Paro, 1990). The other conserved domain is a 65 amino acids region at the carboxyl-terminus which has recently been designated as the "chromo shadow domain" because of its similarity to the chromodomain in primary and secondary structures (Aasland and Stewart, 1995). The chromo shadow domain is not present in Pc proteins (Aasland and Stewart, 1995). The chromodomain of HP1 is involved in heterochromatin binding and is important for gene silencing in *Drosophila* (Messmer *et al.*, 1992; Powers *et al.*, 1993; Platero *et al.*, 1995). The chromo shadow domain of HP1 contains nuclear localization information and also has heterochromatin binding affinity (Powers and Eissenberg, 1993). HP1 proteins and Pc proteins cannot bind to DNA directly (Paro, 1990), suggesting that other proteins may be involved that mediate the interaction of these chromodomain proteins with heterochromatin. Other heterochromatin-specific proteins that have been identified in mammals are CENP-A, -B and -C, which are found associated with the centromeric heterochromatin of mitotic chromosomes (Earnshaw and Rothfield, 1985). CENP-B has been shown to bind to a 17 bp segment of the alphoid DNA repeat *in vitro* (Masumoto *et al.*, 1990). Despite these findings, the biochemical composition of heterochromatin is largely unknown.

It is not known how heterochromatin is localized to the nuclear periphery. Lamins are able to associate with chromatin and DNA (Glass and Gerace, 1990; Shoeman and Traub, 1990; Yuan *et al.*, 1991; Luderus *et al.*, 1992), however, it has not been shown that

they can specifically associate with heterochromatin. Transcriptionally silent regions of the genome are similarly localized to the nuclear envelope in *Saccharomyces cerevisiae* (Palladino *et al.*, 1994; Cockell *et al.*, 1995; Hecht *et al.*, 1995), an organism in which a nuclear lamina has not been clearly identified, suggesting that integral inner nuclear membrane proteins may mediate the attachment of heterochromatin to the nuclear periphery. In fact, several integral proteins of inner nuclear membrane of higher eukaryotic cells have been shown to have binding affinities to lamins, chromosomes or double-stranded DNA (Worman *et al.*, 1988; 1990; Senior and Gerace, 1988; Harel *et al.*, 1989; Courvalin *et al.*, 1990; Padan *et al.*, 1990; Bailer *et al.*, 1991; Foisner and Gerace, 1993; Harris *et al.*, 1994; Schuler *et al.*, 1994; Ye and Worman, 1994; Furukawa *et al.*, 1995; Martin *et al.*, 1995; see also Chapter 3). Among these proteins, the rat proteins LAPs 1A, 1C and LAP2 can bind to mitotic chromosomes (Foisner and Gerace, 1993) but interactions of these proteins with specific chromatin proteins or with DNA have not been described. These interactions cannot therefore explain the specific association of the heterochromatin with the inner nuclear membrane in interphase.

LBR is an integral protein of the inner nuclear membrane previously shown to bind to B-type lamins (Worman *et al.*, 1988; Simos and Georgatos 1992; Smith and Blobel 1994; Ye and Worman, 1994) and to double-stranded DNA (Ye and Worman, 1994). We used the amino-terminal domain of human LBR (see Chapter 3) to perform a yeast two-hybrid screen and isolated two human homologues of *Drosophila* HP1. We further demonstrated that the interactions between LBR and human HP1 proteins can occur *in vitro* and *in vivo*. This interaction thus provides a potential link between heterochromatin and the nuclear inner membrane.

Results

Identification of Human Homologues of *Drosophila* HP1 that Interact with the Nucleoplasmic Domain of LBR

We performed a yeast two-hybrid screen (Fields and Song 1989; Chien *et al.*, 1991) using the amino-terminal domain of human LBR fused to the DNA binding domain of GAL4 in pGBT9 as a bait. Yeast strain Y190 (Durfee *et al.*, 1993; Harper *et al.*, 1993) was used in the library screening. The library screened was a Hela cell cDNA library fused to the GAL4 transcription activation domain in pGADGH (Clontech Laboratories, Inc.). From an estimated 10^6 yeast cotransformants, ten colonies were isolated that grew on the synthetic media lacking histidine, tryptophan and leucine in the presence of 25 mM 3-AT and all tested positive for ß-galactosidase activity (see Materials and Methods). Only two pGADGH recombinant plasmids rescued from these ten clones were able to give positive β-galactosidase activity when they were cotransformed again with pGBT9-LBRAT (the bait plasmid). These two plasmids were termed ATBP115 and ATBP8132. When ATBP115 and ATBP8132 were cotransformed with control plasmid pGBT9 that has the DNA binding domain of GAL4 alone, the transformants did not demonstrate β-galactosidase activity. Cotransformants of the bait plasmid (pGBT9-LBRAT) and control vector plasmid pGADGH gave no β-galactosidase activity, indicating that the amino-terminal domain of LBR does not activate transcription by itself (Table 5-1). These results demonstrated that ATBP115 and ATBP8132 expressed proteins that can specifically interact with the amino-terminal domain of LBR in the two-hybrid system.

Table 5-1. ß-Galactosidase activities for yeast cotransformed with LBR amino-terminal domain-GAL4 DNA binding domain fusion protein and cDNA clones isolated in the two-hybrid screen and controls

Plasmids transformed	Filter assay	Liquid assay
pGBT9 + pGADGH	White	0.67 ± 0.15
pGBT9-LBRAT + pGADGH	White	0.61 ± 0.01
pGBT9-LBRAT + ATBP115	Blue	4.50 ± 0.39
pGBT9 + ATBP115	White	0.57 ± 0.01
pGBT9-LBRAT + ATBP8132	Blue	1.20 ± 0.15
pGBT9 + ATBP8132	White	0.60 ± 0.01

pGBT9 is the plasmid that expresses the DNA binding domain of GAL4. pGADGH is the plasmid that expresses the transcription activation domain of GAL4. pGBT9-LBRAT is the plasmid that expresses the amino-terminal domain of LBR fused to the DNA binding domain of GAL4. ATBP115 and ATBP8132 are the cDNA clones in a pGADGH-derived plasmid that were isolated in the two hybrid screen using pGBT9-LBRAT as the "bait." In the colorimetric filter assay, blue indicates positive ß-galactosidase activity and white negative. Enzyme activities for liquid culture assays of co-transformed yeast are given in units as described elsewhere (Guarente, 1983). Values for ß-galactosidase activities are means ± standard errors (n = 3).

Sequencing of the cDNA insert of clone ATBP115 and a GenBank search showed that it encoded a protein 100% identical to $HP1^{Hs\alpha}$ (Fig. 5-1a), a human homologue of *Drosophila* heterochromatin protein HP1. $HP1^{Hs\alpha}$ had been identified as a nuclear antigen recognized by autoantibodies from some patients with scleroderma and is localized to the heterochromatin (Saunders *et al.*, 1993). Sequencing of the cDNA insert of clone ATBP8132 showed that it encodes a novel human polypeptide 65% identical to $HP1^{Hs\alpha}$, which we termed $HP1^{Hs\gamma}$ to distinguish it from another homologous human protein that has been designated $HP1^{Hs\beta}$ (Singh *et al.*, 1991; Saunders *et al.*, 1993). The ATBP8132 cDNA sequence overlapped with two human nucleotide sequences of unknown function in GenBank. One of the sequences (accession number Z15820) has 345 nucleotides which were 98% identical, over a stretch of 209 nucleotides, to ATBP8132. The nonoverlapping nucleotides of Z15820 extend in the 5' direction of ATBP8132. We thus deduced the first 17 putative amino acids of $HP1^{Hs\gamma}$ from this GenBank sequence. The position of the amino-terminal methionine is based on the highly conserved sequences among other HP1 proteins (Clark and Elgin, 1992; Eissenberg *et al.*, 1990; James and Elgin, 1986; Saunders *et al.*, 1993; Singh *et al.*, 1991). The amino acid at position 11 (X) of $HP1^{Hs\gamma}$ cannot be deduced from the nucleotide sequence data in GenBank (Fig. 5-1b).

Over their entire lengths, $HP1^{Hs\gamma}$ is 98% identical to the mouse HP1-like protein M32 and 72% identical to M31 (Singh *et al.*, 1991). $HP1^{Hs\alpha}$ is 65% identical to M31 and M32. In addition, $HP1^{Hs\gamma}$ is 38% identical and $HP1^{Hs\alpha}$ is 29% identical to SWI6 of *Schizosaccharomyces pombe* (Lorentz *et al.*, 1994), which is a chromodomain protein located in fission yeast centromeric heterochromatin and is required for functions of centeromeres (Ekwall *et al.*, 1995). A partial sequence encoding a chromodomain protein has also been identified in *Caenorhabditis elegans* (Waterston *et al.*, 1992). In addition, the *Drosophila melanogaster* protein polycomb contains the conserved chromodomain or HP1/Pc box (Paro and Hogness 1991) from amino acids 24 to 60 in HP1 and 20 to 56 in $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$.

a

```
   1  ATGGGAAAGAAAACCAAGCGGACAGCTGACAGTTCTTCTTCAGAGGATGAGGAGGAGTAT
   1   M  G  K  K  T  K  R  T  A  D  S  S  S  S  E  D  E  E  E  Y
  61  GTTGTGGAGAAGGTGCTAGACAGGCGCGTGGTTAAGGGACAAGTGGAATATCTACTGAAG
  21   V  V  E  K  V  L  D  R  R  V  V  K  G  Q  V  E  Y  L  L  K
 121  TGGAAAGGCTTTTCTGAGGAGCACAATACTTGGGAACCTGAGAAAAACTTGGATTGCCCT
  41   W  D  G  F  S  E  E  H  N  T  W  E  P  E  K  N  L  D  C  P
 181  GAGCTAATTTCTGAATTTATGAAAAAGTATAAGAAGATGAAGGAGGGTGAAAATAATAAA
  61   E  L  I  S  E  F  M  K  K  Y  K  K  M  K  E  G  E  N  N  K
 241  CCCAGGGAGAAGTCAGAAAGTAACAAGAGGAAATCCAATTTCTCAAACAGTGCCGATGAC
  81   P  R  E  K  S  E  S  N  K  R  K  S  N  F  S  N  S  A  D  D
 301  ATCAAATCTAAAAAAAAGAGAGAGCAGAGCAATGATATCGCTCGGGGCTTTGAGAGAGGA
 101   I  K  S  K  K  K  R  E  Q  S  N  D  I  A  R  G  F  E  R  G
 361  CTGGAACCAGAAAAGATCATTGGGGCAACAGATTCCTGTGGTGATTTAATGTTCCTAATG
 121   L  E  P  E  K  I  I  G  A  T  D  S  C  G  D  L  M  F  L  M
 421  AAATGGAAAGACACAGATGAAGCTGACCTGGTTCTTGCAAAAGAAGCTAATGTGAAATGT
 141   K  W  K  D  T  D  E  A  D  L  V  L  A  K  E  A  N  V  K  C
 481  CCACAAATTGTGATAGCATTTTATGAAGAGAGACTGACATGGCATGCATATCCTGAGGAT
 161   P  Q  I  V  I  A  F  Y  E  E  R  L  T  W  H  A  Y  P  E  D
 541  GCGGAAAACAAAGAGAAAGAAACAGCAAAGAGCTAAAGGAGGGGATGGTCTCTGTCATTT
 181   A  E  N  K  E  K  E  T  A  K  S  ***
 601  CTCTTTGTACATAATACATTCACCTCCCTGCCTCCTCTCCTTTCTACCCACCCCCC ATAA
 661  AAAATGTGCTTATTTCTATCCTAAACACATTCACTGTGCTCCACGG
```

b

```
   1  ATGGGAAAAAAACAGAATGGAAAGAGTAAAAANGTTGAAGAGGACGAGCCTGAAGAATTT
   1   M  G  K  K  Q  N  G  K  S  K  X  V  E  E  A  E  P  E  E  F
  61  GTCGTGGAAAAAGTACTAGATCGACGTGTAGTGAATGGGAAAGTGGAATATTTCCTGAAG
  20   V  V  E  K  V  L  D  R  R  V  V  N  G  K  V  E  Y  F  L  K
 121  TGGAAGGGATTTACAGATGCTGACAATACTTGGGAACCTGAAGAAAATTTAGATTGTCCA
  41   W  K  G  G  T  D  A  D  N  T  W  E  P  E  E  N  L  D  C  P
 181  GAATTGATTGAAGCGTTTCTTAACTCTCAGAAAGCTGGCAAAGAAAAAGATGGTACAAAA
  61   E  L  I  E  A  F  L  N  S  Q  K  A  G  K  E  K  D  G  T  K
 241  AGAAAATCTTTATCTGACAGTGAATCTGATGACAGCAAATCAAAGAAGAAAAGAGATGCT
  81   R  K  S  L  S  D  S  E  S  D  D  S  K  S  K  K  K  R  D  A
 301  GCTGACAAACCAAGAGGATTTGCCAGAGGTCTTGATCCTGAAAGAATAATTGGTGCCATA
 101   A  A  D  K  P  R  G  F  A  R  G  L  D  P  E  R  I  I  G  A
 361  GACAGCAGTGGAGAATTGATGTTTCTCATGAAATGGAAAGATTCAGATGAGGCAGACTTG
 121   I  D  S  S  G  E  L  M  F  L  M  K  W  K  D  S  D  E  A  D
 421  GTGCTGGCGAAAGAGGCAAATATGAATTGTCCTCAAATTGTAATTGCTTTTTATGAAGAG
 141   L  V  L  A  K  E  A  N  M  C  P  Q  I  V  I  A  F  Y  E  E
 481  AGACTAACTTGGCATTCTTGTCCAGAAGATGAAGCTCAATAATTGTTCACATTGTTCTTT
 161   R  L  T  W  H  S  C  P  E  D  E  A  Q  ***
 541  TATATATATTTATATATATATATTAAAAATTGGGTCTTAGATTTTGATTTACTAGTGTGA
 601  CAAAATAACTACATCCTAATGAAAATCAAGTTTGATATGTTTGTTTTGAAAGTAGCGTTG
 661  GAAGAGTTGTTGGGGGTTTTTTGCATCCATAGCACTGGTTACTTTGAACA AATAATAA AA
 721  GCTTTCTGTAGTTGCTTCCTTTATCAG
```

Fig. 5-1. DNA sequence and deduced amino acid sequence of ATBP115 (a) and ATBP8132 (b). Sequences obtained from GenBank Database of Z18520 overlapping with ATBP8132 and S62077 overlapping with ATBP115 are underlined. Protein sequences are shown in single letter code. Potential signal sequence for polyadenylation are in bold letters. Poly(A) tails were found after the last nucleotide sequences in both ATBP115 and ATBP8132 cDNAs. The sequences of $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$ have been submitted to GenBank with accesssion numbers U26311 and U26312, respectively.

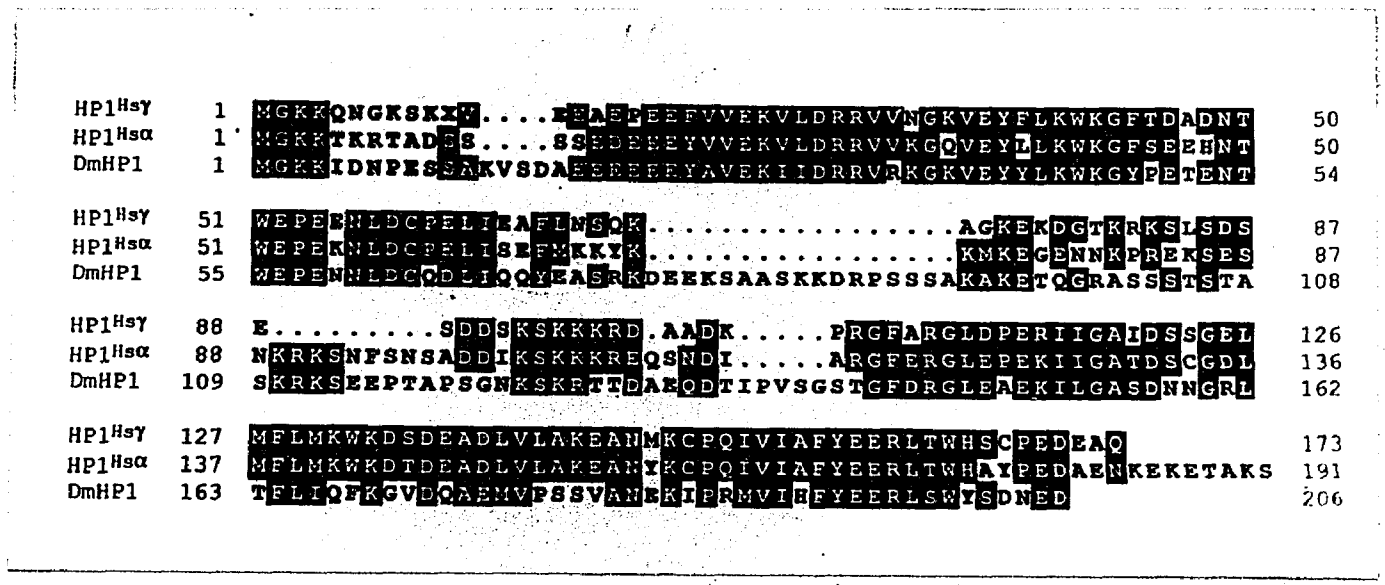


Fig. 5-2. Alignment of the amino acid sequences of HP1$^{Hs\gamma}$, HP1$^{Hs\alpha}$ and *Drosophila melanogaster* (Dm) HP1. Identical or conservative amino acids are shown as white on black. Conservative amino acid substitutions used are: aliphatic hydrophobic L/I/V/M/A; aromatic hydrophobic F/Y; basic R/K/H; acidic E/D; aliphatic alcoholic S/T; polar amides Q/N.

Human HP1 Proteins Bind to LBR *In Vitro*

To confirm the interactions detected in the yeast two-hybrid assay, we showed that the amino-terminal domain of LBR binds to $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$ *in vitro* (Fig. 5-3). ^{35}S-labeled, *in vitro* translated, $HP1^{Hs\alpha}$ (Fig. 5-3, lanes 1-3) and $HP1^{Hs\gamma}$ lacking its first 17 amino acids (Fig. 5-3, lanes 4-6) were incubated with a GST fusion protein of the amino-terminal domain of human LBR (gtLBR-AT, see Chapter 3) or GST protein alone coupled on glutathione-Sepharose beads. After two hours of incubation and extensive washing with binding buffer, the proteins retained on beads were eluted, analyzed on SDS-PAGE and subjected to autoradiography. The results showed that both *in vitro* translated proteins remained bound on gtLBR-AT beads (Fig. 5-3, lane 3 and lane 6) but not on GST beads (Fig. 5-3, lane 2 and lane 4). The amount of $HP1^{Hs\gamma}$ lacking its first 17 amino acids that associated with the gtLBR-AT appeared to be less than that of $HP1^{Hs\alpha}$ (Fig. 5-3, compare lanes 3 and 6). This is consistent with the lower ß-galactosidase activity this polypeptide gave in the yeast two-hybrid assay (see Table 5-1). This may be because $HP1^{Hs\gamma}$ has lower affinity for the amino-terminal domain of LBR than $HP1^{Hs\alpha}$ or because this protein lacks 17 amino acids at its amino-terminus.

The *in vitro* binding of translated HP1 proteins to gtLBR-AT was also examined in buffers of increasing ionic strengths and detergent concentrations (Fig. 5-4). The amount of $HP1^{Hs\alpha}$ that bound to gtLBR-AT started to decrease slightly at NaCl concentrations greater than 0.5 M but significant binding was still observed in buffers containing 1.0 M (Fig. 5-4a, lanes 4-8). Binding of $HP1^{Hs\alpha}$ to the amino-terminal domain of LBR was also observed in the presence of the non-ionic detergent NP40 at concentrations up to 1% (Fig. 5-4b, lanes 4-6). The binding was sensitive to ionic degergents as 0.05% SDS in the binding buffer significantly reduced the interaction with only trace amount of HP1 remaining bound to gtLBR-AT (Fig. 5-4b, lanes 7 and 8). These data show that the binding of human HP1 proteins to LBR is not mediated by weak, non-specific electrostatic or hydrophobic interactions.

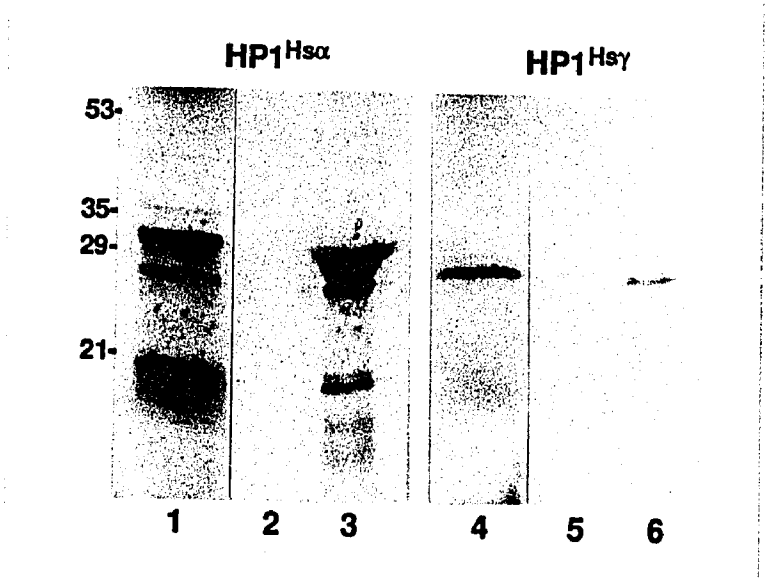


Fig. 5-3. The amino-terminal domain of LBR binds to HP1$^{Hs\alpha}$ and HP1$^{Hs\gamma}$ *in vitro*. The autoradiogram shows ^{35}S-labeled HP1$^{Hs\alpha}$ (lane 1) and HP1$^{Hs\gamma}$ lacking its first 17 putative amino acids (lane 4) synthesized by *in vitro* translation in reticulocyte lysates. HP1$^{Hs\alpha}$ (lanes 2 and 3) or HP1$^{Hs\gamma}$ (lanes 4 and 5) were incubated with either GST (lanes 2 and 5) or the gtLBR-AT fusion protein (lanes 3 and 6) coupled to glutathione-Sepharose. The glutathione-Sepharose was pelleted by centrifugation, washed and the bound proteins eleuted with SDS. Eluted proteins were separated by SDS-PAGE and examined by autoradiography. HP1$^{Hs\alpha}$ and HP1$^{Hs\gamma}$ bound to the gtLBR-AT (lanes 3 and 6) but not to GST (lanes 2 and 5). Migration of molecular mass standards is indicated in kilodaltons.

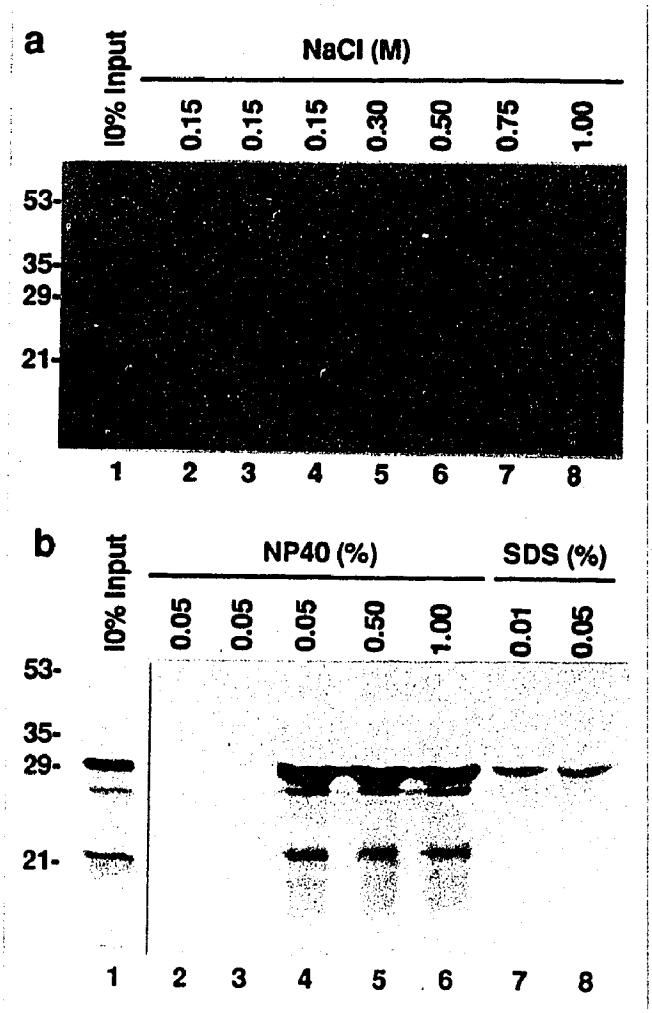


Fig. 5-4. Effect of ionic strength and detergents on the *in vitro* binding of HP1$^{Hs\alpha}$ to the amino-terminal domain of LBR. **(a)** A standard amount of ^{35}S-labeled HP1$^{Hs\alpha}$, 10% of which is shown (lane 1), was used in each binding assay. This amount was incubated with glutathione-Sepharose alone (lane 2) or GST coupled to glutathione-Sepharose (lane 3) in binding buffer containing 150 mM NaCl. This amount was also incubated with the same amount of gtLBR-AT coupled to glutathione-Sepharose (lanes 4-8) in buffers containing the NaCl concentrations indicated above each lane. Glutathione-Sepharose was pelleted by centrifugation, washed in buffer containing the NaCl concentration indicated above each lane and the bound proteins eluted with SDS. Eluted proteins were separated by SDS-PAGE and examined by autoradiography. Migration of molecular mass standards is indicated in kilodaltons. **(b)** A standard amount of ^{35}S-labeled HP1$^{Hs\alpha}$, 10% of which is shown (lane 1), was used in each binding assay. This amount was incubated with glutathione-Sepharose alone (lane 2) or GST coupled to glutathione-Sepharose (lane 3) in binding buffer containing 0.5% NP40. This amount was also incubated with the same amount of gtLBR-AT coupled to glutathione-Sepharose in buffers containing NP40 at the concentrations indicated above each lane (lanes 4-6) or SDS at the concentrations indicated above each lane (lanes 7 and 8).

We demonstrated that cellular HP1 proteins prepared from human Hela cells also bound to the gtLBR-AT fusion protein (Fig. 5-5). Hela cell extracts were used to perform *in vitro* binding assays with the same procedures as the *in vitro* translated HP1 proteins binding to gtLBR-AT. The eluted proteins were analyzed by western blots. Two proteins of approximately 30 kDa, recognized by autoantibodies from a patient with scleroderma against $HP1^{Hs\alpha}$ and related chromodomain proteins (Saunders *et al.*, 1993), were present in Hela cell extracts (Fig. 5-5a, lane 1). These two polypeptides were retained on glutathione-Sepharose attached to gtLBR-AT (Fig. 5-5a, lane 3). These two polypeptides did not bind to GST (Fig. 5-5a, lane 2) or to a truncated LBR amino-terminal domain (amino acid 1 to 100) fused to GST (see Chapter 3) (Fig. 5-5a, lane 4), demonstrating that non-specific interactions of HP1 proteins with components of the binding matrix or control polypeptides did not occur. The scleroderma autoantibodies used to detect the cellular HP1 proteins recognized recombinant proteins 6xHis-$HP1^{Hs\alpha}$ and 6xHis-$HP1^{Hs\gamma}$ expressed from the cDNAs isolated in the two-hybrid screen (Fig. 5-5b), indicating that proteins encoded by ATBP115 and ATBP 8132 were the proteins detected in the cellular extracts. These results demonstrated that the LBR amino-terminal domain associated *in vitro* with HP1 proteins from human cells.

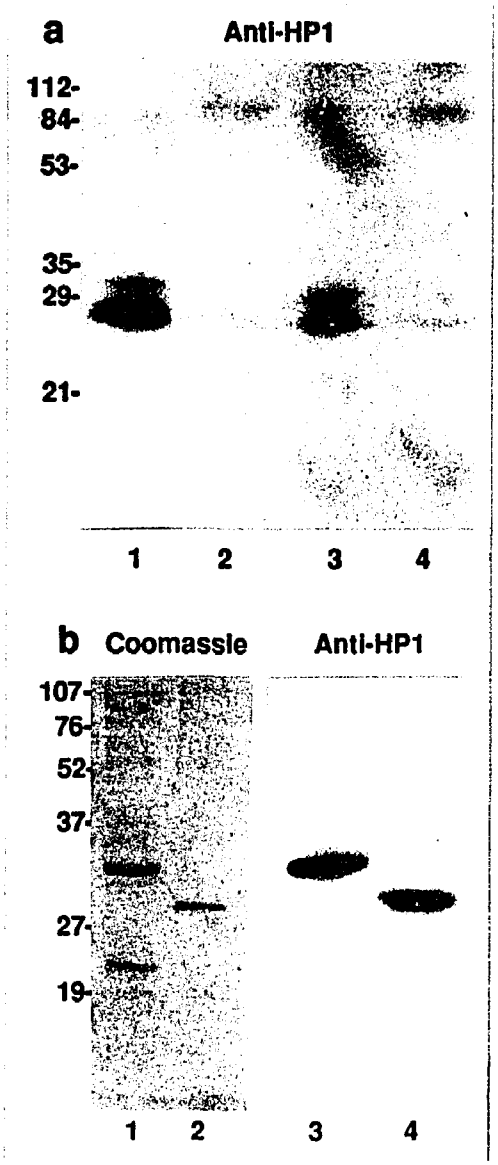


Fig. 5-5. Binding of HP1 proteins in cell extracts to the amino-terminal domain of LBR. (a) Autoradiogram of immunoblot probed with autoantibodies from a patient with scleroderma that recognize human HP1 proteins is shown. These autoantibodies recognized two polypeptides with molecular masses of approximately 30 kilodaltons in Hela cell extracts (lane 1). Hela cell extracts were incubated with GST coupled to glutathione-Sepharose (lane 2), the amino-terminal domain of LBR fused to GST coupled to glutathione-Sepharose (lane 3) and the first 100 amino acids of LBR fused to GST coupled to glutathione-Sepharose (lane 4). The glutathione-Sepharose was washed and the bound proteins eluted with SDS, separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting with the anti-HP1 antibodies. The same proteins recognized by the anti-HP1 antibodies in the cell extract bound only to the fusion protein that contained the entire amino-terminal domain of LBR. Migration of molecular mass standards is indicated in kilodaltons. **(b)** Confirmation that the autoantibodies used to detect HP1 proteins in the cell extracts and immunoprecipitates recognize $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$. The Coomassie-blue stained gel shows $HP1^{Hs\alpha}$ (lane 1) and $HP1^{Hs\gamma}$ (lane 2) polyhistidine fusion proteins. The autoradiogram of the immunoblot shows that the antibodies used to detect HP1 proteins in the cell extracts and immunoprecipitates recognized the $HP1^{Hs\alpha}$ (lane 3) and $HP1^{Hs\gamma}$ (lane 4) fusion proteins. Migration of molecular mass standards is indicated in kilodaltons.

Interactions of LBR and HP1 *In Vivo*

To demonstrate that the LBR-HP1 interaction can occur under biologically relevant conditions, we examined whether HP1 proteins could be coimmunoprecipitated from human cell extracts by anti-LBR antibodies. Because full-length LBR contains a large hydrophobic domain with eight putative transmembrane segments, this protein was difficult to solublize and immunoprecipite from cells without the use of detergents that disrupt protein-protein interactions. When Hela cells were broken by sonication and extracted with buffer containing 1% of the detergent NP40, a concentration which did not completely abolish the interaction between gtLBR-AT and HP1 proteins *in vitro* (see Fig. 5-4b), only about 20% of the total cellular LBR was present in the soluble extract. Immunoprecipitation with anti-LBR antibodies yielded a small amount of LBR that was detectable on immunoblots and only barely detectable amounts of two polypeptides of approximately 30 kilodaltons that reacted with the antibodies against HP1 proteins.

Because of the low efficiency with which LBR and HP1 proteins were coimmunoprecipitated Hela cells extracts, we switched to a human cell line 293T from which that LBR was more easily extractable. When 293T cells were disrupted by sonication and extracted with buffer containing 0.5% NP40 and low salt, about 70% of the cellular LBR was present in the extract. Serum from a patient with PBC that contained anti-LBR antibodies (Nickowitz *et al.*, 1994; chapter 3, Fig. 3-4) immunoprecipitated LBR from cell extracts (Fig. 5-6a, lanes 1 and 3). No detectable LBR was present in immunoprecipitates when control serum from another patient with PBC without anti-LBR autoantibodies was used (Fig. 5-6a, lane 2). Two polypeptides of approximately 30 kDa that were recognized by anti-HP1 antibodies were present in immunoprecipitates obtained with the experimental serum (Fig. 5-6b, lane 3) but not in immunoprecipitates obtained with the control serum (Fig. 5b, lane 2). The experimental serum with anti-LBR antibodies did not label the interior of the nucleus when used in immunofluorescence microscopy

(Chapter 3, Fig. 3-5) and did not recognize HP1 proteins on immunoblots. Thus it is unlikely that it would directly immunoprecipitate HP1 proteins. Both the experimental and control sera contained antimitochondrial autoantibodies (Nickowitz *et al.*, 1994), and immunoblotting (data not shown) showed that immunoprecipitates from both serum contained the major mitochondrial autoantigen pyruvate dehydrogenase E2 (Coppel *et al.*, 1988), indicating that they had no differences in the efficiency of immunoprecipitation. These coimmunoprecipitation experiments showed that HP1 proteins and LBR were associated in human cells.

Co-localization of HP1 Proteins and LBR at the Nuclear Envelope

An *in vivo* interaction between LBR and HP1 proteins was further supported by showing that a portion of $HP1^{Hs\alpha}$ containing a FLAG epitope tag was co-localized with LBR at the nuclear envelope when expressed in Hela cells by transient transfection (Fig. 5-7). Hela cells transfected with pSVK3-FLAG-$HP1^{Hs\alpha}$ were examined by confocal immunofluorescence microscopy. Labeling of Hela cells with rabbit anti-LBR antibodies showed that LBR was exclusively localized to the nuclear envelope in a confocal section (Fig. 5-7a, red). In the same cell and same section labeling with anti-FLAG antibodies showed that this polypeptide was expressed in discrete areas throughout the nucleus and that a significant amount was localized at the nuclear periphery (Fig. 5-7b, green). Superimposition of confocal images showed that a portion of the expressed $HP1^{Hs\alpha}$ co-localized with LBR at the nuclear envelope (Fig. 5-7c, yellow). Hence, HP1 proteins were present in the appropriate subnuclear location to interact with LBR.

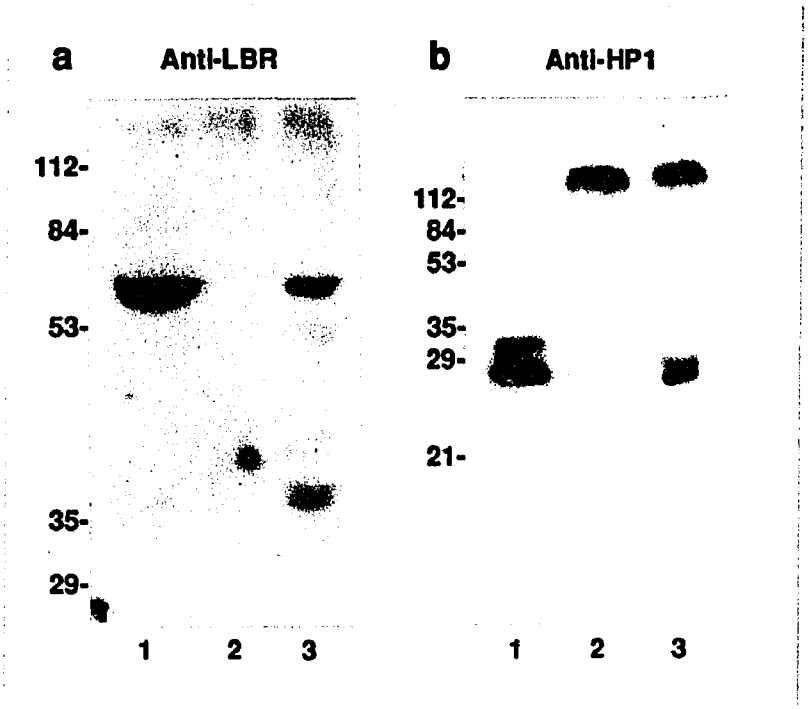


Fig. 5-6. Co-immunoprecipitation of HP1 proteins from cell extracts by anti-LBR antibodies. **(a)** Autoradiogram of immunoblot of 8.5% SDS-polyacrylamide slab gel probed with rabbit anti-LBR antibodies shows LBR in 293 T cell extracts (lane 1). LBR was not precipitated from extracts by control serum from a patient with PBC (lane 2) but was immunoprecipitated from extracts using serum from a patient with the same disease with autoantibodies against LBR (lane 3). **(b)** Autoradiogram of immunoblot of 12.5% SDS-polyacrylamide slab gel using autoantibodies against HP1 (see Fig. 5-5b). Two proteins with apparent molecular masses of approximately 30 kilodaltons were detected in 293 T cell extracts (lane 1). These proteins were not detected in immunoprecipitates obtained with the control serum from a patient with PBC (lane 2) but were detected in immunoprecipitates obtained with serum from a patient with the same disease with autoantibodies against LBR (lane 3). The slowly migrating bands in lanes 2 and 3, which are less well visualized at the top of the immunoblot of the lower concentration gel in panel a, correspond to cross-linked complexes of immunoglobulin (generated during protein A coupling, see Materials and Methods) that were present in the reaction and recognized by the protein A used to reveal the immunoblots. Migration of molecular mass standards is indicated in kilodaltons at the left of each autoradiogram.

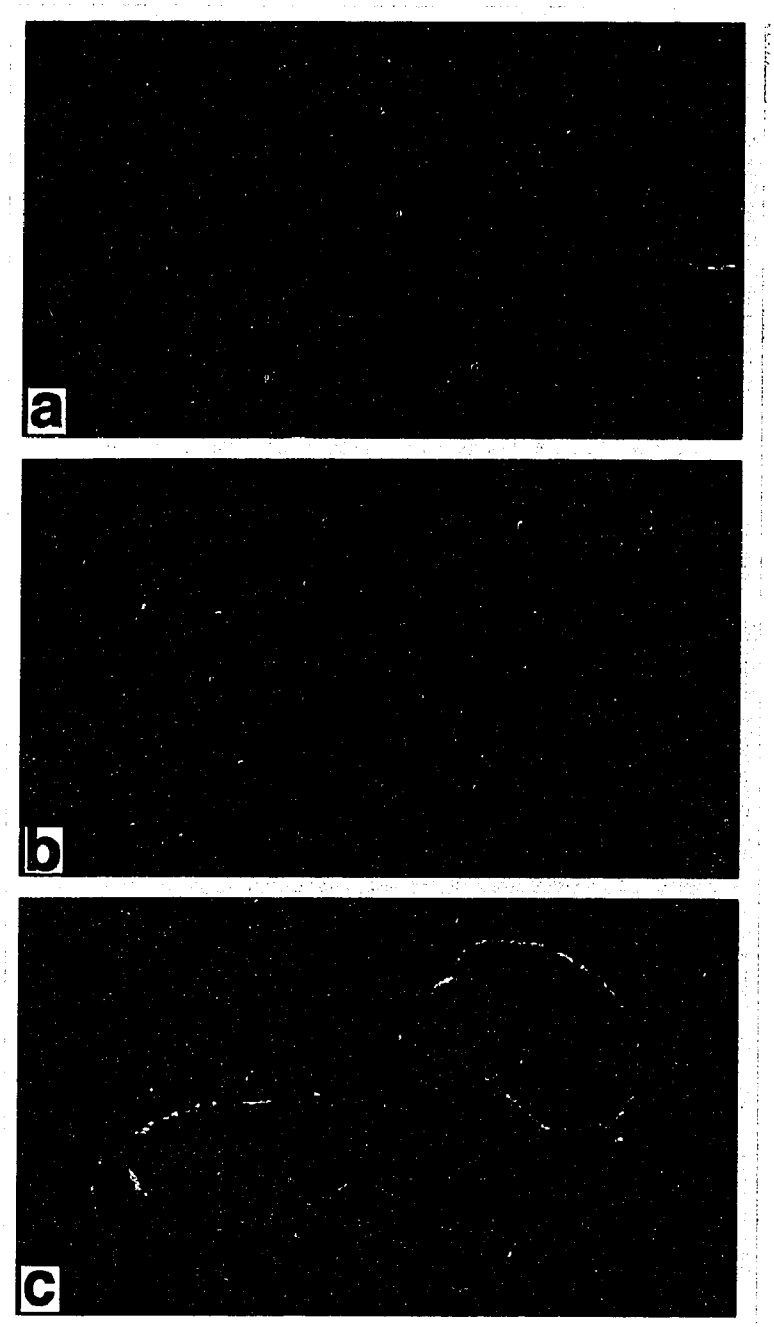


Fig. 5-7. Co-localization of HP1$^{Hs\alpha}$ and LBR in cells. **(a)** Confocal immunofluorescence micrograph of Hela cells showing LBR in the nuclear envelope (red). **(b)** Confocal immunofluorescence micrograph of the same optical section of the same cells in panel a showing localization of FLAG-tagged HP1$^{Hs\alpha}$ (green). **(c)** Superimposed images from panels a and b showing areas where LBR and HP1$^{Hs\alpha}$ are co-localized (yellow). Cells were transfected to express HP1$^{Hs\alpha}$ with a FLAG epitope tag and fixed cells were processed for immunofluorescence microscopy using rabbit anti-LBR antibodies and mouse anti-FLAG antibodies. Secondary antibodies were Texas red-conjugated goat anti-rabbit (red) and fluorescence isothiocyanate-conjugated goat anti-mouse (green). Bar indicates 10 μm.

The Chromo Shadow Domain of HP1 Proteins is Necessary and Sufficient for LBR-HP1 Interaction.

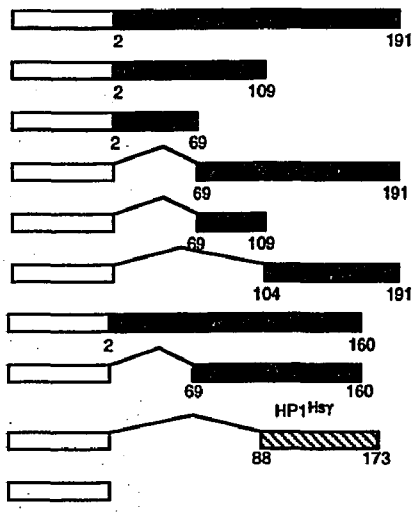
According to secondary structure analysis (Aasland and Stewart, 1995; Dr. J.-C. Courvalin, personal communication), HP1 proteins can be divided into two major domains. The amino-terminal region contains the chromodomain that is highly conserved among chromodomain proteins. The carboxyl-terminus (the chromo shadow domain, Aasland and Stewart, 1995) is also highly conserved among HP1 proteins. The chromodomain and chromo shadow domain are separated by less conserved amino acid stretches that are rich in charged amino acid residues. To examine which domain of HP1 is involved in the HP1-LBR interactions, we cloned cDNAs for various domains of $HP1^{Hs\alpha}$ protein (Fig. 5-8, left panel) fused to the activation domain of GAL4 in a two-hybrid vector pACT2. These pACT2-$HP1^{Hs\alpha}$ constructs were used to cotransform yeast strain Y190 with pGBT9-LBRAT. The β-galactosidase activities of the cotransformants were examined by both filter assays and liquid assays (Fig. 5-8, right panel). A result of "blue" on filter assay indicates a positive protein-protein interaction while "white" is negative. Enzyme activities similar or lower than the negative control (pACT2 alone) also indicates a negative protein-protein interaction. The results showed that $HP1^{Hs\alpha 2\text{-}69}$, $HP1^{Hs\alpha 69\text{-}109}$ and $HP1^{Hs\alpha 2\text{-}109}$, which represents the chromodomain alone, the charged region alone and the chromodomain plus charged region respectively, do not interact with the amino-terminal domain of LBR. The polypeptides of $HP1^{Hs\alpha 2\text{-}191}$, $HP1^{Hs\alpha 69\text{-}191}$ and $HP1^{Hs\alpha 104\text{-}109}$, which represents the full length $HP1^{Hs\alpha}$, charged region plus the chromo shadow domain and the chromo shadow domain alone respectively, can interact with the amino-terminal domain of LBR. $HP1^{Hs\gamma 88\text{-}173}$, the corresponding polypeptide of $HP1^{Hs\alpha 104\text{-}109}$ in $HP1^{Hs\gamma}$, is also able to interact with the amino-terminal domain of LBR. When the last 30 amino acids of the carboxyl-terminal domain were deleted ($HP1^{Hs\alpha 1\text{-}160}$ and $HP1^{Hs\alpha 69\text{-}160}$), protein-protein interactions were abolished. There were no significant difference among the positive enzyme activities measured. These results demonstrated that the

polypeptide of HP1$^{Hs\alpha 104\text{-}109}$, which contains the chromo shadow domain, is necessary and sufficient for HP1-LBR interaction.

The B-Type Lamin Binding, DNA Binding, and HP1-Binding Domain of the Amino-Terminus of LBR are Contiguous but not Overlapped

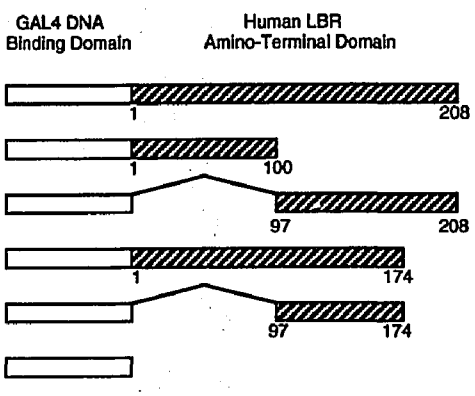
We have previously demonstrated that the stretch of amino acids from 70 to 100 of the amino-terminal domain of LBR is essential for its binding to double stranded-DNA (Chapter 3, Fig. 3-8). Although *in vitro* binding experiments indicated that the full-length amino-terminal domain of LBR may be required for its *in vitro* binding to B-type lamins (Ye and Worman, 1994), immunological evidence (Lassoud *et al.*, 1991; Lin *et al.*, 1996) suggested that the first 60 amino acids of LBR may be involved in B-type lamin binding. The first 60 amino acids of human LBR contains the epitope of autoantibodies that recognize human LBR (Lin *et al.*, 1996) and some of these autoantibodies have been shown to be anti-idiotypic to certain antibodies directed against B-type lamins (Lassoud *et al.*, 1991). To determine the HP1 binding domain of LBR, we cloned cDNAs into pGBT9 to express different parts of the LBR amino-terminal domain and analyzed their interactions with HP1$^{Hs\alpha}$ in the two-hybrid system (Fig. 5-9). The results demonstrated that the polypeptide LBRAT$^{1\text{-}100}$, which contains the DNA binding domain and the probable B-type lamin binding domain, do not interact with HP1. The polypeptide LBRAT$^{97\text{-}208}$ interacts with HP1 with the same level of β-galactosidase activities as the full length amino-terminal domain of LBR (LBRAT$^{1\text{-}208}$). Similar levels of enzyme activity were obtained when pGBT9-LBRAT$^{97\text{-}208}$ was cotransformed with pACT2-HP1$^{Hs\alpha 104\text{-}191}$ (data not shown), demonstrating that the interactions between HP1 and LBR occurred through these two defined regions on both proteins. LBRAT$^{1\text{-}174}$ and LBRAT$^{97\text{-}174}$, of which the stretch of amino acids from 175 to 208 was deleted, did not interact with HP1$^{Hs\alpha}$ at all. These results demonstrate that the stretch of amino acids from 97 to 208 of the amino-terminal domain of LBR is sufficient and essential for the interaction between HP1 and

LBR. This domain does not overlap with the DNA binding domain and the domain that may be important in binding to B-type lamins (amino acids 1-60). When 10 μg of lambda phage DNA or 100 μg of sonicated salmon sperm DNA were incubated with *in vitro* translated HP1 proteins in the *in vitro* binding experiments, the binding of HP1 protein on gtLBR-AT beads was not affected (data not shown), suggesting that the amino-terminal domain of LBR can bind with DNA and HP1 protein at the same time. Thus the amino-terminal domain of LBR may bind to its three identified ligands through three contiguous but non-overlapping domains.



GAL4 Activation Domain Constructs	β-Gal Activity Cotransformed with pGBT9-LBRAT Filter Assay	Liquid Assay
pACT2-HP1$^{Hs\alpha 1\text{-}191}$	Blue	11.1 ± 0.4
pACT2-HP1$^{Hs\alpha 1\text{-}109}$	White	0.6 ± 0.1
pACT2-HP1$^{Hs\alpha 1\text{-}69}$	White	0.6 ± 0.1
pACT2-HP1$^{Hs\alpha 69\text{-}191}$	Blue	14.7 ± 1.2
pACT2-HP1$^{Hs\alpha 69\text{-}104}$	White	0.85 ± 0.1
pACT2-HP1$^{Hs\alpha 104\text{-}191}$	Blue	8.1 ± 0.5
pACT2-HP1$^{Hs\alpha 1\text{-}160}$	White	0.2 ± 0.1
pACT2-HP1$^{Hs\alpha 69\text{-}160}$	White	0.2 ± 0.1
pACT2-HP1$^{Hs\gamma 88\text{-}173}$	Blue	9.4 ± 1.6
pACT2	White	0.8 ± 0.3

Fig. 5-8. The carboxyl-terminal half of HP1$^{Hs\alpha}$ is necessary and sufficient for HP1-LBR interactions. The left panel shows diagrams representing the various HP1$^{Hs\alpha}$ domains fused to the GAL4-activation domain. Names of each corresponding construct are given and listed. The HP1 amino acids included in the fusion proteins are indicated. cDNAs for HP1 domains are cloned in-frame downstream of GAL4 activation domain in plasmid pACT2. The right panel shows the β-galactosidase activities of the cotransformants of pACT2-HP1 constructs and pGBT9-LBRAT. "Blue" indicates a positive protein-protein interaction. "White" indicates a negative result. pACT2 was used as a negative control cotransformed with pGBT9-LBRAT. The values of the liquid assay, in standard units, are means ± standard errors (n = 3).



GAL4 DNA Binding Domain Constructs	β-Gal Activity Cotransformed with pACT2-HP1 Hsα	
	Filter Assay	Liquid Assay
pGBT9-LBRAT$^{1-208}$	Blue	12.9 ± 2.5
pGBT9-LBRAT$^{1-100}$	White	0.3 ± 0.1
pGBT9-LBRAT$^{97-208}$	Blue	13.1 ± 1.3
pGBT9-LBRAT$^{1-174}$	White	0.2 ± 0.1
pGBT9-LBRAT$^{97-174}$	White	0.2 ± 0.1
pGBT9	White	0.2 ± 0.1

Fig. 5-9. The second half of the amino-terminal domain of LBR is necessary and sufficient for HP1-LBR interactions. The left panel shows the diagrams representing various amino-terminal domain of human LBR fused with GAL4-DNA binding domain. Names of each corresponding construct are given and the amino acids included in the fusion proteins are indicated. cDNAs for LBRAT domains are cloned in-frame downstream of the GAL4 activation domain in plasmid pGBT9. The right panel shows β-galactosidase activities of the cotransformants of pGBT9-LBRAT constructs and pACT2-HP1$^{Hs\alpha 2-191}$. "Blue" indicates a positive protein-protein interaction. "White" indicates a negative result. pGBT9 was used as a negative control cotransformed with pACT2-HP1$^{Hs\alpha 2-191}$. The values of the liquid assay, in standard units, are means ± standard errors (n = 3).

Discussion

Our findings show that human heterochromatin proteins homologous to *Drosophila* HP1 interact with LBR, an integral membrane protein of the inner nuclear membrane. This molecular interaction can potentially explain the observed association of the heterochromatin with the inner nuclear membrane in higher eukaryotic cells. These two human heterochromatin proteins, $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$, were the only two proteins that interacted with the amino-terminal domain of LBR from approximately 10^6 recombinant clones in a yeast two-hybrid screen. This selectivity suggests a high specificity of the interactions. The specificity of the interaction was further addressed by the examination of direct *in vitro* interactions between $HP1^{Hs\alpha}$ and the amino-terminal domain of LBR under stringent conditions utilizing various salt and detergent concentrations. Identification of the essential domains for HP1-LBR interactions suggests that these two proteins more likely bind via interactions between secondary structural motifs and not ionic binding. In addition, we demonstrated that LBR fusion proteins can extract HP1 proteins from cell lysates, and antibodies that immunoprecipitate LBR co-precipitate HP1 proteins. Finally, a portion of $HP1^{Hs\alpha}$ expressed in human cells is co-localized with LBR at the nuclear envelope. These data demonstrate that HP1-LBR interactions can occur in higher eukaryotic cells.

In *Drosophila melanogaster*, heterochromatin is associated with PEV. In PEV, when an active gene in euchromatin is translocated near constitutive heterochromatin, transcription of this gene can be variably repressed (Muller 1930; Henikoff 1990). PEV has also been demonstrated to be associated with altered chromatin structure (Wallrath and Elgin, 1995). Heterozygous HP1 mutant proteins in *Drosophila* can demonstrate suppression of PEV, probably because the formation of stable heterochromatin is defective (Eissenberg *et al.*, 1990; 1992; Henikoff, 1990; Paro, 1990; Paro and Hogness 1991). Furthermore, *Drosophila* mutants in polycomb (Pc) demonstrate developmental abnormalities because homeotic selector genes active in embryogenesis are not

appropriately repressed, also presumably because heterochromatin formation is defective (Paro, 1990; Paro and Hogness, 1991). These genetic studies and localization of HP1 and other chromodomain proteins demonstrate that they are important in the formation and function of heterochromatin. None of the HP1 proteins or other chromodomain proteins can bind to DNA directly, suggesting that they are associated with heterochromatin via protein-protein interactions (Paro, 1990). Partners of HP1 proteins have not been identified. LBR therefore represents the first binding partner for HP1 proteins. Since LBR can also bind to double-stranded DNA (Ye and Worman, 1994), it may provide a link between DNA and chromodomain proteins that are attached to the nuclear envelope.

In higher eukaryotic cells, some heterochromatin is associated with the nuclear envelope, but most is distributed in the nuclear core. Our observation that only a potion of HP1 staining overlap with LBR is consistent with previous findings (Franke *et al.*, 1981; Bouteille *et al.*, 1983; Mathog *et al.*, 1984). The differential distribution of heterochromatin suggests that its three-dimensional organization in the nucleus may arise from multiple hierarchical protein-protein and DNA-protein interactions. Certain intranuclear substructures, for example, the nucleoli, may provide other sites for heterochromatin attachment. At these site, proteins with similar function as LBR, or even other proteins homologous to LBR, may bind to HP1 proteins. In *Drosophila* polytene chromosomes, HP1 is located in several different heterochromatic regions including centromeres, telomeres and throughout polytene chromosome IV (James *et al.*, 1989). The homologous SWI6 protein of *Schizosaccharomyces pombe* has also been localized to centromeres, telomeres and silent mating-type loci (Ekwall *et al.*, 1995). It is possible that these different heterochromatin regions contain different proteins to target them to different domains of the nucleus, including the inner nuclear membrane, to generate a complex three-dimensional chromatin organization. Therefore, the binding of HP1 proteins to LBR may be only one of the multiple protein-protein interactions involved in the formation of large DNA-protein complexes responsible for heterochromatin packaging, stabilization and

targeting to different domains of the nucleus. This model is supported by studies on transcription silencing at telomeres in budding yeast, in which the heterochromatic silencing complexes contain multiple DNA-binding and other proteins including histone H3, histone H4, RAP1, SIR3 and SIR4 (Palladino *et al.*, 1993; Moretti *et al.*, 1994; Cockell *et al.*, 1995; Hecht *et al.*, 1995). These silencing complexes are also localized to the nuclear envelope in yeast and although an interaction between them and the inner nuclear membrane has been observed, the membrane proteins(s) has not been identified (Cockell *et al.*, 1995; Hecht *et al.*, 1995). Since telomere silencing in yeast is analogous to gene repression by heterochromatin in higher eukaryotic cells, it can be inferred that proteins other than HP1 and LBR are involved in the formation of heterochromatin at the nuclear envelope in higher eukaryotic cells. Likewise, it is possible the proteins functionally analogous to LBR exist in the yeast inner nuclear membrane that are responsible for the localization of telomere silencing complexes to the nuclear periphery.

In addition to HP1 proteins, LBR also binds to B-type lamins (Worman *et al.*, 1988; Simos and Georgatos, 1992; Smith and Blobel, 1994; Ye and Worman 1994), thus LBR may have a duel function in anchoring both the lamina and the heterochromatin to the inner nuclear membrane in higher eukaryotic cells. These two functions may be carried out by the independent domains of the amino-terminal domain of LBR. The first 60 amino acids may be involved in the B-type lamin binding, the following 30 amino acids Ser-Arg-rich stretch binds to DNA, and the last 100 amino acids binds to HP1. Whether the B-type lamin binding and heterochromatin binding are mutually exclusive or co-operative is not known. To summarize these findings, a diagram illustrating interactions of LBR with the nuclear lamina and heterochromatin is shown in Fig. 5-10.

The nuclear lamina has also been proposed to function in attaching the chromatin to the nuclear envelope, and interactions between chromatin and A-type and B-type lamins have been reported to occur via histones (Yuan *et al.*, 1991; Taniura *et al.*, 1995). However, these interactions cannot explain the specific association of heterochromatin at

the nuclear envelope. Furthermore, A-type lamins are not expressed in many cells (Guilly *et al.*, 1987; Lebel *et al.*, 1987; Stewart and Burke 1987) suggesting their role in chromatin binding *in vivo* may not be essential. Other integral membrane proteins may also function in chromatin attachment to the inner nuclear membrane. LAP2 has been shown to interact with whole chromosomes (Foisner and Gerace, 1993) but its specificity for heterochromatin has not been demonstrated. Thus, it is possible that LBR plays the pivotal role in the attachment of heterochromatin to the inner nuclear membrane by binding to HP1 and DNA. The interactions of lamins and other inner nuclear membranes with heterochromatin may be less specific and function to strengthen the chromatin-nuclear envelope interaction in some cells.

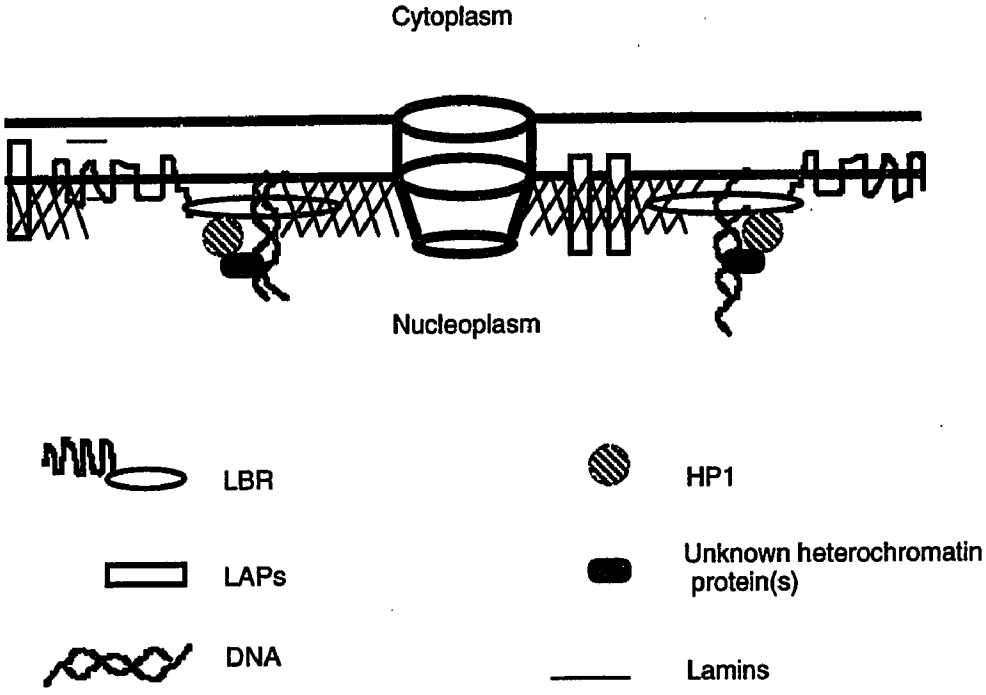


Fig. 5-10. Diagram showing the interactions of LBR with the lamina and heterochromatin at the nuclear envelope. The symbols in the diagram representing different components are indicated. The nuclear envelope is illustrated in cross-section, showing a nuclear pore complex and the outer and inner nuclear membranes. This diagram is not drawn in proportion to the size of different components.

Acknowledgments

We thank J.-C. Courvalin for invaluable advice and for production of anti-LBR antibodies; R. A. Lazzarini for use of the confocal microscope; V. L. Friedrich and X. Zhang for help with confocal microscopy; W. C. Earnshaw, S. J. Elledge, S. Fields and J. Licht for reagents, P. Küssel for help with sequence alignments and S. C. R. Elgin for helpful discussions.

Chapter 6. General Discussion on Protein-Protein and Protein-Chromatin Interactions at the Nuclear Envelope

1. Interactions of the Integral Proteins of the Inner Nuclear Membrane with the Nuclear Lamina

The nuclear lamina associated with the inner nuclear membrane is a meshwork of intermediate filament proteins (Aebi *et al.*, 1986). Lamins are the protein components of the nuclear lamina (Gerace *et al.*, 1978). Four different lamins including lamins A, B1, B2 and C have been found in most terminally differentiated mammalian somatic cells (Nigg, 1989). Several integral inner nuclear membrane proteins with lamin binding affinity have been proposed to be responsible for the association of the lamina to the inner nuclear membrane (Worman *et al.*, 1988b; 1990; Senior and Gerace, 1988; Foisner and Gerace, 1993). Among these proteins, LBR has been identified protein that specifically binds to B-type lamins *in vitro* and *in vivo* (Worman *et al.*, 1988b; Simos and Georgatos, 1992; Smith and Blobel, 1994). In the present study, we demonstrated that the nucleoplasmic amino-terminal domain of human LBR can bind to B-type lamins independently *in vitro*. The amino-terminal domains of other lamin binding proteins (LAP1C and LAP2) are also localized at the nucleoplasm (Furukawa *et al.*, 1995; Martin *et al.*, 1995). It is likely that the nucleoplasmic domains of these proteins are also responsible for their association with lamins.

The significance of the presence of multiple lamin binding proteins at the inner nuclear membrane is not clear. LBR specifically binds to B-type lamins (Worman *et al.*, 1988b). LAP1A, 1B, 1C binds to lamins A, B1 and C while LAP2 associates with lamin B1 only (Senior and Gerace, 1988; Foisner and Gerace, 1993). The presence of specific B-type lamin binding proteins and the constitutive expression of B-type lamins suggest that B-type lamins may have functions that are different from lamins A and C. It is possible that B-type lamins are the core components in the organization of the nuclear lamina and the incorporation of lamins A and C contributes to the complexity of

structure and functions of the nuclear lamina. Integral membrane proteins such as LAP1A, LAP1B and LAP1C that have general lamin binding affinities may also contribute to the attachment of the nuclear lamina at the inner nuclear membrane.

The nucleoplasmic domains of LBR, LAP1C and LAP2 all have consensus sites for phosphorylation by $p34^{cdc2}$ protein kinase (Worman *et al.*, 1990, Ye and Worman, 1994; Furukawa *et al.*, 1995; Martin *et al.*, 1995). One of the $p34^{cdc2}$ phosphorylation sites of LBR is cell cycle-dependent (Courvalin *et al.*, 1992; Chapter 3). Phosphorylation of LBR affects it's B-type lamin binding affinity (Appelbaum *et al.*, 1990) and mitotic phosphorylation of LAP2 inhibits its B-type lamin binding as well as chromosome binding. (Foisner and Gerace, 1993). $p34^{cdc2}$ is the key protein kinase that promotes mitosis and is responsible for the disassembly of the nuclear lamina (Peter *et al.*, 1990; Dessev *et al.*, 1991). Thus the simultaneous phosphorylation of the lamin binding proteins and lamins by $p34^{cdc2}$ kinase may weaken the association of the nuclear lamina with the nuclear inner membrane and lead to the breakdown of the nuclear envelope at the onset of mitosis.

2. Interactions of Chromatin with Nuclear Envelope Proteins

In interphase cells, chromatin, especially heterochromatin, is associated with the nuclear envelope. The association of chromatin with the nuclear envelope is important for cellular functions including replication of chromatin and regulation of gene expression (Blobel, 1985; Goldberg, 1995). The transcriptionally active euchromatin is localized to the NPCs and the interior of the nuclear core (Blobel, 1985). One NPC protein Nup153 has been demonstrated to be able to associate with DNA (Sukegawa and Blobel, 1993) suggesting that NPC components can directly involved in the organization of chromatin in the cells. This finding is a preliminary evidence of the "gene gating" hypothesis that NPC components bind to transcription-active genes to facilitate the export of transcripts into the cytoplasm (Blobel, 1985).

Chromatin associated with the nuclear envelope is mainly the transcriptionally inactive heterochromatin (Mathog *et al.*, 1984; Blobel, 1985; Hochstrasser *et al.*,

1986). The formation and maintenance of heterochromatin is important for the repression of gene expression in cell differentiation and development (Henikoff, 1990). The best example is PEV in *Drosophila* (Muller, 1930). Lamins have been demonstrated to be able to associate with chromatin or DNA (Glass and Gerace, 1990; Shoeman and Traub, 1990; Yuan *et al.*, 1991; Luderus *et al.*, 1992; 1995; Glass *et al.*, 1993; Taniura *et al.*, 1995). LAP2 of the inner nuclear membrane can also associate with chromosomes (Foisner and Gerace). However, specific interactions between these proteins and chromatin or heterochromatin components have not been demonstrated. The present findings that LBR interacts with both DNA (Chapter 3) and heterochromatin binding proteins (Chapter 5) suggest that heterochromatin associates the inner nuclear membrane via both protein-protein and DNA-protein interactions. Thus, LBR, in addition to functioning as an anchoring site for nuclear lamina, may also play an important role in the formation and maintenance of heterochromatin at the nuclear envelope. Lamins may interact with chromatin in less specific ways or with other components of chromatin. Whether LAP2 interacts chromatin during interphase is not known.

Chromatin undergoes constant changes in cells. Changes in histones, DNA and heterochromatin packaging constantly occur to regulate the activation or repression of gene expressions (Lewin, 1994). Alterations in the interaction of LBR with HP1 proteins, possibly induced by covalent modifications, could potentially result in dramatic changes in chromatin organization. LBR undergoes phosphorylation catalyzed by $p34^{cdc2}$ protein kinase in mitosis (Courvalin *et al.*, 1992; Chapter 3), when the inner nuclear membrane breaks down into vesicles and dissociates from the lamina and the chromatin (Chaudhary and Courvalin, 1993). LBR is also phosphorylated by other protein kinases in interphase when the membrane is associated with these structures (Appelbaum *et al.*, 1990; Simos and Georgatos, 1992). Hence, it is possible that the phosphorylation of LBR, along with the phosphorylation of HP1 proteins (Eissenberg *et al.*, 1994), can induce profound variations in heterochromatin formation, nuclear structure and gene expression at various times during the cell cycle. In

addition, the mRNA level of human LBR can be regulated by IL-6 (Chapter 3). The human LBR gene has binding sites for several transcription factors (Schuler *et al.*, 1994). These findings suggest that LBR expression can be regulated and the differential expression of LBR may regulate the organization and function of heterochromatin.

3. Nuclear Envelope Proteins and the Reassembly of Nuclear Envelope at the End of Mitosis

The interactions of integral membrane proteins with lamins and chromatin are also likely involved in the reassembly of the nuclear envelope at the end of mitosis. Several studies have suggested that lamins are responsible for the targeting of nuclear membrane vesicles to chromosomes to initiate the reassembly of nuclear envelope (Burke and Gerace, 1986; Daubavalle *et al.*, 1991; Ulitzur *et al.*, 1992). In contrast, other work have suggested a lamin-independent model in which a membrane protein(s) is responsible for the membrane targeting (Wilson and Newport, 1988; Newport *et al.*, 1990; Meier *et al.*, 1991). In undifferentiated mammalian cells and cancer cells, only B-type lamins are expressed (Guilly *et al.*, 1987; Stewart and Burke, 1987; Worman *et al.*, 1988a). In these A-type lamin deficient cells, it could only be the B-type lamins that initiate the nuclear membrane targeting, if the lamin-dependent mechanism is true. However, it has been demonstrated that B-type lamins are targeted to chromatin at the same time as lamins A and C, which is later than the targeting of LBR-containing vesicles to the chromatin (Chaudhary and Courvalin, 1993). LAP2 also localizes to the surfaces of chromosomes earlier than B-type lamins and lamins A and C (Foisner and Gerace, 1993). Thus, nuclear envelope reassembly is likely initiated by the targeting of integral membrane proteins such as LBR and LAP2 to chromosomes. Whether LBR and LAP2 target the same population of membrane vesicles or whether they target at the same time to chromatin are not known. LAP2 can bind to mitotic chromosomes and the binding is regulated by phosphorylation (Foisner and Gerace, 1993), but the ligand(s) of LAP2 on chromosomes is not known. In the present study, we have demonstrated

that LBR can bind to DNA and heterochromatin components. Thus, these interactions can be responsible for the targeting of LBR-containing membrane vesicles to reassemble the nuclear envelope at the end of mitosis.

Chapter 7. References

Aaronson, R., and Blobel, G. (1974). On the attachment of the nuclear pore complex. *J. Cell Biol.* 62: 746-754.

Aasland, R., and Stewart, A.F. (1995). The chromo shadow domain, a second chromo domain in heterochromatin-binding protein 1, HP1. *Nucleic Acids Res.* 23: 3168-3173.

Aebi, U., Cohn, J., Buhle, L., and Gerace, L. (1986). The nuclear lamina is a meshwork of intermediate filaments. *Nature* 323: 560-564.

Aitchison, J.D., Blobel, G., and Rout, M.P. (1995). Nup120p: a yeast nucleoporin required for NPC distribution and mRNA transport. *J. Cell Biol.* 131: 1677-1697

Akey, C.W., and Goldfarb, D.S. (1989). Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.* 109: 971-982.

Albers, K., and Fuchs, E. (1987). The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma cell lines. *J. Cell Biol.* 106: 791-806.

Albers, K., and Fuchs, E. (1992). The molecular biology of intermediate filament proteins. *Int. Rev. Cytol.* 134: 243-279.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1989). *Molecular Biology of the Cell* 2nd ed. Garland Publishing, Inc. New York & London.

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., and Berthet, J. (1974). Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* 61: 201-212.

Appelbaum, J., Blobel, G., and Georgatos, S.D. (1990). *In vivo* phosphorylation of the lamin B receptor: Binding of lamin B to its nuclear membrane receptor is affected by phosphorylation. *J. Biol. Chem.* 265: 4181-4184.

Atschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic alignment search tool. *J. Mol. Biol.* 215: 403-241.

Bailer, S.M., Eppenberger, H.M. , Griffiths, G., and Nigg. E.A. (1991). Characterization of a 54-KD protein of the inner nuclear membrane: evidence for cell cycle-dependent interaction with the nuclear lamina. *J. Cell Biol.* 114: 389-400.

Beck, L.A., Hosick, T.J., and Sinensky, M. (1988). Incorporation of a product of mevalonic acid metabolism into proteins of chinese hamster ovary cell nuclei. *J. Cell Biol.* 107: 1307-1316

Beck, L.A., Hosick, T.J., and Sinensky, M. (1990). Isoprenylation is required for the processing of lamin A precursor. *J. Cell Biol.* 110: 1489-1499.

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.* 120: 1671-1685.

Benavente, R., and Krohne, G. (1985). Involvement of nuclear lamins in postmitotic reorganization of chromatin as demonstrated by mciroinjection of lamin antibodies. *J. Cell Biol.* 103: 1847-1854.

Blobel, G. (1985). Gene gating: a hypothesis. *Proc. Natl. Acad. Sci. USA.* 82: 8527-8529.

Boman, A.L., Delannoy, M.R., and Wilson, K.L. (1992). GTP hydrolysis is required for vesicle fusion during nuclear envelope assembly *in vitro*. *J. Cell Biol.* 116: 281-294.

Bouteille, M., Bouvier, D., and Seve, A.P. (1983). Heterogeneity and territorial organization of the nuclear matrix and related structures. *Int. Rev. Cytol.* 83: 135-182.

Bowen, B., Steinberg, J., Laemmli, U.K., and Weintraub, H. (1980). Detection of DNA-binding protein by protein blotting. *Nucleic Acids Res.* 8: 1-20.

Broers, J.L.V., Raymond, Y., Rot, M.K., Kuijpers, H., Wagenaar, S., and Ramaekers, F.C.S. (1993). Nuclear A-type lamins are differentially expressed in human lung cancer subtypes. *Amer. J. Pathol.* 143: 211-220.

Brown, S.W. (1966). Heterochromatin. *Science* 151: 417-425.

Brown, M.S., and Golstein, J. L. (1993). Mad bet for Rab. *Nature* 366: 14-15

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.

Cance, W.G., Chaudhary, N., Worman, H.J., Blobel, G., and Cordon-Cardo, C. (1992). Expression of the nuclear lamins in normal and neoplastic human tissues. *J. Exp. Clin. Cancer Res.* 11: 233-246.

Chardhary, N., and Courvalin, J.-C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. *J. Cell Biol.* 122: 295-306.

Chen, W.N., Capieaux, E., Balzi, E., and Goffeau, A. (1991). The YGL022 gene encodes a putative transport protein. *Yeast* 7: 305-308.

Chen-Kiang, S., Hsu, W., Natkunam, Y., and Zhang, X. (1993). Nuclear signaling by interleukin-6. *Curr. Opin. Immnunol.* 5: 124.

Chien, C., Bartel, P.L., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA.* 88: 9578-9582.

Clark, R.F., and Elgin, S.C.R. (1992). Heterochromatin protein 1, a known suppressor of position-effect variegation, is highly conserved in *Drosophila*. *Nucleic Acids Res.* 22: 6067-6074.

Cockell, M., Palladino, R., Laroche, T., Kyrion, G., Lui, C. L., Lustig, A.J., and Gasser, S.M. (1995). The carboxyl termini of SIR4 and RAP1 affect SIR3 localization: evidence of a multiprotein complex required for yeast telemetric silencing. *J. Cell Biol.* 129: 909-924.

Coppel, R.L., NcNeilage, L.J., Surh, C.D., Van de Water, J., Spithill, T.W., Whittingham, S., and Gershwin, M.E. (1988). Primary structure of the human M2 mitochondrial autoantigen of primary biliary cirrhosis: dihydrolipoamide acetyltransferase. *Proc. Natl. Acad. Sci. USA.* 85: 7317-7321.

Cordes, V., Waizenegger, I., and Krohne, G. (1991). Nuclear pore complex glycoprotein p62 of *Xenopus laevis* and mouse: cDNA cloning and identification of its glycosylated region. *J. Cell Biol.* 121: 1-9.

Coulombe, P. A., and Fuchs, E. (1990). Tailless keratins assemble into regular intermediate filaments *in vitro*. *J. Cell Biol.* 111: 153-169

Courvalin, J.-C., Lassoued, K., Bartnik, E., Blobel, G., and Wozniazk, R.W. (1990a). The 210-kD nuclear envelope polypeptide recognized by human autoantibodies in primary biliary cirrhosis is the major glycoprotein of the nuclear pore. *J. Clin. Invest.* 86: 279-285.

Courvalin, J.-C., Lassoued, K., Worman, H.J., and Blobel, G. (1990b). Identification and characterization of autoantibodies against the nuclear envelope lamin B receptor from patients with primary biliary cirrhosis. *J. Exp. Med.* 172: 961-967.

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 $p34^{cdc2}$-type protein kinase. *J. Biol. Chem.* 267: 19035-19038.

Crevel, G., and Cotterill, L. (1991). DNA replication in cell-free extracts from *Drosophila melanogaster*. *EMBO J.* 10: 4361-4369.

Dabauvalle, M.C., Loos, K., Merkert, H., and Scheer, U. (1991). Spontaneous assembly of pore complex-containing membranes ("annulate tamellae") in *Xenopus* egg extract in the absence of chromatin. *J. Cell Biol.* 112: 1073-1082.

Dargemont, C., and Kuhn, L.C. (1992). Export of mRNA from microinjected nuclei of *Xenopus laevis* oocytes. *J. Cell Biol.* 118: 1-9.

Darnell, J., Lodish, H., and Baltimore, D. (1990). *Molecular Cell Biology* 2nd ed. W.H. Freeman and Company, New York and London.

Davis, L., and Blobel, G. (1986). Identification and characterization of a nuclear pore complex protein. *Cell* 45: 699-709.

Davis, L., and Blobel, G. (1987). Nuclear pore complex contains a family of glycoproteins that include p62: glycosylation through a previously unidentified cellular pathway. *Proc. Natl. Acad. Sci. USA.* 84: 7552-7556.

Dessev, G., Iovcheva-Dessev, C., Bischoff, J.R., Beach, D., and Goldman, R.D. (1991). A complex containing $p34^{cdc2}$ and cyclin B phosphorylates the nuclear lamins and disassembles nuclei of clam oocytes *in vitro*. *J. Cell Biol.* 112: 523-533.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the vax. *Nucleic Acids Res.* 12: 387-395.

Dingwall, C. (1993). Fingers in the pore. *Curr. Biol.* 3: 297-299.

Dodemont, H., Riemer, D., and Weber, K. (1990). Structure of an invertebrate gene encoding cytoplasmic intermediate filament (IF) proteins: implications for the origin and the diversification of IF proteins. *EMBO J.* 9: 4083-4094.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A., Lee, W.-H., and Elledge. S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* 7: 555-569.

Earnshaw, W.C. (1988). Mitotic chromosome structure. *Bioessays* 9: 147-150.

Earnshaw, W.C. (1991). Large scale chromosome structure and organization. *Curr. Topics. Struct. Biol.* 1: 237-244.

Earnshaw, W.C., and Rothfield, N.C. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with sceleroderma. *Chromosoma.* 91: 313-321.

Eissenberg, J.C., Ge, Y.W., and Hartnett, T. (1994). Increased phosphorylation of HP1, a heterochromatin-associated protein of *Drosophila*, is correlated with heterochromatin assembly. *J. Biol. Chem.* 269: 21315-21321.

Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V., and Elgin, S.C.R. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA.* 87: 9923-9927.

Eissenberg, J.C., Morris, G.D., Reuter, G., and Hartnett, T. (1992). The heterochromatin-associated protein is an essential protein in *Drosophila* with dosage-dependent effects on position-effect variegation. *Genetics* 131: 345-352.

Ekwall, K., Javerzat, J.-P., Lorentz, A., Schmidt, H., Cranston, G., and Allshire, R. (1995). The chromodomain protein SWI6: a key component at fission yeast centromeres. *Science* 269: 1429-1431.

Enoch, T., Peter, M., Nurse, P., and Nigg, E.A. (1991). $p34^{cdc2}$ acts as a lamin kinase in fission yeast. *J. Cell. Biol.* 112: 797-807.

Farbre, E., and Hurt, E.C. (1994). Nuclear Transport. *Curr. Opin. Cell Biol.* 6: 335-342.

Farnsworth, C.C., Wolda, S.L., Gelb, M.H., and Glomset, J.A. (1989). Human lamin B contains a farnesylated cysteine residue. *J. Biol. Chem.* 264: 20422-20429.

Featherstone, C., Darby, M.K., and Gerace, L. (1988). A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA *in vivo. J. Cell Biol.* 107: 1289-1297.

Fields, S., and Song, O.K. (1989). A novel genetic system to detect protein-protein interactions. *Nature* 340: 245-246.

Finlay, D.R., Newmeyer, D.D., Price, T.M., and Forbes, D.J. (1987). Inhibition of *in vitro* nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* 104: 189-200.

Firmbach-Kraft, I., and Stick, R. (1993). The role of CaaX-dependent modifications in membrane association of *Xenopus* nuclear lamin B3 during meiosis and the fate of lamin B3 in transfected mitotic cells. *J. Cell Biol.* 123: 1611-1670.

Fisher, D.Z., Chaudhary, N., and Blobel, G. (1986). cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc. Natl. Acad. Sci. USA.* 83: 6450-6454.

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.

Forbes, D.G. (1992). Structure and function of the nuclear pore complex. *Annu Rev. Cell Biol.* 8: 495-527.

Franke, W.W., Scheer, U., Krohne, G., and Jarasch, E.D. (1981). The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91: 39-50.

Franke, W.W. (1974). Structure, biochemistry, and functions of the nuclear envelope. *Int. Rev. Ctyol. Suppl.* 4: 71-236.

Fritz, C.C., Zapp, M.L., and Green, M.R. (1995). A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature* 376: 530-533.

Furukawa , K., Panté, N., Aebi, U., and Gerace, L. (1995). Cloning of a cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope. *EMBO J.* 14: 1626-1636.

Furukawa, K., and Hota, 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., 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.

Garcia-Bustos, J., Heitman, J., and Hall, M.N. (1991). Nuclear protein localization. *Biochem. Biophys. Acta* 1071: 83-101.

Georgatos, S.D., Maroulakou, I., and Blobel, G. (1989). Lamin A, lamin B and lamin B receptor analogues in yeast. *J. Cell Biol.* 108: 2069-2082.

Gerace, L., Appeaublum, B., 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., and Blobel, G. (1980). The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell* 19: 277-287.

Gerace, L., Comeau, C., and Benson, M. (1984). Organization and modulation of nuclear lamina structure. *J. Cell Sci. Suppl.* 1:137-160.

Gerace, L., and Foisner, R. (1994). Integral membrane proteins and dynamic organization of the nuclear envelope. *Trends Cell Biol.* 4: 127-131.

Gerace, L., Ottaviano, Y., and Kondor-Koch, C. (1982). Identification of a polypeptide of the nuclear pore complex. *J. Cell Biol.* 95: 826-837.

Gieffers, C., and Krohne, G. (1991). *In vitro* reconstitution of recombinant lamin A and a lamin A mutant lacking the carboxyl-terminal tail. *Eur. J. Cell Biol.* 55: 191-199.

Gill, S., Wong, P.C., Monteiro, M.J., and Cleveland, D.W. (1990). Assembly proterties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. *J. Cell Biol.* 111: 2005-2019.

Glass, C.A., Glass, J.R., Taniura, H., Hasel, K.W., Blevitt. J.M., and Gerace, L. (1993). The α-helical rod domain of human lamins A and C contains a chromatin binding site. *EMBO J.* 11: 4413-4424.

Glass, J.R., and Gerace, L. (1990). Lamins A and C bind and assemble at the surface of mitotic chromosomes. *J. Cell Biol.* 111: 1047-1057.

Goldberg, M.W., and Allen, T.D. (1995). Structural and functional organization of the nuclear envelope. *Curr. Opin. Cell Biol.* 7: 301-309.

Goldman, A.E., Moir, R.D., Montag-Lowy, M., Stewart, M., and Goldman, R.D. (1992). Pathway of incorporation of microinjected lamin A into the nuclear envelope. *J. Cell Biol.* 119: 725-735.

Goss, V.L., Hocevar, B.A., Thompson, L.J., Stratton, C.A., Burns, D.J., and Fields, A.P. (1994). Identification of nuclear beta II protein kinase C as a mitotic lamin kinase. *J. Biol. Chem.* 269: 19074-19080.

Greber, U.F., Senior, A., and Gerace, L. (1990). A major glycoprotein of the nuclear pore complex is a membrane spanning polypeptide with a large luminal domain and a small cytoplasmic tail. *EMBO. J.* 9: 1495-1502.

Gruenbaum, Y., Landesman, Y., Drees, B., Bare, J.W., Saumweber, H., Paddy, M.R., Sedat, J.W., Smith, D.E., Benton, M.M., and Fisher, P.A. (1988). *Drosophila* nuclear lamin precursor Dmo is translated from either of two developmentally regulated mRNA species apparently encoded by a single gene. *J. Cell Biol.* 106: 585-597.

Guarente, L. (1983). Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* 101: 181-191.

Guilly, M.-N., Bensussan, A., Bourge, J.F., Bornens, M., and Courvalin J.-C. (1987). A human lymphoblastic cell line lacks lamins A and C. *EMBO. J.* 6: 3795-3799.

Guilly, M.-N., Kolb, J.P., Gosti, F., Godeau, G., and Courvalin, J.-C. (1990). Lamin A and C are not expressed at early stages of human lymphocyte differentiation. *Exp. Cell Res.* 189: 45-148.

Hallberg, E., Wozniak, R.W., and Blobel, G. (1993). An integral membrane protein of the pore membrane domain of the nuclear envelope contains a nucleoporin-like region. *J. Cell Biol.* 122: 513-521.

Hancock, R., and Boulikas, T. (1982). Functional organization in the nucleus. *Int. Rev. Cytol.* 79: 165-214.

Harel, A., Zlotkin, E., Nainudel-Epsztyn, S., Feinstein, N., Fisher, P. A., and Gruenbaum, Y. (1989). Persistence of a major nuclear envelope antigen in an envelope-like structure during mitosis in *Drosophila melanogaster* embryos. *J. Cell Sci.* 94: 463-470.

Harper, J.W., Adami, G.R., Wei, N., Keyomasi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75: 805-816.

Harris, C., A., Andryuk, P.J., Cline, S., Chan, H.K., Natarajan, N., 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.

Hatzfeld, M., and Weber, K. (1990). The coiled coil of *in vitro* assembled keratin filament is a heterodimer of type I and type II keratins: use of site mutagensis and recombinant protein expression. *J. Cell Biol.* 110: 1199-1210.

Heald, R., and Mckeon, F. (1990). Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* 61: 579-589.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. (1995). Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. *Cell* 80: 583-592.

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

Heitlinger, E., Peter, M., Villiger, W., Nigg, E.A., and Aebi, U. (1992). The role of the head and tail domain in lamin structure and assembly: analysis of baterially expressed chicken lamin A and truncated B2 lamins. *J. Struct. Biol.* 108:74-89.

Henikoff, S. (1990). Position-effect variegation after 60 years. *Tends Genet.* 6: 422-426.

Hennekes, H., and Nigg, E.A. (1994). The role of isoprenylation in membrane attachment of nuclear lamins: a single point mutation prevents proteolytic cleavage of the lamin A precursor and confers membrane binding properties. *J. Cell Sci.* 107: 1019-1029.

Hinshaw, J.E., Carragher, B.O., and Milligan, R.A. (1992). Architecture and the design of the nuclear pore complex. *Cell* 69: 1133-1141.

Hochstrasser, M., Mathog, D., Gruenbaum, Y., Saumweber, H., and Sedat, J. (1986). Spatial organization of chromosomes in the salivary gland nuclei of *Drosophila melanogaster*. *J. Cell Biol.* 102: 112-121.

Höger, T. H. , Krohne, G. and Franke, W.W. (1988). Amino acid sequence and molecular characterization of murine lamin B as deduced from cDNA clones. *Eur. J. Cell Biol.* 47: 283-290.

Höger, 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* 99: 379-390.

Holtz, T.H., Tanaka, R.A., Hartwig, J., and McKeon, F. (1989). The CaaX motif of lamin A functions in conjunction with the nuclear localization signal to target assembly to the nuclear envelope. *Cell* 59: 969-97.

Hozak, P., Sasseville, M-J., Raymond, Y., and Cook, P.R. (1995). Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. *J. Cell Sci.* 108: 635-644.

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.

Hytiroglou, P., Choi, S.W., Theise, N.D., Chaudhary, N., Worman, H.J., and Thung, S.N. (1993). The expression of nuclear lamins in human liver: an immunohistochemical study. *Hum. Pathol.* 24: 169-172.

Ito, H., Jukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153: 163-168.

James, T. C., Eissenberg, J.C., Craig, C., Deitrich, V., Hobson, A., and Elgin, S.C.R. (1989). Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of *Drosophila*. *Eur. J. Cell Biol.* 50: 170-180.

James, T.C. and Elgin, S.C.R. (1986). Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. *Mol. Cell Biol.* 6: 3862-3872.

Kalderon, D., Roberts, B.L., Richardson, W.D., and Smith, A.E. (1984). A short amino acid sequence able to specify nuclear localization. *Cell* 39: 499-509.

Kaufmann, S.H. Mabry, M., Jasti, R., and Shaper, J.H (1994). Differential expression of nuclear envelope lamins A and C in human lung cancer cell lines. *Cancer Res.* 51: 581-586.

Kitten, G.T., and Nigg, E.A. (1991). The CaaX motif is required for isoprenylation, carboxyl methylation, and nuclear membrane association of lamin B2. *J. Cell. Biol.* 113: 13-23.

Klymkowsky, M.W., Bachant, J.B., and Domingo, A. (1989). Function of intermediate filaments. *Cell Motil. Ctyoskeleton* 14: 109-331.

Krohne, G., Waizenegger, I., and Hoger, T.H. (1989). The conserved carboxyl-terminal cysteine of nuclear lamins is essential for lamin association with the nuclear envelope. *J. Cell Biol.* 109: 2003-2011.

Krohne, G., Wolin, S.L., Mckeon, F.D., Franke, W.W., and Kirschner, M.W. (1987). Nuclear lamin L1 of *Xenopus laevis*: cDNA cloning, amino acid sequence and binding specificity of a member of the lamin B subfamily. *EMBO J.* 6: 3801-3808.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lassoued, K., Danon, F., and Brouet, J.-C. (1991). Human autoantibodies to lamin B receptor are also anti-idiotypic to certain anti-lamin B antibodies. *Eur. J. Immunol.* 21: 1959-1962.

Lebel, S., Lampron, C., Royal, A., and Raymond, Y. (1987). Lamins A and C appear during retinoic acid-induced differentiation of mouse embryonal carcinoma cells. *J. Cell Biol.* 105: 1099-1104.

Lewin, B. (1994). Chromatin and gene expression: constant questions but changing answers. *Cell* 79: 397-406.

Lin, F., Noyer, C.M., Ye, Q., Courvalin, J.-C., and Worman, H.J. (1996). Autoantibodies from patients with primary biliary cirrhosis recognize a region within the nucleoplasmic domain of inner nuclear membrane protein LBR. *Hepatology* 23: 57-61.

Lin, F., and Worman, H.J. (1993). Structural organization of the human gene encoding nuclear lamin A and nuclear lamin C. *J. Biol. Chem.* 268: 16321-16326.

Lin, F., and Worman, H.J. (1995). Structural organization of the human gene (LMB1) encoding nuclear lamin B1. *Genomics* 27: 230-236.

Lohka, M.J., and Masui, Y. (1983). Formation *in vitro* of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science* 220: 719-721.

Loo, S., and Rine, J. (1994). Silencers and domains of generalized repression. *Science* 264: 1768-1771.

Lorentz, A., Ostermann, K., Fleck, O., and Schmidt, H. (1994). Switching gene SWI6, involved in repression of silent mating-type loci in fission yeast, encodes a homologue of chromatin-associated proteins from *Drosophila* and mammals. *Gene* 143: 139-143.

Lorenz, R.T., and Parks, L.W. (1992). Cloning, sequencing, and disruption of the gene encoding sterol C-14 reductase in *Saccharomyces cerevisiae*. *DNA Cell Biol.* 11: 685-692.

Lourim, D., and Krohne, G. (1993). Membrane-associated lamins in *Xenopus* egg extracts: identification of two vesicle populations. *J. Cell. Biol.* 123: 501-512.

Low, P.S. (1986). Structure and function of the cytoplasmic domain of band3: center of erythrocyte membrane-peripheral protein interactions. *Biochim. Biophys. Acta* 864: 145-167.

Luderus, M.E., den-Blaauwen. J.L., de-Smit, O.J., Compton, D.A., and van-Driel, R. (1995). Binding of matrix attachment regions to lamin polymers involves single-stranded regions and the minor groove. *Mol. Cell Biol.* 14: 6297-6305.

Luderus, M.E., Graaf, A.D, Mattia, E., den-Blaauwen, J.L., Grande, M.A., Jong, L.D., and van-Driel, R. (1992). Binding of matrix attachment regions to lamin B1. *Cell* 70: 949-959.

Lutz, R. J., Trujillo, M.A., Denham, K.S., Wenger, L., and Sinensky, M. (1992). Nucleoplasmic localization of prelamin A: implication for prenylation-dependent lamin A assembly into the nuclear lamina. *Proc. Natl. Acad. Sci. USA.* 89: 3000-3004.

Manuelidis, L. (1990). A view of interphase chromosomes. *Science* 250: 1533-1540.

Masumoto, H., Masukata, H., Maro, Y., Nozaki, N., and Okazaki, T. (1989). A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J. Cell Biol.* 190: 1963-1973.

Martin, L., Crimaudo, 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.

Mathog, D., Hochstrasser, M., Gruenbaum, Y., Saumweber, H., and Sedat, J. (1984). Characteristic folding pattern of the polytene chromosomes in *Drosophila* salivary glands nuclei. *Nature* 308: 414-421.

Maul, G.G. (1977). The nuclear and the cytoplasmic pore complex: structure, dynamics, distribution and evolution. *Int. Rev. Cytol. Suppl.* 6: 75-186.

McKeon, F.D., Kirschner, M.W., and Caput, D. (1986). Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature* 319: 463-468.

Meier, J., Campbell, K.H.S., 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.

Meier J., and Georgatos, S.D. (1994). Type B lamins remain associated with the integral nuclear envelope protein p58 during mitosis: implications for nuclear reassembly. *EMBO J.* 13: 1888-1898.

Meng, J.J., Khan, S., and Ip, W. (1996). Intermediate filament protein domain interactions as revealed by two-hybrid screens. *J. Biol. Chem.* 271: 1599-1604.

Messmer, S., Franke, A., and Paro, R. (1992). Analysis of the function role of the polycomb chromo domain in *Drosophila melanogaster. Genes & Dev.* 6: 1241-1254.

Miake-Lye, R., and Kirschner, M.W. (1985). Induction of early mitotic events in a cell-free system. *Cell* 58: 361-372.

Moir, R.D, Donaldson, A.D., and Stewart, M. (1991). Expression in *Escherichia coli* of human lamin A and C: influence of head and tail domains on assembly properties and paracrystal formation. *J. Cell Sci.* 99: 363-372.

Moir, R.D., Montag-Lowy, M., and Goldman, R.D. (1994). Dynamics properties of nuclear lamins: lamin B is associated with sites of DNA replication. *J. Cell Biol.* 125: 1201-1212.

Moore, G.D., Procunier, J.D., Cross, D.P., and Grigliatti, T.A. (1979). Histone deficiencies in position-effect variegation in *Drosophila. Nature* 282: 312-314.

Moore, M.S., and Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell* 69: 939-950

Moore, M.S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* 365: 661-663.

Moore, M.S., and Blobel, G. (1994). Purification of Ran-interacting that is required for protein import into the nucleus. *Proc. Natl Acad. Sci. USA.* 91: 10212-10216.

Moreno, S., and Nurse, P. (1990). Substrates for $p34^{cdc2}$: *in vivo* veritas? *Cell* 61: 549-551.

Moretti, P., Freeman, K., Coodly, L., and Shore, D. (1994). Evidence that a complex of SIR proteins interacts with the silencer and telomere binding protein RAP1. *Genes & Dev.* 8: 2257-2269.

Muller, H. J. (1930). Types of visible variations induced by X-rays in *Drosophila. J. Genet.* 22: 299-334.

Newport, J. (1987). Nuclear reconstitution *in vitro*: stages of assembly around protein-free DNA. *Cell* 48: 205-217

Newport, J., and Dunphy, W. (1992). Characterization of the membrane binding and fusion events during nuclear envelope assembly using purified components. *J. Cell Biol.* 116: 295-306.

Newport, J., and Spann, T. (1987). Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell* 48: 219-230.

Newport, J., Wilson, K.L., and Dunphy, W.G. (1990). A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* 111: 2247-2259.

Nickowitz, R.E., and Worman, H. J. (1993). Autoantibodies from patients with primary biliary cirrhosis recognize a restricted region within the cytoplasmic tail of nuclear pore membrane glycoprotein gp210. *J. Exp. Med.* 178: 2237-2242.

Nickowitz, R.E., Wozniak, R.W., Schaffner, F., and Worman, H.J. (1994). Autoantibodies against integral membrane proteins of the nuclear envelope in patients with primary biliary cirrhosis. *Gastroenterology.* 106: 193-199.

Nicolini, C., Vernazza, G., Chiabrera., A., Maraldi, I.N., and Capitani, S. (1984). Nuclear pores and interphase chromatin: high resolution image analysis and freeze etching. *J. Cell Sci.* 72: 75-87.

Nigg, E.A. (1989). The nuclear envelope. *Curr. Opin. Cell Biol.* 1: 435-440.

Nigg, E.A. (1992). Assembly and cell cycle dynamics of the nuclear lamina. *Sem. Cell Biol.* 3: 245-253.

Nigg, E.A. (1993). Targets of cyclin-dependent protein kinases. *Curr. Opin. Cell Biol.* 5: 187-193.

Ottaviano, Y., and Gerace, L. (1985). Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* 266: 624-632.

Padan, R., Nainudel-Epszteyn, S., Goitein, R., Fainsod, A., and Gruenbaum, Y. (1990). Isolation and characterization of *Drosophila* nuclear envelope otefin cDNA. *J. Biol. Chem.* 265: 7808-7813.

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.

Paine, P.L., Moore, L.C., and Horowitz, S.B. (1975). Nuclear envelope permeability. *Nature* 254: 109-114.

Palladino, F., Laroche, T., Gilson, E., Axelrod, A., Pillus, L., and Gasser, S.M. (1993). SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. *Cell* 75: 531-542

Panté, M. and Aebi, U. (1993). The nuclear pore complex. *J. Cell Biol.* 122: 977-984.

Panté, N. and Aebi, U. (1994). Towards understanding the three dimensional structure of the nuclear pore complex at the molecular level. *Curr. Opin. Struct. Biol.* 4: 187-196.

Paro, R., and Hogness, D. (1991). The polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 88: 262-267.

Paulin-Levasseur, M., Scherbarth, A., Traub, U., and Traub, P. (1988). Lack of lamins A and C in mammalian hemopoietic cell lines devoid of intermediate filament proteins. *Eur. J. Cell Biol.* 47: 121-131.

Paro, R. (1990). Imprinting a determined state into the chromatin of *Drosophila*. *Trends Genet.* 6: 416-421.

Peter, M., Heitlinger, E., Haner, M., Aebi, U. and Nigg, E.A. (1991). Disassembly of *in vitro* formed lamin head-to-tail polymers by cdc2 kinase. *EMBO J.* 10: 1535-1544.

Peter, M., Nakagawa, J., Doree, M., Labbe, J.-C., and Nigg, E.A. (1990). *In vitro* disassembly of the nuclear lamina and M phase phosphorylation of lamins by cdc2 kinase. *Cell* 61: 591-602.

Pfaller, R., Smythe, C., and Newport, J.W. (1991). Assembly/disassembly of the nuclear membrane vesicles to chromatin *in vitro*. *Cell* 65: 209-217.

Platero, J.S., Hartnett, T., and Eissenberg, J.C. (1995). Functional analysis of the chromo domain of HP1. *EMBO J.* 14: 3977-3986.

Pollard, K.M., Chan, E.K.L, Grant, B.J., Sullivan, K.F., Tang, E,M., and Glass, C.A. (1990). *In vitro* posttranslational modification of lamin B cloned from a human T-Cell line. *Mol. Cell. Biol.* 10: 2164-2175.

Powers, J. A., and Eissenberg, J.C. (1993). Overlapping domains of heterochromatin-associated protein HP1 mediate nuclear localization and heterochromatin binding. *J. Cell Biol.* 120: 291-299.

Radu, A., Blobel, G., and Moore, M.S. (1995). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92: 1769-1773.

Riemer, D., Stuurman, N., Berrios, M., Hunter, C., Fisher, P.A., and Weber, K. (1995). Expression of *Drosophila* lamin C is developmentally regulated: analogies with vertebrate A-type lamins. *J. Cell Sci.* 108: 3189-3196.

Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* 64:615-623.

Rober, R.-A., Stauter, H., Weber, K., and Osborn, M. (1990). Cells of the cellular immune and hemopoietic system of the mouse lack lamins A/C: distinction versus other somatic cells. *J. Cell Sci.* 95: 587-598.

Rober, R-A., Weber, K., and Osborn, M. (1989). Differential timing of nuclear lamin A/C expression in the various organ of the mouse embryo and the young animal: a developmental study. *Development* 105: 365-378.

Rose, M.D., Winston, F., and Hieter, P. (1990). *Methods in Yeast Genetics: A Laboratory Course Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Rout, M.P., and Wente, S.R. (1994). Pores for thought: nuclear pore complex proteins. *Trends Cell Biol.* 4: 357-365

Saiki, R.K., Gelfand, D.H., Stofffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1987). *Science* 239: 487-491.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A laboratory Manual.* 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., Nicklen,S., and Couslson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74: 5463-5467.

Sapp, M., Knippers, R., and Richter, A. (1986). DNA binding properties of a 110 KDa nucleolar protein. *Nucleic Acids. Res.* 14: 6803-6821.

Saunders, W.S., Chue, C., Goebl, M., Craig, G., Clark, R.F., Powers, J.A., Eissenberg, J.C., Elgin, S.C.R., Rothfield, N.F., and Earnshaw, W.C. (1993). Molecular cloning of a human homologue of *Drosophila* heterochromatin protein HP1 using anti-centromere antibodies with anti-chromo specificity. *J. Cell Sci.* 104: 573-582.

Schuler, E., Lin, F., and Worman, H.J. (1994). Characterization of the human gene encoding LBR, an integral protein of the nuclear envelope inner membrane. *J. Biol. Chem.* 269: 11312-11317.

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.

Sherman, F., Fink, G.R., and Hicks, J.B., eds. (1986). *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shimanuki, M., Goebl, M., Yanagida, M., and Toda, T. (1992). Fission yeast *Sts1*+ gene encodes a protein similar to the chicken lamin B receptor and is implicated in drug pleiotropic, drug sensitivity, divalent cation-sensitivity, and osmoregulation. *Mol. Biol. Cell* 3: 263-273.

Shoeman, R., and Traub, P. (1990). The *in vitro* DNA-binding properties of purified nuclear lamin and vimentin proteins. *J. Biol. Chem.* 265: 9055-9061.

Silver, P.A. (1991). How proteins enter the nucleus. *Cell* 64: 489-497.

Simos, G., and Georgatos, S.D. (1992). The inner nuclear membrane protein p58 associates *in vivo* with a p58 kinase and the nuclear lamins. *EMBO J.* 11: 4027-4036.

Simos, G. and Georgatos, S.D. (1994). The lamin B receptor-associated protein p34 shares sequence homology and antigenic determinants with the splicing factor 2-associated protein p32. *FEBS Lett.* 346: 225-228.

Sinensky, M., McLain, T., and Fantle, K. (1994). The processing pathway of prelamin A. *J. Cell Sci.* 107: 2215-2218.

Singh, P.B. (1994). Molecular mechanisms of cellular determination: their relation to chromatin structure and parental imprinting. *J. Cell. Sci.* 107: 2653-2668.

Singh, P.B., Miller, J.R., Pearce, J., Kothary, R., Nurton, R.D., Paro, R., James, T.C., and Gaunt, S.J. (1991). A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acid Res.* 19: 789-794.

Skalli, O., and Goldman, R.D. (1991). Recent insights into the assembly, dynamics, and function of intermediate filament networks. *Cell Motil. Cytoskelteon.* 19: 67-79.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-S-transferase. *Gene* 67: 31-40.

Smith, D.E., and Fisher, P.A. (1984). Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* 99: 20-28.

Smith, D.E., and Fisher, P.A. (1989). Interconversion of *Drosophila* nuclear lamin isoform during oogenesis, early embryogenesis, and upon entry of cultured cells into mitosis. *J. Cell Biol.* 108: 255-265.

Smith, S. (1995). Cloning and sequence analysis of an ERG24 homolog from *Schizosaccharomyces pombe. Gene* 155: 139-140..

Smith, S., and Blobel, G. (1993). The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. *J. Cell Biol.* 120: 631-637.

Smith, S., and Blobel, G. (1995). CO localization of vertebrate lamin B and lamin B receptor (LBR) in nuclear envelopes and in LBR-induced membrane stacks of the yeast Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA.* 91: 10124-10128.

Soullam, B., and Worman, H.J. (1993). The amino-terminal domain of the lamin B receptor is a nuclear envelope targeting signal. *J. Cell Biol.* 120: 1093-1100.

Soullam, B., and Worman, H.J. (1995). Signals and structural features involved in integral membrane protein targeting to the inner nuclear membrane. *J. Cell Biol.* 130: 15-27.

Starr, C.M., D'Onoferio, M., Park, M.K., and Hanover, J.A. (1990). Primary sequence and heterologous expression of nuclear pore glycoprotein p62. *J.Cell Biol.* 110: 1861-1871.

Steinert, P.M., and Roop, D.R. (1988). Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* 57: 593-625.

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.

Stewart, M. (1990). Intermediate filaments: structure, assembly and molecular interactions. *Curr. Opin. Cell Biol.* 2: 91-100.

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.

Stick, R., and Schwarz, H. (1983). Disappearance and reformation of the nuclear lamina structure during specific stages of meiosis in oocytes. *Cell* 33: 949-953.

Sukegawa, J. and Blobel, G. (1993). A nuclear pore complex glycoprotein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell* 72: 29-38.

Suzuki, M. (1989). SPXX, a frequent sequence motif in gene regulatory proteins. *J. Mol. Biol.* 207: 61-84.

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.

Tartof, K.D., Hobbs, C., and Jones, M. (1984). A structural basis for variegation position effect. *Cell* 37: 869-878.

Ulitzur, N., and Gruenbaum, Y. (1989). Nuclear envelope assembly around sperm chromatin in cell-free preparations from *Drosophila* embryos. *FEBS. Lett.* 259: 113-116.

Ulitzur, N., Harel, A., Feinstein, N., and Gruenbaum, Y. (1992). Lamin activity is essential for nuclear envelope assembly in a *Drosophila* embryo cell free system. *J. Cell Biol.* 119: 17-25.

Varshavsky, A. (1987). Electrophoretic assay for DNA-binding proteins. *Methods Enzymol.* 151: 551-565.

Vigers, G.P.A., and Lohka, M.J. (1991). A distinct vesicle population targets membranes and pore complexes to the nuclear envelope in *Xenopus* eggs. *J. Cell Biol.* 112: 545-554.

Vorburger, K., Lehner, C.F., Kitten, G.T., Eppenberger, H.M., and Nigg. E.A. (1989). A Second higher vertebrate B-type lamin cDNA sequence determination and *in vitro* processing of chicken lamin B2. *J. Mol. Biol.* 208: 405-415.

Wallrath, L. L., and Elgin, S.C.R. (1995). Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes & Dev.* 9: 1263-1277.

Wang, N.P., Chen, P.-L., Huang, S., Donoso, L.A., Lee, W.-H., and Lee, E.Y. (1990). DNA binding activity of retinoblastoma protein is intrinsic to its carboxyl-terminal region. Cell Growth *Differentiation.* 1: 233-239.

Ward, G.E., and Krischner, M.W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell* 61: 579-590.

Waterston, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R.K., Green, P., Shownkeen, R., Halloran, N., Hawkins, T., Wilson, R., Berks, M., Du, Z., Tomas, K., Thierry-Mieg, J., and Sulston, J. (1992). A survey of expressed genes in *Caenorhabditis elegans. Nature Genet.* 1: 114-123.

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.

Weber, K., Plessmann, U., and Traub, P. (1990). Protein chemical analysis of purified murine lamin B identifies two distinct polypeptides B1 and B2. *FEBS Lett.* 2: 361-364.

Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* 82: 463-473.

Wente, S.R., Rout, M.P., and Blobel, G. (1992). Identification of genes encoding novel nucleoporins of the yeast nuclear pore complex. *J. Cell Biol.* 119: 705-723.

Wiese, C., and Wilson, K.C. (1993). Nuclear membrane dynamics. *Curr. Opin. Cell Biol.* 5: 387-394.

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.

Wolda, S.L., and Glomset, J.A. (1988). Evidence for modification of lamin B by a product of mevalonic acid. *J. Biol. Chem.* 263: 5997-6000.

Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M.F., Polakis, L., and McCormick, F. (1992). Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phospho-protein p62. *Cell* 69: 551-558.

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: 1534-1542.

Worman, H.J., Lazaridis, I., Georgatos, S.D. (1988a). Nuclear lamina heterogeneity in mammalian cells: differential expression of the major lamins and variations in lamin B phosphorylation. *J. Biol. Chem.* 263: 12135-12141.

Worman, H.J., Yuan, J., Blobel, G., and Georgatos, D.S. (1988b). A lamin B receptor in the nuclear envelope. *Proc. Natl. Acad. Sci. USA.* 85: 8531-8534.

Wozniak, R.W., Bartnick, E., and Blobel, G. (1989). Primary structure analysis of an integral membrane glycoprotein of the nuclear pore. *J. Cell Biol.* 108: 2083-

Wu., J., Matunis, M., Kraemer, D., Blobel, G, and Coutavas, E. (1995). Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A-homologous domain, and a leucine-rich region. *J. Biol. Chem.* 270: 14209-14213.

Wydner, 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: (in press).

Ye, Q., and Worman, H.J. (1994). Primary structure analysis and lamin B and DNA binding of human LBR, an integral protein of the nuclear envelope inner membrane. *J. Biol. Chem.* 269: 11306-11311.

Ye, Q., and Worman, H.J. (1995). Protein-protein interactions between human nuclear lamins expressed in yeast. *Exp. Cell Res.* 219: 292-298.

Yokoyama, N., Hayashi, N., Seki, T., Pante, N., Ohba, T., Nishii, K, Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M., and Nishimoto, T. (1995). A giant nucleopore protein that binds Ran/TC4. *Nature* 376:184-188.

Yuan, J., G., Simos, G. Blobel, and S. D. Georgatos. (1991). Binding of lamin A to polynucleosomes. *J. Biol. Chem.* 266: 9211-9215.

Zeligs, J., and Wollman, S. (1979). Mitosis in rat thyroid epithelial cells *in vivo*. 1. Ultralstructural changes in cytoplasmic organelles during the mitotic cycle. *J. Ultrastruct. Res.* 66: 53-77.