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MOLECULAR CHARACTERIZATION OF THE MAN ANTIGENS

by

© Deborah L. Blake

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LIST OF ABBREVIATIONS

3D	Three-dimensional
6-His	6-histidine
β-gal	β-galactosidase
A	Adsorbed
BrDU	Bromodeoxyuridine
CaPo4	Calcium phosphate
cpm	Counts per minute
dCTP	2'-deoxycytidine 5'-triphosphate
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleoside triphosphate
EC	Embryonal Carcinoma
ER	Endoplasmic Reticulum
FCS	Fetal Calf Serum
FITC	Fluorescein isocyanate
HI	Heat Inactivated
GTP	Guanosine 5'-triphosphate
hr	Hour
HRP	Horseradish Peroxidase
IF	Intermediate Filament
Ig	Immunoglobulin
IPTG	Isopropylthiogalactoside
kb	Kilobase
LAP	Lamin-associated Polypeptide
LB	Luria-Bertani
LBR	Lamin B receptor
LiCl	Lithium cloride
MAR	Matrix Attachment Region
MEM	Minimum Essential Medium
min	Minute
NB	Non-adsorbed
NE	Nuclear Envelope
NLS	Nuclear Localization Signal
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PEG	Polyethelene glycol
PKC	Protein Kinase C
RA	Retinoic acid
RB	Retinoblastoma
RT	Room Temperature
SAR	Scaffold Attachment Region
SB	Sample Buffer
sec	Second
SDS	Sodium Dodecyl Sulfate
TCA	Trichloro-acetic acid
UV	Ultra violet

ABSTRACT

The nuclear lamina consists of a filamentous network of proteins situated beneath the inner nuclear membrane and apposed to peripheral chromatin. As a consequence of its location, the nuclear lamina has been proposed to be involved in a number of different cellular functions, including structural support, signal transduction and higher order chromatin organization. With the exception of the family of nuclear lamin proteins, few other constituents of the lamina have been described.

I have used a human antiserum to further characterize a novel set of nuclear lamina proteins, termed the MAN antigens. These antigens comprise three major polypeptides with relative mobilities of 78, 58 and 40 kDa. During interphase, the MAN antigens co-localized with the lamins at the nuclear periphery, but were absent from intranuclear foci of lamin B. In cells which possessed micronuclei, both the MAN antigens and lamins A/C were observed to segregate within these structures, separate from lamin B. Through mitosis, lamins A/C were seen to disassemble in late prophase and reassemble in telophase. Conversely, lamin B and the MAN antigens began to disassemble only during late prometaphase and then reformed around segregating chromosomes in anaphase, prior to lamins A/C.

The human antiserum was used to screen a P19 embryonal carcinoma cDNA expression library for clones which encoded polypeptides immunologically related to the MAN antigens. Two cDNA fragments were isolated, designated MAN #1 and MAN #2. These clones

were identical except for an additional two nucleotides at the 5′ end of MAN #2. Sequence analysis of the MAN #1 clone revealed no homology to any previously published cDNAs. However, the amino acid sequence of the encoded polypeptide contained several motifs, including a potential nuclear localization signal, a hydrophobic domain, and an eleven amino acid glutamine stretch. I have shown that the MAN #1 polypeptide bears at least two epitopes in common with the three major cellular MAN antigens, and that one of these epitopes maps to a region encompassing the glutamine stretch. Using immunofluorescence microscopy, these epitopes were detected at the nuclear periphery. By genomic southern, the DNA sequence of the MAN #1 fragment was shown to be related to a single gene and not a family of genes. Moreover, the MAN #1 transcript was detected by RNase protection in a variety of mouse cell types, including both differentiated and undifferentiated P19 cells. This data complements the ubiquitous expression pattern observed for the MAN antigens across vertebrate species (Paulin-Levasseur *et al.*, 1996). In transient transfectants, a myc-tagged MAN #1 polypeptide was found to contain sequences that facilitate its translocation into the nucleus. In this thesis, my results are discussed in relation to our current knowledge of nuclear lamina proteins, as well as, nuclear lamina function.

RÉSUMÉ

La lamina nucléaire est composée d'un réseau filamenteux de protéines situé sous la membrane nucléaire interne et apposé à la chromatine périphérique. Sa position amène donc à suggérer qu'elle serait associée à diverses fonctions cellulaires telles que le soutien structural, la transmission de signaux et l'organisation tridimensionnelle de la chromatine. Mis à part la famille des lamines nucléaires, peu de constituants de la lamina ont été décrits. Dans la présente étude, nous avons utilisé un antisérum humain afin de poursuivre la caractérisation d'un nouvel ensemble de protéines de la lamina nucléaire appelées antigènes MAN. Ces antigènes comprennent trois principaux polypeptides avec des mobilités relatives de 78, 58 et 40 kDa. Au cours de l'interphase, les antigènes MAN se retrouvent avec les lamines à la périphérie du noyau, mais ils sont absents des centres nucléoplasmiques contenant la lamine B. De plus, dans les cellules qui possèdent des micronoyaux, on a pu observer que les antigènes MAN et les lamines A/C se retrouvent dans ces structures qui sont autrement dépourvues de lamine B. Pendant la mitose, on note que les lamines A/C se désassemblent à la fin de la prophase pour se réassembler en télophase. D'autre part, la lamine B et les antigènes MAN commencent à se désassembler seulement à la fin de la prométaphase pour se reformer autour de chromosomes en anaphase, avant que les lamines A/C ne se réassemblent.

L'antisérum humain a été utilisé pour cribler une banque d'expression d'ADNc de carcinomes embryonnaires P19 afin d'obtenir des clones codant des polypeptides immunologiquement apparentés aux antigènes MAN. Deux fragments d'ADNc ont été isolés, soient MAN 1 et MAN 2. Les deux clones sont identiques, sauf que MAN 2 porte deux nucléotides additionnels à l'extrémité 5'. L'analyse de la séquence de MAN 1 n'a pas révélé d'homologie avec les ADNc ayant déjà fait l'objet de publication. Toutefois, on a trouvé que la séquence en acides aminés du polypeptide codé contient plusieurs motifs, dont un signal de localisation nucléaire potentiel, un domaine hydrophobe et une extension d'onze glutamines. Nous avons démontré que le polypeptide MAN 1 possède au moins deux épitopes en commun avec les trois principaux antigènes cellulaires MAN et que l'un de ces épitopes peut être retracé dans la région comportant l'extension de glutamines. Aussi, ces épitopes sont détectés par immunofluorescence à la périphérie du noyau. En utilisant la technique de southern génomique, on note que le fragment d'ADNc de MAN 1 ne s'apparente qu'à un seul gène, et non à une famille de gènes. De plus, les transcrits d'ARNm de MAN 1 sont exprimés dans une variété de cellules de souris, y compris les cellules P19 tant différenciées que non-différenciées. Ces données corroborent des observations préalables qui montrent l'expression des antigènes MAN chez tous les vertébrés (Paulin-Levasseur *et al.*, 1996). Enfin, lors de transfections transitoires, nous avons observé que le polypeptide MAN 1 portant une "étiquette myc" contient des séquences qui facilitent le transport vers le noyau. Les résultats

sus-mentionnés sont discutés dans le présent mémoire en relation avec les connaissances actuelles sur les protéines de la lamina nucléaire ainsi que les fonctions de cette structure.

1. INTRODUCTION

During interphase, the nuclear envelope (NE) is an essential membrane system that functions as a selective barrier separating cellular activities involved in transcription and translation. As well, there is evidence to support the involvement of the NE in organizing nuclear architecture (for reviews: Nigg, 1992; Georgatos *et al.*, 1994). Structurally, the NE has three major constituents: 1) the inner and outer nuclear membranes; 2) the nuclear pore complexes; and 3) the nuclear lamina (Gerace and Burke, 1988). The two membranes are continuous, joined where the nuclear pores have integrated. The outer membrane is continuous with the rough endoplasmic reticulum (ER) and is decorated with ribosomes while the inner membrane is smooth and may serve as an anchorage site for chromatin via interactions with the nuclear lamina, a filamentous network of proteins ubiquitously found at the periphery of eukaryotic nuclei (figure 1). During mitosis, the NE is transiently dismantled. In prometaphase, the nuclear pore complexes are dispersed into the cytoplasm, the nuclear membranes are fragmented into vesicles and the nuclear lamina is disassembled. At the end of mitosis, these components are recycled and reassembled around daughter chromosomes.

The molecular mechanisms underlying the function of the NE are still poorly understood. Considerable research efforts have been devoted towards understanding the composition of the nuclear pores, as well as the process of nucleocytoplasmic transport (for reviews: Goldfarb, 1989; Maquat, 1991; Izaurralde and Mattaj, 1992).

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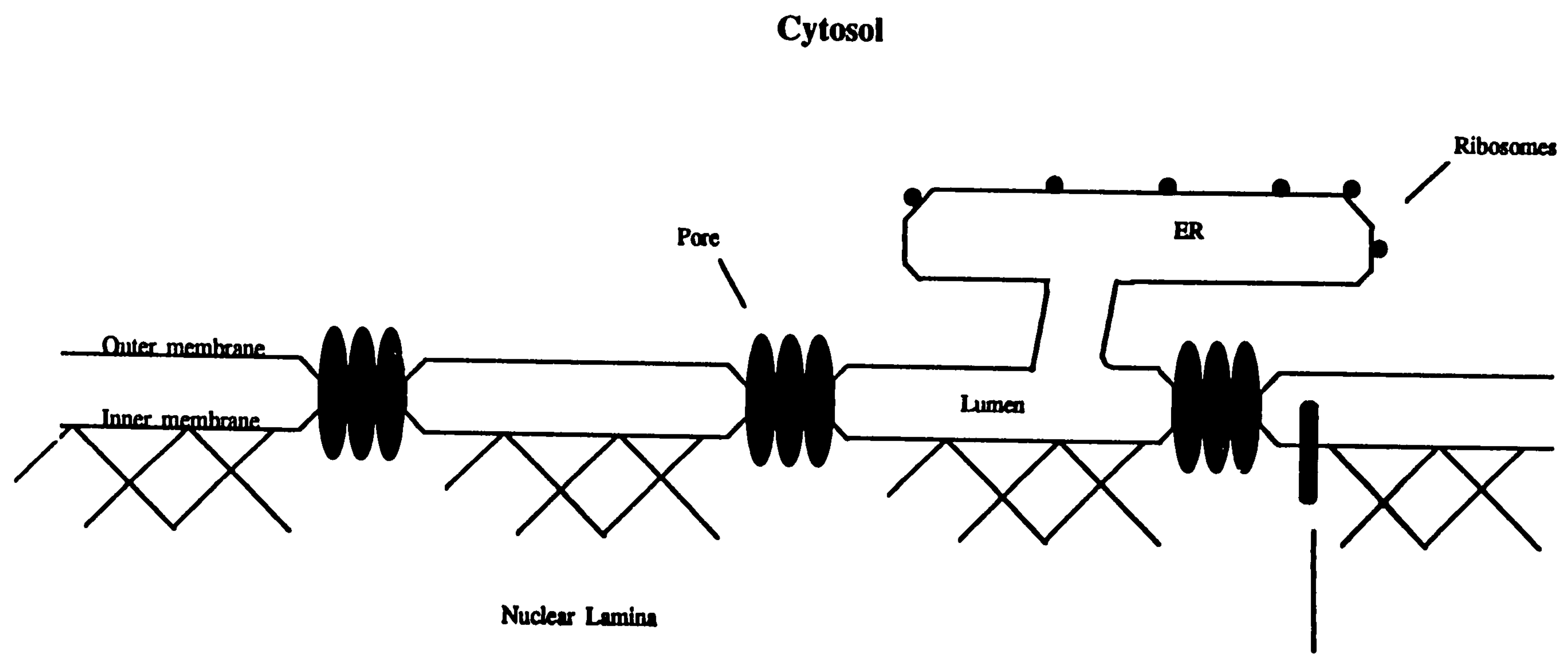
Figure 1. Schematic representation of the structural organization of the NE.

The constituents of the NE include: 1) the inner and outer nuclear membranes; 2) the nuclear pore complexes; and 3) the nuclear lamina. In mammalian cells, the nuclear lamina is described as a filamentous structure underlying the inner nuclear membrane and apposed to peripheral chromatin.


Cytosol
Ribosomes
Pore
ER
Outer membrane
Inner membrane
Lumen
Nuclear Lamina
Integral membrane protein
Nucleoplasm

Recently, research groups have characterized integral membrane proteins specifically targeted to the inner NE (Senior and Gerace, 1988; Worman *et al.*, 1988; Simos and Georgatos, 1992, 1994; Foisner and Gerace, 1993). The nuclear lamina itself has been shown to be a polymorphic protein lattice (reviewed by Nigg, 1989; Burke, 1990). Since my research project involved the molecular characterization of the nuclear lamina proteins, designated MAN antigens, I will briefly review our current knowledge of this lattice structure.

1.1 THE NUCLEAR LAMINA IS A MAJOR COMPONENT OF THE NUCLEAR ENVELOPE AND THE NUCLEAR MATRIX

The nuclear lamina was first identified by electron microscopy in the unicellular organism *Amoeba proteus*, where it appears as a prominent honeycomb-like structure associated with the nucleoplasmic surface of the inner nuclear membrane (Pappas, 1956; Mercer, 1959; Schmidt *et al.*, 1995). Early studies examining the ultrastructure of the nuclear lamina were performed on *Xenopus* oocytes, due to their large volume and easy manipulation. In this cellular system, the nuclear lamina was visualized by electron microscopy as a semi-regular orthogonal network of fibrils interconnecting the nuclear pores (Scheer *et al.*, 1976; Aebi *et al.*, 1986). Although a similar arrangement was observed in rat hepatocytes (Dwyer and Blobel, 1976), most other studies of mammalian NEs have described the nuclear lamina as an irregular fibrous structure apposed to the inner nuclear membrane (Fawcett, 1966; Patrizi and Poger, 1967; Aaronson and Blobel, 1975; Capco *et*

al., 1982). When stained with specific antibodies, the nuclear lamina appears, by immunofluorescence, as a smooth continuous structure at the nuclear periphery (Gerace *et al.*, 1978; Krohne *et al.*, 1978). However, as supported by three-dimensional (3D) reconstitution analysis of data collected on both *Drosophila* and mammalian cells, the nuclear lamina may form a discontinuous fibrillar network, with thicker regions aligning with peripheral chromatin (Paddy *et al.*, 1990; Belmont *et al.*, 1993).

Biochemical fractionation of mammalian nuclei revealed that the nuclear lamina is a major component of the nuclear matrix, a subnuclear fraction resistant to high and low salt buffers, non-ionic detergents and nuclease treatment (Berezney and Coffey, 1974). Typically, nuclear matrices retain many of the architectural features recognized in the intact cell, including residual components of the NE, nucleolus and internal fibrous network. Recently, nuclear functions, such as DNA replication and transcription, have been mapped to sites of interactions between active chromatin and the nuclear matrix (Fakan and Hancock, 1974; Berezney, 1984). This has prompted a new model in which the nucleus is divided into functionally distinct domains organized and maintained through interactions with the nuclear matrix (Berezney, 1984). It has been reported in some cell types that the nuclear matrix comprises over 200 proteins (Fey and Penman, 1988). Although poorly characterized, it has been found that the total number of proteins can vary depending on the cell type or state of differentiation (Fey and Penman, 1988; Stuurman *et al.*, 1990).

1.2 BIOCHEMICAL COMPOSITION OF THE NUCLEAR LAMINA

1.2.1 The family of nuclear lamin proteins

A. Nuclear lamins are major constituents of the nuclear lamina

Initially, the existence of a nuclear matrix was not universally accepted. In fact, it was believed to be an artifact of the isolation procedure and not representative of the *in vivo* nuclear architecture. The confusion was due, in part, to the different protocols (detergent, high salts and nucleases) used for nuclear matrix extraction. Several investigators reported "empty" nuclear matrices comprised exclusively of the nuclear lamina and residual nuclear pore complexes. Kaufmann and colleagues (1981) demonstrated that the internal matrix is inherently more sensitive to extraction than the nuclear lamina-pore fraction. This resulted in optimized protocols for isolating purified nuclear lamina components, devoid of contaminating internal structures (Belgrader *et al.*, 1991). Numerous studies have shown since that the primary components of the nuclear lamina in mammalian cells are the nuclear lamins A, B and C (Gerace *et al.*, 1978; Krohne *et al.*, 1978; Gerace and Blobel, 1980), immunologically and structurally related to the multigene family of intermediate filament (IF) proteins (Zackroff *et al.*, 1984; Aebi *et al.*, 1986; Fisher *et al.*, 1986; Goldman *et al.*, 1986; McKeon *et al.*, 1986).

B. The molecular structure of the nuclear lamins

The nuclear lamins are classified as type V intermediate filament proteins (Parry and Steinert, 1992) and share a common

tri-partite organization with cytoplasmic IF proteins (Steinert and Roop, 1988). This structure consists of a highly conserved central rod domain of 350 amino acids with a repeat sequence characteristic of an α-helical coiled-coil conformation (Steinert and Roop, 1988). The conserved distribution of alternating positively and negatively charged residues along this domain are thought to contribute to the interactions between molecules, resulting in the assembly of dimers, tetramers and ultimately 10 nm-diameter IFs (Aebi *et al.*, 1986; Steinert and Roop, 1988). Flanking the rod domain are less conserved non-helical N- and C-terminal sequences of variable lengths (30-40 and 210-300 amino acids respectively), which contain lamin specific motifs involved in lamin dynamics and assembly (reviewed by McKeon, 1991). Compared to vertebrate cytoplasmic IF proteins, the rod domains of nuclear lamins have an additional 42-amino acid insertion (Fisher *et al.*, 1986; McKeon *et al.*, 1986). Several invertebrate cytoplasmic IF proteins contain a similar 42-amino acid segment, suggesting that the nuclear lamins are the ancestors to all IF proteins (Weber *et al.*, 1991; Stick, 1992).

The first lamin cDNA sequences to be cloned were human lamins A and C (Fisher *et al.*, 1986; McKeon *et al.*, 1986). Presently, over 25 lamin sequences have been isolated from *Caenorhabditis elegans* to *Homo sapiens* (reviewed by Moir *et al.*, 1995). Based on these cDNA sequences, lamins are classified into two groups, A- and B-types. In mammalian cells, nuclear lamins consist of four major polypeptides, lamins A, B_1/B_2 and C_1 (70, 65 and 60 kDa respectively). Lamins A and C_1 (A-type) are generated by alternative splicing

of the same primary transcript (Fisher *et al.*, 1986; Lin and Worman, 1993), whereas lamins B_1 and B_2 (B-type) are the products of two separate and distinct genes (Höger *et al.*, 1988 and 1990). Additional minor lamin polypeptides (termed lamins D and E) have also been identified within mammalian cells. These polypeptides have been shown to possess a common epitope with lamin B_1, but are the expression products of different genes (Lehner *et al.*, 1986; Kaufmann, 1989). As well, a minor alternative splicing variant of the lamin A/C gene, termed lamin AΔ10, has been identified in a variety of carcinoma cell lines and normal colon tissues (Machiels *et al.*, 1996). Lamins A/C and B can be further distinguished according to their different isoelectric properties and mitotic fate (Gerace and Blobel, 1980; Burke and Gerace, 1986). At mitosis, nearly neutral (pI 7.5) A-type lamins are solubilized, whereas acidic (pI 6.0) B-type lamins remain associated with membrane vesicles (Gerace and Blobel, 1980). Recently, germ cell-specific lamins B_3 and C_2 have been cloned from mouse spermatocytes (Furukawa and Hotta, 1993; Furukawa *et al.*, 1994).

C. Variable expression of nuclear lamins in mammalian cells

Mammalian somatic cells constitutively express B-type lamins but differ in their expression of A-type lamins. Mammalian lamins B_1 and B_2 are expressed at similar levels in somatic cells (Höger *et al.*, 1990). Interestingly, this is not representative of chicken lamins B_1 and B_2. In this case, lamin B_1 is expressed ubiquitously in somatic cells, while lamin B_2 is expressed at high levels only in embryonic tissues (Lehner *et al.*, 1986).

The major A-type lamins are expressed strictly within differentiated cells (Stewart and Burke, 1987; Lanoix *et al.*, 1992; Mattia *et al.*, 1992). In fact, the timing of lamin A/C expression coincides with tissue development. Most mammalian adult tissues contain four major lamin proteins, A, B_1, B_2 and C_1 (Höger *et al.*, 1988, 1990). The notable exceptions are intestinal epithelial and hemopoietic cells which do not express lamins A/C (Rober *et al.*, 1989; 1990 a,b). During early mouse development, A-type lamins are present within the fertilized egg. As the embryo progresses through the first 2-4 cell divisions, lamins A/C disappear and only begin to be expressed again after day 8 post-implantation (Stewart and Burke, 1987). These observations which link induction of lamin A/C expression with organogenesis have led investigators to speculate that A-type lamins are required for maintaining a differentiated phenotype, possibly through reorganization of chromatin (Rober *et al.*, 1989; Peter and Nigg, 1991). Attempts to stably transfect lamin A in undifferentiated mouse cell lines revealed that the protein could be targeted successfully to the nuclear periphery, but it did not induce expression of differentiation-specific genes (Peter and Nigg, 1991). These results indicated that, while the expression of lamins A and C is temporally correlated with differentiation, it is not sufficient to initiate a change in phenotypic expression.

The compositional change of the nuclear lamina upon differentiation has been documented within many different cell types, including several tumor cell lines (Haas *et al.*, 1990; Peter and

Nigg, 1991; Horton *et al.*, 1992). Typically, in the undifferentiated state, these tumor cell lines only express B-type lamins (Stewart and Burke, 1987). Some of the cell lines have been ideal for studying lamin expression, because they can often be induced to differentiate after treatment with various chemical agents (Stewart and Burke, 1987; Lanoix *et al.*, 1992; Mattia *et al.*, 1992; Paulin-Levasseur *et al.*, 1989a,b). For instance, P19 embryonal carcinoma cells can be induced along a pathway of muscle development after exposure to dimethylsulfoxide (DMSO), resulting in an increase of both lamins A/C mRNA and protein levels (Stewart and Burke, 1987; Lanoix *et al.*, 1992; Mattia *et al.*, 1992).

Therefore, due to their ubiquitous expression, it is likely that B-type lamins contribute to some housekeeping function(s). On the other hand, since cells expressing only lamin B are completely viable, it is conceivable that A-type lamins exert a more subtle influence, possibly related to cellular differentiation (Nigg, 1989).

D. Post-translational modifications of nuclear lamins

The two major A-type (A and C_1) lamins have been reported to be the products of the same gene (Lin and Worman, 1993; Furukawa *et al.*, 1994). The cDNA sequences for somatic lamins A and C_1 are identical except for a unique 18 bp extension present in lamin C_1, and a 270 bp segment in lamin A (Fisher *et al.*, 1986), a consequence of differential splicing of C-terminal exons from lamin A/C_1 pre-mRNAs (Lin and Worman, 1993). The C-terminals of the lamin A

precursor and lamin B_1 contain a consensus target sequence (CaaX; C:cysteine, a:aliphatic residue, X:any residue) for protein isoprenylation and carboxymethylation, which is not found in lamin C_1 (Beck *et al.*, 1988; Chelsky *et al.*, 1989; Farnsworth *et al.*, 1989; Vorburger *et al.*, 1989). Subsequent proteolytic cleavage causes the removal of the modified cysteine from lamin A precursors, resulting in mature lamin A (Figure 2). Therefore, only lamin B retains the modified isoprenylated and carboxymethylated cysteine residue. Similar motifs have been characterized in ras-related proteins (reviewed by Marshall, 1993) and are thought to be necessary for association with the plasma membrane (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989).

During mitosis, the nuclear lamins disassemble and then reassemble around daughter nuclei. This process of nuclear lamina breakdown and subsequent reformation is correlated with the phosphorylation state of nuclear lamins (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985). Sequence analysis has shown that nuclear lamins possess two recognition consensus sites for the mitotic kinase, $p34^{cdc2}$, immediately flanking the central rod domain (Heald and McKeon, 1990). Subsequent *in vitro* experiments have demonstrated that purified $p34^{cdc2}$ kinase is sufficient for lamin depolymerization. However, other cellular components are required for complete breakdown of the nuclear envelope (Newport and Spann, 1987; Peter *et al.*, 1990; Dessev *et al.*, 1991).

Protein kinase C (PKC) has been shown to phosphorylate B-type lamins both during mitosis and interphase (Hennekes *et al.*, 1993;

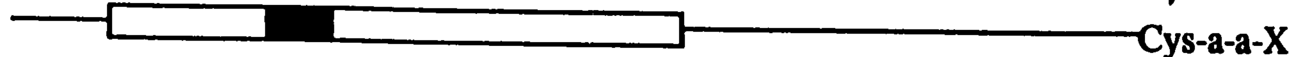
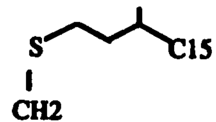
Goss *et al.*, 1994). Phosphorylated serine residues in chicken lamin B_2 have been mapped adjacent to the C-terminal p34^{cdc2} site, in close proximity to the nuclear localization signal (NLS; Hennekes *et al.*, 1993). *In vitro*, phosphorylation of lamin A and lamin B_2 by PKC does not interfere with proper assembly of lamin dimers, as would be predicted. Instead, PKC hyper-phosphorylation results in the inhibition or complete lack of nuclear lamin transport into the nucleus of interphase cells (Hennekes *et al.*, 1993; Haas and Jost, 1993).

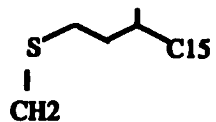
E. The nuclear lamins mediate interactions between the nuclear envelope and chromatin

The reassembly of the NE at the end of mitosis is poorly understood. Both rat lamin B_1- and *Xenopus* lamin B_3-associated vesicles are capable of binding chromatin *in vitro* (Höger *et al.*, 1991; Ludérus *et al.*, 1992). This suggests that B-type lamins could be sufficient for targeting nuclear vesicles to chromosomes. However, in similar experiments, it was demonstrated that soluble lamins A and C can interact with chromosomes in the absence of lamin B. The assembly of lamins A/C is proposed to be effected through specific protein-DNA binding sites on chromosomes and would facilitate the docking of lamin B-bearing vesicles (Burke, 1990; Glass and Gerace, 1990). Although the mechanism of

Figure 2. Post-translational modifications leading to the production of mature lamin A

The C-terminal CaaX motif within the lamin A precursor undergoes numerous modifications, these include: 1) isoprenylation of the cysteine residue; 2) proteolytic cleavage of the -aaX sequence; 3) carboxymethylation of the cysteine; and 4) proteolytic cleavage at residue 646.



Targeted Cysteine residue
Lamin A precursor
Cys-a-a-X
1.
S
C15
CH2
Cys -a-a-X
2.
Step 1. Isoprenylation
Step 2. Proteolytic cleavage
S
C15
CH2
Cys-o-CH3
3.
4.
Step 3. Carboxymethylation
Step 4. Proteolytic cleavage (residue 646)
Mature Lamin A
Tyr(646)

nuclear assembly *in vivo* needs further investigation, there is growing support for a lamin-dependent model (Lourim and Krohne, 1994).

The nuclear lamins can also interact with chromatin during interphase. Recent studies have revealed that lamin A is capable of binding to isolated polynucleosomes (Yuan *et al.*, 1991). As well, the ectopic expression of the germ cell specific lamin B_3 in somatic cells has been shown to result in a complete rearrangement of chromatin (Furukawa and Hotta, 1993). With the recent discovery of intranuclear lamin foci (Goldman *et al.*, 1992; Bridger *et al.*, 1993; Moir *et al.*, 1994), it has been suggested that nuclear lamins may influence the internal organization of chromatin. For example, lamin A/C foci have been localized to sites of heterochromatin in G1 (Bridger *et al.*, 1993), and lamin B foci have been mapped to replication centres during S phase (Moir *et al.*, 1994).

There is little known about the DNA sequences that interact with nuclear lamins. The only exception would be the matrix attachment regions (MARs) or the scaffold attachment regions (SARs) (Ludérus *et al.*, 1992, 1994). Capable of binding to the nuclear matrix, these elements are believed to form the base of chromatin loops (Gasser and Laemmli, 1987).

1.2.2 Integral proteins of the inner nuclear membrane

A. The lamin B receptor

In addition to the nuclear lamins, there are several proteins which are believed to influence the organization of the lamina

lattice (Senior and Gerace, 1988; Worman *et al.*, 1988; Powell and Burke, 1990; Bailer *et al.*, 1991; Foisner and Gerace, 1993). The majority of these polypeptides are classified as integral membrane proteins of the inner nuclear envelope. The first of these integral membrane proteins to be characterized was p58. cDNA sequence analysis revealed that p58 has eight potential transmembrane domains and possesses a large highly charged N-terminal domain, extending into the nucleoplasm (Worman *et al.*, 1990). *In vitro*, this N-terminal domain has been shown to bind both DNA and lamin B (Ye and Worman, 1994). As a consequence, p58 is also referred to as the lamin B receptor (LBR)(Worman *et al.*, 1988). The LBR has been found to be constitutively expressed in different cell types (Worman *et al.*, 1988; Georgatos *et al.*, 1989; Bailer *et al.*, 1991; Shimanuki *et al.*, 1992; Chaudhary and Courvalin, 1993) and is believed to be as conserved across species as lamin B. In fact, analogues to the LBR have been identified in yeast (Georgatos *et al.*, 1989). Curiously, several other cloned yeast genes share sequence homology to chicken p58. Of particular interest is the ergosterol enzyme product of the ERG24 gene in *S. cerivisiae*. This protein shares 42% identity in a segment encompassing part of the transmembrane domain and carboxy terminus of p58. However, these clones differ from p58 in one important aspect: they all lack the long amino terminal domain. Consequently, these yeast proteins are not considered to be functional homologues of p58. Yet, the structural similarities between these proteins implies that they are derived from a common ancestor, most likely the result of a

recombination event, producing a chimeric protein with the properties of p58 (Georgatos *et al.*, 1994).

Through immunoprecipitation of the LBR, several other nuclear proteins have been identified (Simos and Georgatos 1992, 1994). This multimeric complex, termed the "LBR complex", consists of both A- and B-type lamins, a kinase which specifically phosphorylates the LBR, and polypeptides p18, p34 and p150 (Simos and Georgatos, 1992). The p150 polypeptide has not since been characterized. However, p18 has recently been described as a new integral membrane protein of the inner nuclear envelope of avian erythrocytes (Simos *et al.*, 1996). p34 is reported to be the homologue to human p32, a nuclear protein which co-isolates with splicing factor 2 (Krainer *et al.*, 1991), suggesting that the nuclear envelope, via the LBR complex, may be involved in mRNA splicing (Simos and Georgatos, 1994).

B. The lamin-associated polypeptides

Another subset of proteins, collectively termed lamin-associated polypeptides (LAPs), were initially identified in rat liver nuclear envelopes (Senior and Gerace, 1988). The family of LAP proteins, including LAP 2 and immunologically related LAPs 1A, 1B and 1C, show differential expression patterns (Senior and Gerace, 1988; Foisner and Gerace, 1993; Furukawa *et al.*, 1995; Martin *et al.*, 1995). Interestingly, only LAP 1C is expressed in undifferentiated cell types (Senior and Gerace, 1988; Foisner and Gerace, 1993; Furukawa *et al.*, 1995; Martin *et al.*, 1995). *In vitro*

binding experiments indicate that LAPs 1A and 1B bind to both lamins A and B whereas LAP 2 exclusively binds to lamin B and mitotic chromosomes, depending on its phosphorylation state (Senior and Gerace, 1988; Foisner and Gerace, 1993; Furukawa *et al.*, 1995). These three polypeptides remain associated with the lamina after nuclear envelope extraction. LAP 1C does not bind to nuclear lamins *in vitro* and is solubilized during isolation of nuclear envelopes. The published cDNA sequences for both LAPs 1C and 2 have confirmed their structural similarities. Both proteins possess a single transmembrane domain and a large hydrophilic N-terminal domain exposed to the nucleoplasm. The latter is believed to contribute to the interactions between the nuclear membrane and the nuclear lamina (Furukawa *et al.*, 1995; Martin *et al.*, 1995). In the case of LAP 2, analysis of the derived amino acid sequence revealed that it shares 91% identity with a family of human thymopoietin proteins, particularly thymopoietin β (Furukawa *et al.*, 1995; Harris *et al.*, 1995). These proteins have been implicated in T cell differentiation (Goldstein, 1974; Sunshine *et al.*, 1978) and it has been suggested that LAP 2 may represent the rat homologue (Furukawa *et al.*, 1995).

1.2.3 Other non-lamin components of the nuclear lamina

Two proteins have been identified as non-lamin constituents of the nuclear lamina, these include P1 and perichromin. Both proteins are localized to the NE in interphase cells and become redistributed to the periphery of chromosomes during mitosis (Chaly *et al.*,

1984; McKeon *et al.*, 1984). The possibility that these proteins may play a role in chromatin organization (Chaly *et al.*, 1984; McKeon *et al.*, 1984; Hernandez-Verdun and Gautier, 1994; Chaly *et al.*, 1996) would be consistent with their cell cycle fate but has not yet been addressed experimentally.

1.3 PROPOSED FUNCTIONS OF THE NUCLEAR LAMINA

The functions of the nuclear lamina remain to be ascertained. Based on structural data and the differential expression of its constituents, different suggestions have been formulated.

As a consequence of its location, apposed to both the inner nuclear membrane and chromatin (Gray and Guillery, 1963; Coggeshall and Fawcett, 1964), the nuclear lamina has been proposed to play a role in the maintenance of nuclear integrity. This suggestion is supported by several lines of evidence. For instance, the nuclear lamins are incorporated into insoluble polymers (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976; Gerace *et al.*, 1978; Gerace and Blobel, 1980), with similar physical and biochemical properties as the cytoplasmic IF proteins (Moir *et al.*, 1991). It is believed that these polymers would contribute to the stability of the NE during interphase (Zackroff *et al.*, 1984; Aebi *et al.*, 1986; Fisher *et al.*, 1986; Goldman *et al.*, 1986). In experiments where nuclear assembly extracts from *Xenopus* oocytes were immunodepleted of lamin B_3, it was found that the resulting nuclei were mechanically fragile (Newport *et al.*, 1990; Meier *et al.*, 1991). These results suggested that lamin B_3, and any component immunoprecipitating with

lamin B_3, are required to stabilize the NE. Similarly, it has been reported that nuclei of cultured cells which are devoid of lamins A/C were consistently more sensitive to mechanical stress than nuclei with lamins A/C (Wang and Traub, 1991). Considered together, these data promote the role of the nuclear lamina (nuclear lamin proteins) as a structure which strengthens the NE.

Coggeshall and Fawcett (1964) were the first to suggest that the nuclear lamina may interact with chromatin, due to the close proximity of peripheral heterochromatin with the inner nuclear membrane. Since then, the nuclear lamins have been shown to interact *in vitro* with both telomeric (Shoeman and Traub, 1990) and MAR/SAR sequences (Ludérus *et al.*, 1992, 1994). As previously described, these interactions would not occur strictly at the nuclear periphery. Lamin B is believed to interact with chromatin in replication granules during S phase (Moir *et al.*, 1994). Hozák and colleagues (1995) have proposed that the nuclear lamins are not confined to the nuclear periphery and intranuclear foci, but are also part of a diffuse internal network. Although more work has to be done to reconcile these results with previously published data, the concept of an intricate IF network existing throughout the nucleoplasm is intriguing. The differential expression of nuclear lamins during development has led to theories regarding their role in higher order chromatin structure. Lamins A/C are expressed only in differentiated cell types (Stewart and Burke, 1987; Rober *et al.*, 1989), and have been proposed to play a role in the reorganization of chromatin during differentiation (Rober *et al.*, 1989;

Peter and Nigg, 1991). It is interesting to consider that integration of newly synthesized lamins A/C into an internal network would have an effect on the overall reorganization of chromatin.

The existence of an internal IF network in the nucleus (Hozák *et al.*, 1995) lends support to the idea that interactions between nuclear lamins and cytoplasmic IFs could form a signal transduction pathway from the plasma membrane to the nucleus (Bissell *et al.*, 1982; Goldman *et al.*, 1985). Cytoplasmic IF proteins have already been shown to associate with the NE (Fey *et al.*, 1984; Jones *et al.*, 1985). In addition, results of *in vitro* binding assays have shown that cytoplasmic IFs can interact directly with nuclear lamins (Georgatos and Blobel, 1987). These interactions would allow external signals to traverse the IF network in the cytoplasm into the nucleus, ultimately affecting gene expression. Associations between lamins and cytoplasmic IFs have been observed *in vivo*. During mitosis, lamin B bearing vesicles have been found to bind vimentin IF proteins (Maison *et al.*, 1993). It is believed that these interaction would allow the vesicles to be sequestered to specific locations, excluded from the mitotic spindle.

1.4 THE USE OF AUTOANTIBODIES IN THE IDENTIFICATION OF NUCLEAR ENVELOPE PROTEINS

Autoantibodies directed against nuclear antigens have long been a characteristic feature of patients with autoimmune and liver diseases (reviewed by Worman and Courvalin, 1991). These autoantibodies often target subcellular organelles or particles and

have been used to identify antigens involved in cellular activities such as pre-mRNA splicing and DNA replication (Tan, 1991). A subset of these autoantibodies, when used in immunofluorescence microscopy, stain the nuclear periphery with a rimlike pattern (Gerace *et al.*, 1978; Tan, 1989; Senécal *et al.*, 1991). Frequently, these antibodies recognize nuclear envelope associated proteins, such as the nuclear lamins (Senécal *et al.*, 1991), LBR (Worman *et al.*, 1988) and nuclear pore proteins (Gerace and Blobel, 1982; Davis and Blobel, 1986).

Our laboratory has acquired a human antiserum (MAN) from a patient suffering from a form of rheumatoid arthritis called collagenosis. By immunofluorescence microscopy, these autoantibodies recognize components of the nuclear envelope, present in both lamin A/C-positive and -negative cells. Partial characterization of the antiserum has demonstrated that the MAN antigens are conserved among vertebrate species, from humans to fish. Through immunoblotting techniques and *in situ* isolation of nuclear matrices, the MAN antigen(s) are distinguishable from the nuclear lamins, LAPs and the LBR (Paulin-Levasseur *et al.*, 1996). Consequently, the MAN antigens represent a novel set of nuclear lamina proteins.

1.5 RESEARCH PROPOSAL

Although, the nuclear lamins have been implicated in various nuclear processes (e.g. DNA replication and organization), there is little evidence on how they may interact with other nucleoplasmic

proteins, particularly other nuclear matrix proteins. An exception would be the retinoblastoma (RB) protein, a nuclear matrix component. RB has been found to bind lamins A/C *in vitro* (Mancini *et al.*, 1994). This data support the notion of interdependence between the lamina and the internal matrix. This relationship has been further substantiated by recent experiments. It was found that the proper assembly of matrices from sperm pronuclei is dependent on the presence of lamin B_3 in *Xenopus* egg extracts (Zhang *et al.*, 1996). While a number of nuclear matrix components may form protein-protein interactions with the nuclear lamins, the most likely candidates are still other lamina proteins. To date, the only reported non-lamin components of the nuclear lamina have been perichromin, P1 and the MAN antigens.

The MAN antiserum has allowed the identification of a novel set of NE proteins. One to three major polypeptides, as well as several minor proteins, are recognized by the serum depending on the species and cell types. The degree to which these MAN antigens are conserved across vertebrate species suggests that they have a function which is vital to the cell. It would be of particular interest to determine whether or not the MAN antigens share functions with the nuclear lamins. If the MAN antigens are involved in structural support, chromatin organization or NE reformation, it will be important to study these functions at the molecular level. This will require examining domains within the antigens which are responsible for a specific function. As a tool, the antiserum has limitations. For example, it does not recognize a single protein,

but a subset of polypeptides in mammalian cells. Therefore, to distinguish the fate of the individual antigens, it is necessary to employ molecular techniques. The aim of my research project has been to further characterize the antigens recognized by the MAN antiserum. To achieve this goal, I have concentrated my efforts on: 1) documenting the mitotic fate of the MAN antigens relative to A- and B-type lamins; and 2) isolating a mouse cDNA clone encoding a polypeptide recognized by the antiserum. It is assumed that by studying the mitotic fate of the antigens, as well as characterizing a MAN reactive clone, it will be possible to infer potential functions of the family of immunologically related MAN polypeptides.

2. DOUBLE IMMUNOFLUORESCENCE STUDY OF THE CELL CYCLE FATE OF MAN ANTIGENS IN RELATION TO A- AND B-TYPE NUCLEAR LAMINS IN MAMMALIAN CELLS

2.1 INTRODUCTION

The nuclear lamina of interphase cells is an insoluble structure, classified as part of the nuclear matrix. It is resistant to salt extraction and nuclease treatment (Berezney and Coffey, 1974). Nevertheless, the nuclear lamina has been shown to undergo highly dynamic rearrangements during the cell cycle. In the majority of cells, synthesis of nuclear lamins occurs throughout interphase (Jost and Johnson, 1981; Gerace *et al.*, 1984; Ottaviano and Gerace, 1985; Bludau *et al.*, 1986; Foisy and Bibor-Hardy, 1988). Yet, most of the nuclear lamins partition with the insoluble nuclear matrix. Therefore, the lamina must be capable of rapidly incorporating lamin subunits during all stages of interphase, a process which may be required for the growth of the nucleus prior to cell division (Swanson *et al.*, 1991; Goldman *et al.*, 1992; Bridger *et al.*, 1993). The dynamic nature of the nuclear lamina has been demonstrated in 3T3 cells microinjected with mature lamin A (Goldman *et al.*, 1992). After injection into the cytoplasm, lamin A is seen to accumulate in nucleoplasmic foci prior to its incorporation into the lamina (Goldman *et al.*, 1992). These intranuclear lamin A/C foci have also been seen in uninjected G_1 cells, and are thought to be part of the normal nucleoplasmic architecture (Goldman *et al.*, 1992; Bridger *et al.*, 1993; Sasseville and Raymond, 1995), possibly acting as sites of lamin post-translational modification (Sasseville and Raymond, 1995) or

serving as pools of newly synthesized lamins for growth of the lamina (Goldman *et al.*, 1992; Bridger *et al.*, 1993). Similar foci have been detected with lamin B antibodies. The greatest number of these lamin B intranuclear foci have been found in S phase, co-localizing with sites of DNA replication (Moir *et al.*, 1994).

In mitosis, lamins disassemble at prophase and then reconstitute around the decondensing chromosomes during telophase. Once disassembled, the A-type lamins become soluble in the cytoplasm, whereas B-type lamins remain associated with nuclear vesicles (Gerace and Blobel, 1980; Burke and Gerace, 1986). Lamin B interacts with the lipid membrane through either its isoprenylated carboxy-terminus (Beck *et al.*, 1988) or binding with integral membrane proteins such as the LBR (Appelbaum *et al.*, 1990; Worman *et al.*, 1990) or the LAPs (Senior and Gerace, 1988). In telophase, the components of the nuclear lamina reform around daughter nuclei, but the precise mechanisms involved in this process remain to be determined.

Both the LBR and the LAPs are believed to form tight associations with the nuclear lamina of interphase cells (Senior and Gerace, 1988; Powell and Burke, 1990; Foisner and Gerace, 1993; Meier and Georgatos, 1994; Ye and Worman, 1994). However, the role of these proteins during mitosis remains unclear. This is due, in part, to the conflicting reports describing the fate of these proteins during cell division. As integral membrane proteins, the LBR and LAPs would be expected to segregate with lamin B bearing membrane vesicles. In fact, this appears to be true in mitotic

chicken hepatoma cells, where the LBR and lamins B_1 and B_2 are found to co-localize by immunofluorescence (Meier and Georgatos, 1994). Conversely, in HeLa cells, LBR membrane vesicles were seen to reform around anaphase chromosomes prior to lamin B associated vesicles (Chaudhary and Courvalin, 1993). As well, LAP2 and lamin B_1 did not co-localize during mitosis of NRK cells (Foisner and Gerace, 1993). These observations suggest that the sequence of events responsible for NE reassembly may vary in different cell systems. The role that the different LAPs, nuclear lamins and the LBR play in this process will require further investigation.

In this study, I have used double immunofluorescence microscopy to compare the distributions of the MAN antigens, lamins A/C and lamin B throughout the cell cycle. During interphase the MAN antigens were found to co-localize with both lamins A/C and lamin B at the nuclear periphery. In a subset of cells which possessed lamin B intranuclear foci, the MAN antigens were confined to the NE and did not co-localize with lamin B within these internal structures. In addition, both the MAN antigens and lamins A/C were observed to segregate within micronuclei, separate from lamin B. When followed through mitosis, lamins A/C disassembled in late prophase and reassembled in telophase. In contrast, both lamin B and the MAN antigens began to disassemble only during late prometaphase and then reformed around segregating chromosomes in anaphase, prior to lamins A/C. In conclusion, the MAN antigens appear to preferentially associate with lamins A/C during interphase, possibly through direct interactions. However, the

apparent associations change during mitosis when the MAN antigens and lamin B segregate concomitantly, distinct from lamins A/C. Therefore, I propose that the MAN antigens may play a role in both establishing and maintaining the integrity of the NE.

2.2 MATERIALS AND METHODS

Cell cultures

Both 3T3 (CCL 92) and HeLa (CCL 2) cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were maintained at 1×10^6 cells/ml in Eagle's minimum essential medium (MEM; Flow Laboratories Inc., Mississauga, Ontario, Can.) at 37°C and 5% CO_2. The medium was supplemented with 10% heat-inactivated (HI) fetal calf serum (FCS; Gibco BRL, Burlington, Ontario, Can.) as well as antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Gibco BRL).

Indirect double immunofluorescence

For double labelling, cells were plated onto coverslips and subsequently fixed and stained 24 hours (hr) later as described by Chaly *et al.* (1984). In brief, the cells were fixed in 3% paraformaldehyde for 5 minutes (min) and then washed in phosphate buffered saline (PBS) solution. Before antibody staining, free aldehyde groups were reduced by rinsing cells 3 X 4 min in sodium borohydride (1mg/ml PBS). Cells were subsequently permeabilized in 0.2% Triton X-100/PBS for 20 min. This was followed by a 20 min incubation in 0.15% gelatin/PBS. Double labelling was performed in the following order: 1) the first primary antibody 2) the first secondary antibody 3) the second primary antibody and 4) the second secondary antibody. Each incubation was carried out for 45 min, followed by washes with PBS. For conventional epifluorescence microscopy, cells were counterstained with Hoechst 33258 (1 μg/ml in PBS) and mounted in glycerol containing ρ-phenylenediamine. For

HeLa cells, primary antibodies included: a murine monoclonal immunoglobulin (Ig) M lamins A/C antibody (Burke *et al.*, 1983) at 1:10 (provided by Dr. P. Traub, Max-Plank Institut für Zellbiologie, Heidelberg, Germany); a murine monoclonal anti-lamin B (Matritech Inc., Cambridge, Ma) at 1:800; a murine monoclonal Mab 414 to nuclear pore complex proteins at 1:3,000 (BabCO, Richmond, Ca); and the MAN antiserum diluted 1:15,000. The secondary antibodies used for HeLa cells were: rabbit anti-human Ig conjugated with fluorescein isothiocyanate (FITC) (ICN Immunobiochemicals, Lisle, IL); donkey anti-mouse Ig conjugated with CY3 (Jackson Immunoresearch, West grove, PA, USA); donkey anti-mouse IgM conjugated with CY3 (Jackson Immunoresearch). For 3T3 cells, primary antibodies were: a rabbit polyclonal lamin B antibody at 1:150 (provided by Dr. P. Traub); the MAN antiserum at the same dilution as used for HeLa cells. The secondary antibodies for 3T3 cells were: a donkey anti-rabbit IgG conjugated with CY3 (Jackson Immunoresearch); and a rabbit anti-human Ig conjugated with FITC (ICN immunobiochemicals).

Observations were performed on a Zeiss Axiophot and recorded using Ilford XP2-400 film. Confocal laser scanning microscopy was performed with an upright Leica optical system equipped with a mixed-gas argon-krypton laser and images were printed on Kodak Colorease.

Western blot analysis

For western blotting, populations of HeLa or 3T3 cells were harvested at 1,500 g for 6 min and washed twice with 10 mM Tris-

acetate, pH 7.5, 150 mM NaCl, and 1 mM EGTA. Pellets were resuspended in 1 ml of 10% trichloro-acetic acid (TCA) and incubated at 40°C for 15 min. Protein precipitate was collected by low speed centrifugation and washed with 5% TCA and acetone. Samples were then solubilized in sodium dodecyl sulfate (SDS)-sample buffer (Laemmli, 1970) and run on 5% stacking and 12% resolving SDS-polyacrylamide gels (PAGE) according to the protocol of Laemmli (1970). Samples equivalent to the protein content of 5 x 10^5 cells were loaded onto the gels. Protein profiles were visualized by Coomassie staining of gels. Otherwise, the resolved polypeptides were electrophoretically transferred from the gels to nitrocellulose membranes according to the method of Towbin *et al.* (1979). Immunoblotting was done according to the instructions in the Western Blotting Kit by Amersham Canada Inc. (Oakville, Ontario, Can.). Nitrocellulose membranes were blocked in 5% milk/PBS/Tween-20 solution for 1 hr. All antibodies, including streptavidin coupled to horseradish peroxidase (HRP), were diluted in PBS/Tween-20. The primary antibody was the MAN antiserum diluted 1:20,000. The secondary antibody was a biotinylated sheep anti-human IgG, used at 1:4,000 (whole antibody; Amersham Canada Inc.). Streptavidin-HRP was added at a dilution of 1:3,000 (Amersham, Canada Inc.). All incubations were performed for 1 hr. Immunoreactive bands were detected visually by incubating the blots with 4-chloro-1-naphthol.

2.3 RESULTS

The MAN antiserum recognizes from one to three polypeptides in a variety of vertebrate cells (Paulin-Levasseur *et al.*, 1996). Western blot analysis of homogenates from HeLa and 3T3 cells probed with the MAN antiserum is illustrated in figure 3. In both cell types, three major polypeptide bands with a relative mobility of 78, 58 and 40 kDa were detected. Additional reactive protein bands of 47-54 kDa were also detected in 3T3 cells.

Using the MAN antiserum and antibodies to either lamins A/C, lamin B or nuclear pore proteins, a series of double immunofluorescence experiments were performed on HeLa cells. In all cases, unsynchronized cell populations were used. As determined by the configuration of DNA stained with Hoechst, such populations contained from 4 to 7% mitotic cells. Furthermore, there was consistently a small number of interphase cells (< 1%) exhibiting the presence of micronuclei.

In interphase cells, the MAN antiserum was found to label the nuclear periphery in much the same way as antibodies directed against nuclear lamins A/C and B, presenting a smooth continuous pattern distinct from the punctate staining seen with nuclear pore antibodies (Fig.4). By examining lamin A/C positive cells, it was observed that within cells which possessed micronuclei the MAN antigens consistently segregated with lamins A/C. This is illustrated in panels d and e of figure 4, where the MAN antigens and lamins A/C are observed at the periphery of micronuclei. Lamin B and the nuclear pore proteins are seen to co-localize with

Figure 3. Detection of the MAN antigens in HeLa and 3T3 cell homogenates

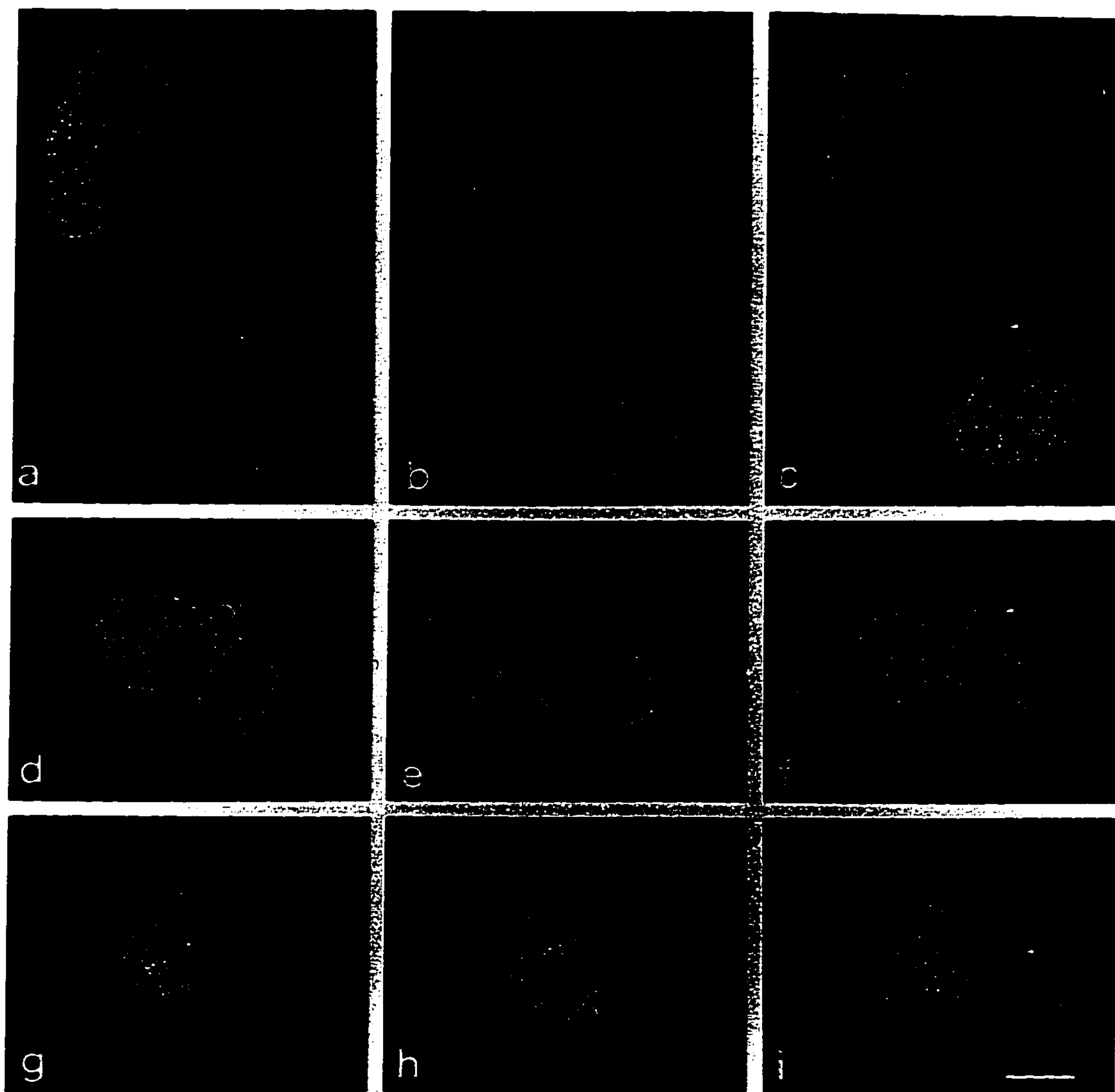
Protein extracted from populations of cycling HeLa (A) and 3T3 (B) cells were prepared for immunoblotting with the MAN antiserum. Specific MAN reactive bands are seen migrating at relative mobilities of 78, 58 and 40 kDa in HeLa cells (A) and 78, 58, 47-54 and 40 kDa in 3T3 cells (B). The black squares represent the relative positions of polypeptides migrating at 78, 58 and 40 kDa.

A

B

Figure 4. Spatial relationship of MAN antigens to nuclear pore proteins and nuclear lamins in micronucleus-bearing interphase HeLa cells

Cells were double labelled for immunofluorescence with the MAN antiserum (a, d and g) and with antibodies to nuclear pore proteins (b), lamins A/C (e) or lamin B (h). Preparations were counterstained with Hoechst 33258 (c, f and i) to visualize DNA and monitor the presence of micronuclei. Arrows point at micronuclei. Bar, 4.7 μm



lamins A/C and the MAN antigens only at the nuclear rim (Fig.4).

In mitosis, the breakdown of the NE results in the depolymerization and redistribution of nuclear lamins into soluble lamins A/C and membrane-associated lamin B (Gerace and Blobel, 1980). As depicted in figures 5 and 6, the MAN antigens redistribute in parallel with lamin B but are segregated from lamins A/C. Upon NE disassembly in early prophase, as the chromosomes begin to condense (Figs.5c and 6c), the MAN antigens are co-localized at the nuclear periphery with lamins A/C and B (Figs.5a-d and 6a-d). In late prophase/prometaphase, lamins A/C begin to disassemble (Fig.5e-h). In contrast, the MAN antigens and lamin B remain at the periphery of the condensing chromosomes, illustrated by a strong continuous rimlike pattern. This only changes in late prometaphase when they disperse throughout the cytoplasm (Fig.6i-l). When the chromosomes align along the metaphase plate (Figs.5k and 6o), both the MAN antigens and lamin B are detected in the cytoplasm but are excluded from the mitotic apparatus (Fig.6m, n). In comparison, lamins A/C became undetectable during metaphase, only to reappear later. As the cells progress into anaphase, the MAN antigens along with lamin B are seen to reassociate with the segregating chromosomes (Fig.6q-t). This is followed by lamins A/C reappearing at the nuclear periphery during telophase (Fig.5q-t).

In another set of double immunofluorescence experiments, mouse 3T3 cells were stained for the MAN antigens and lamins A/C or B. Visualized by confocal microscopy, the MAN antiserum strongly labels the nuclear periphery with no detectable labelling of either

Figure 5. Spatial relationship of MAN antigens to lamins A/C in mitotic HeLa cells

Cells were double labelled for immunofluorescence with the anti-lamins A/C (a, e, i, m, q and u) and with the MAN antiserum (b, f, j, n, r and v). Preparations were counterstained with Hoechst 33258 (c, g, k, o, s and w) and observed by phase contrast (d, h, l, p, t and x) to identify mitotic stages. The pictures show the progression of these cells through early prophase (a-d), late prophase/early prometaphase (e-h), metaphase (i-l), early telophase (m-p), late telophase (q-t) and cytokinesis/early G1 (u-x). Bar, 6 µm.

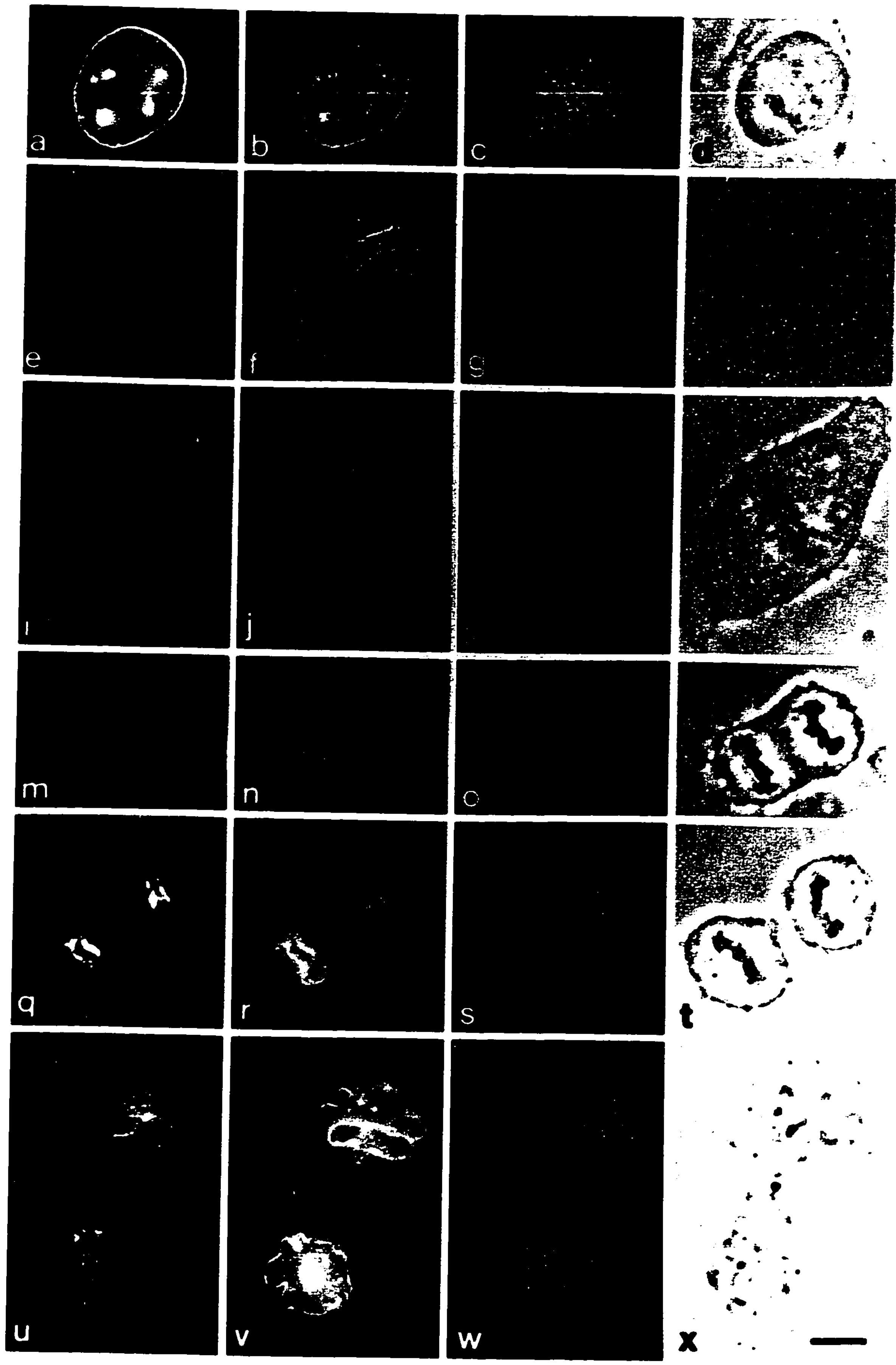


Figure 6. Spatial relationship of MAN antigens to lamin B in mitotic HeLa cells

Cells were double labelled for immunofluorescence with the anti-lamin B (a, e, i, m, q and u) and with the MAN antiserum (b, f, j, n, r and v). Preparations were counterstained with Hoechst 33258 (c, g, k, o, s and w) and observed by phase contrast (d, h, l, p, t and x) to identify mitotic stages. The pictures show the progression of these cells through early prophase (a-d), late prophase/early prometaphase (e-h), late prometaphase (i-l), metaphase (m-p), anaphase (q-t) and cytokinesis/early G1 (u-x). Bar, 6 μm.

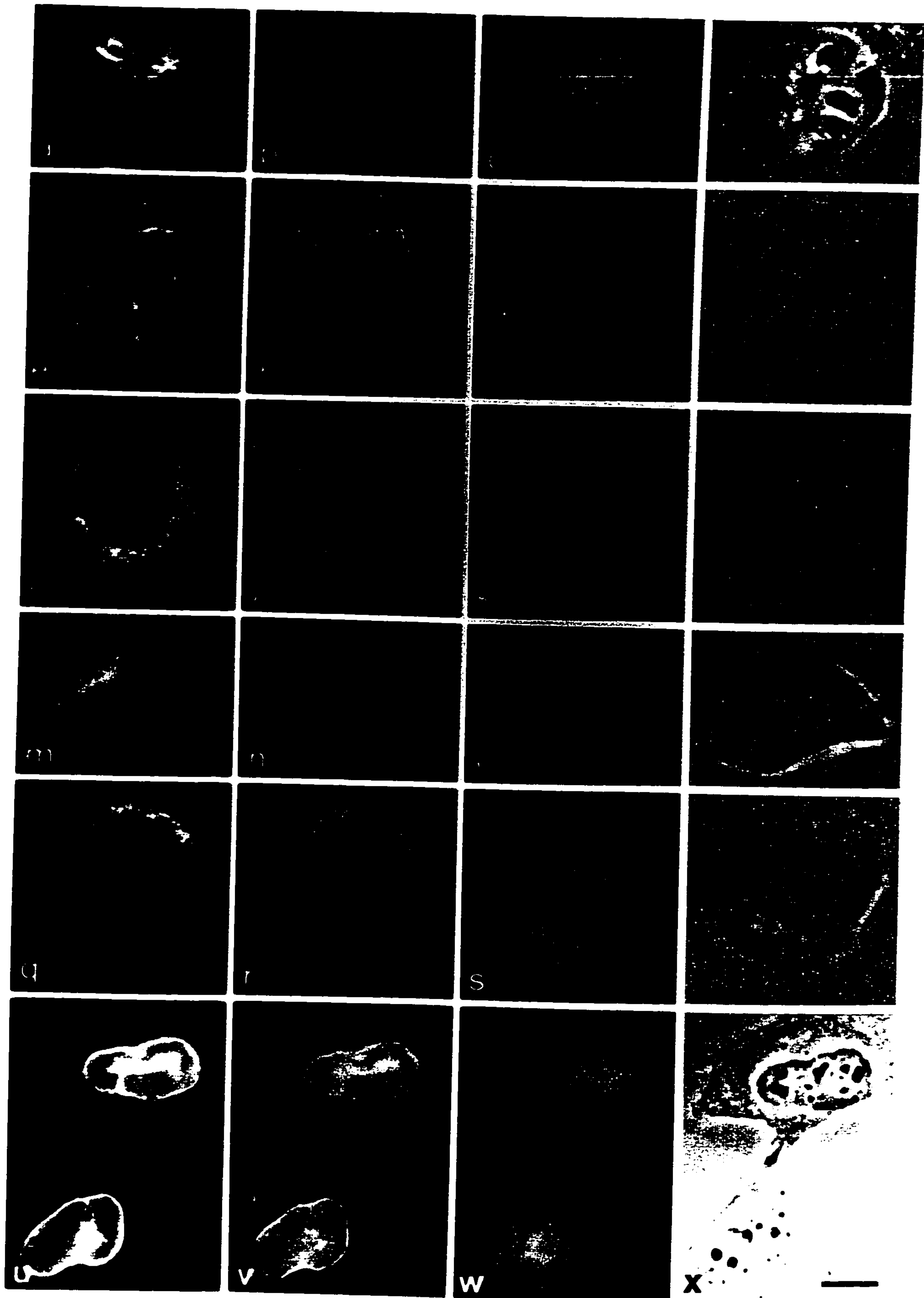
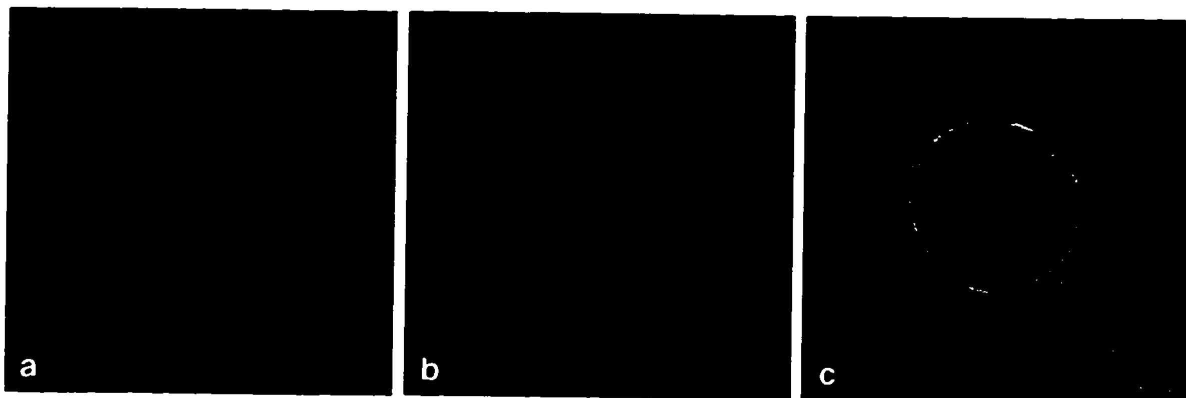


Figure 7. Confocal microscopic observation of the cellular localization of lamin B and the MAN antigens within an interphase 3T3 cell

Cells were double labelled with the MAN antiserum (a) and the anti-lamin B (b). A digital optical section (1 μm) of an interphase cell shows that MAN antigens co-localized with lamin B at the nuclear periphery, but were absent from intranuclear foci. Panel c represents a superimposed image of staining patterns observed in a and b. Colour was added artificially to aid comparison between the staining patterns. Bar, 3.8 μm


a
b
c

the cytoplasm or the internal nucleus (Fig.7a). This closely resembles the pattern seen with lamin B antibodies (Fig.7b). Yet, when the two images are superimposed, MAN antigens are seen to co-localize with lamin B at the nuclear periphery but are absent from lamin B intranuclear foci (Fig.7c).

2.4 DISCUSSION

The human antiserum (MAN) specifically labels the NE and recognizes a small subset of polypeptides with molecular weights ranging from 30-80 kDa in vertebrate cells, from human to fish (Paulin-Levasseur *et al.*, 1996). The MAN serum does not react with isolated nuclear lamins. Moreover, the MAN antigens are not recognized by the Pruss antibody, which targets a conserved epitope in most IF proteins, including the nuclear lamins. Based on these data, it appears that the MAN antigens are not immunologically related to the nuclear lamins, nor are they likely members of the family of IF proteins. The MAN antigens partition with nuclear matrices and therefore are distinct from both the LAPs and LBR which are extracted under detergent-high salt conditions (Worman *et al.*, 1988; Foisner and Gerace, 1993). These data demonstrate that the MAN antiserum recognizes a novel class of highly conserved nuclear lamina proteins found in vertebrate cells.

As described previously, the MAN antigens co-localize with lamins A/C and B at the nuclear periphery of interphase cells. However, in a subset of cells, the MAN antigens were found to be absent from intranuclear lamin B foci. The inhibition of DNA synthesis in *Xenopus* extracts immunodepleted of lamin B_3 (Newport *et al.*, 1990; Meier *et al.*, 1991; Jenkins *et al.*, 1993), and the co-localization of lamin B foci with proliferating cell nuclear antigen (PCNA: a DNA polymerase cofactor) at sites of bromodeoxy-uridine (BrDU) incorporation (Moir *et al.*, 1994) have provided evidence supporting the role of intranuclear lamin B in DNA

replication. This implies that, by not co-localizing with lamin B foci, the MAN antigens are not likely to be directly involved in DNA replication.

In mitotic HeLa cells, the spatial and temporal reorganization of the MAN antigens appears to be coordinated with lamin B. Lamins A/C redistribute to the cytoplasm in late prophase and reform around chromatin at telophase. Conversely, lamin B and MAN antigens only disassemble in late prometaphase and reassemble in anaphase, prior to lamins A/C. These observations provide evidence that A and B-type lamins do not segregate concomitantly during mitosis. This supports a model in which the two types of lamins possess distinct functional roles during NE disassembly/reassembly. In late prophase, lamins A/C disassemble prior to lamin B and the MAN antigens. This implies that lamins A/C may be involved in releasing the NE from its tight associations with the karyoskeleton, subsequently permitting lamin B associated vesicles to detach and redistribute through the cytoplasm. This is supported by the observation that cells devoid of lamins A/C have mechanically fragile nuclei (Wang and Traub, 1991).

The stepwise reconstitution of the NE, at the end of mitosis, is currently under much investigation (Lourim and Krohne, 1994). Our results show that the MAN antigens and lamin B reform around segregating chromosomes in anaphase. This conflicts with the report of Chaudhary and Courvalin (1993), who demonstrated that lamin B begins to reassociate with chromatin only during late telophase. The reasons for the discrepancies remain unclear. However, there

have been other reports documenting the reassembly of lamin B during anaphase (Meier and Georgatos, 1994). I did not study the mitotic fate of either the LBR or the LAPs, but it would be interesting to examine their temporal relationship to the MAN antigens. In NRK rat liver cells, LAP1 and LAP2 co-localize and are seen to reassemble around chromosomes in mid/late anaphase, before either A or B-type lamins (Foisner and Gerace, 1993). This data implies that the LAPs may target membrane vesicles to the chromatin or at least may be involved in early events in NE reformation (Moir *et al.*, 1995). Therefore, it would be important to establish if, within similar cell systems, the MAN antigens co-localize with either the LBR or the LAPs during these early events in NE reassembly.

The mechanisms responsible for the sequential reassembly of proteins, such as the nuclear lamins, LBR and the LAPs, may be cell-type specific (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Meier and Georgatos, 1994). Acknowledging this possibility makes it difficult to propose a mitotic role for the MAN antigens based on our observations. However, our data indicate that the MAN antigens interact closely with the nuclear lamins. For instance, in interphase the MAN antigens segregate in micronuclei with lamins A/C. This may reflect a pathway of reassembly separate from lamin B. In general, little is known about the events leading to the spontaneous formation of micronuclei, although it is often associated with deficiencies in DNA repair. Some investigators have speculated that micronuclei might be the result of non-disjunction

(Vogel and Motulsky, 1979). In this scenario, chromosomal breaks resulting in the loss of a centromere could prevent attachment of the chromosome to the mitotic spindle. In telophase, the lagging chromosome would appear as a distinct micronucleus as it becomes surrounded by a NE. It is feasible that, as the NE reassembles during early telophase, a chromosome which lags too far behind could be physically separated from some of the components reconstituting the NE. The segregation of lamins A/C and the MAN antigens within micronuclei could be the result of a similar process. I have shown that both lamin B and the MAN antigens reassemble around segregating chromosomes during anaphase. Yet, a considerable amount of MAN staining is still visible in the cytoplasm as the cells progress into telophase (Fig.6). Therefore, the surface of lagging chromosomes could be coated by lamins A/C and residual MAN antigens as they form micronuclei in telophase. Conversely, the targeting of lamin B to the nucleus may be indicative of a conserved pathway specific for the nucleus. This is supported by experiments examining the specific targeting of nuclear proteins in *Tetrahymena thermophila*. In this instance, microinjected *Tetrahymena* histone H1 and proteins tagged with the SV40 large T antigen NLS sequence strictly accumulated within the macronucleus, while *Tetrahymena* histone H4 was detected in both macro-and micronuclei (White *et al.*, 1989).

In addition, it was observed that lamin B and the MAN antigens co-localized throughout mitosis. Although it suggests that the MAN antigens and lamin B associate, either through binding to each

other or by mutual affinities for the nuclear envelope, it is also possible that the MAN antigens could segregate with a distinct vesicle population. Again, this would be supported by the lack of lamin B within micronuclei. Examples of separate vesicle populations have been found in many systems (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Lourim and Krohne, 1994). For instance, it was observed that LBR-derived vesicles and nuclear pore-derived vesicles could be distinguished within HeLa cells (Chaudhary and Courvalin, 1993). Whether or not the MAN antigens associate with membrane vesicles will require not only cell fractionation experiments but will also need to be confirmed by DNA sequencing analysis.

An issue which complicates the interpretation of these results has been the fact that within HeLa cells the MAN antiserum recognizes three major proteins. Therefore, it is not possible to use the antiserum to determine which, if not all three, of the MAN antigens are associated with lamins A/C in micronuclei or lamin B during mitosis. What is required are molecular tools which will enable us to tag these proteins and document their fate through the cell cycle. Yet, based on their ubiquitous expression and distribution through mitosis, the function of the MAN antigens might be associated with events of NE reassembly, possibly related to initial attachment of the lamina to decondensing chromosomes.

3. ISOLATION OF A MURINE cDNA ENCODING FOR A MAN REACTIVE POLYPEPTIDE, UBIQUITOUSLY EXPRESSED IN MOUSE TISSUES

3.1 INTRODUCTION

The nuclear lamina is a fibrous layer underlying the inner nuclear membrane of eukaryotic cells (Nigg, 1992). This structure and its primary components, the nuclear lamins, have been studied extensively at the biochemical and molecular level (reviewed by Moir *et al.*, 1995). In spite of this, the organization and function of the nuclear lamina remains unclear. Difficulties arise to a large extent from the differential expression patterns of the nuclear lamins and the lamina-associated proteins, the LAPs. In mammalian cells, as many as five different lamin isotypes may be expressed depending on the cell type and state of differentiation (Fisher *et al.*, 1986; McKeon *et al.*, 1986; Stewart and Burke, 1987; Höger *et al.*, 1988, 1990; Furukawa and Hotta, 1993; Furukawa *et al.*, 1994). Similarly, there is variable expression of integral membrane proteins between cells. LAP1C is the only member of the LAP family which is present in both differentiated and undifferentiated cells (Senior and Gerace, 1988; Martin *et al.*, 1995). The recent identification of a novel set of nuclear lamina proteins, the MAN antigens (Paulin-levasseur *et al.*, 1996), adds further complexity to this area of research. Undoubtedly, the dogmatic view of the nuclear lamina as an invariable structure needs to be revised.

While I was investigating the cell cycle fate of the MAN antigens, it became evident that molecular tools would be required

to examine how these proteins contribute to the organization and function of the nuclear lamina. Hence, I used the MAN antiserum to probe a P19 embryonal carcinoma cDNA expression library in order to isolate clones encoding for polypeptides immunologically related to the MAN antigens. Two cDNA clones, designated MAN #1 and MAN #2 (997 bp and 999 bp respectively), were isolated. Sequence analysis demonstrated that MAN #1 and MAN #2 are identical DNA sequences except for an additional two nucleotides at the 5′ end of MAN #2. The amino acid sequence of the MAN #1 polypeptide contains several motifs including: 1) a stretch of eleven glutamine residues, a characteristic of transactivation domains found in several transcription factors; 2) a short basic domain, which may represent a NLS sequence; and 3) a region of 18 hydrophobic amino acids, which could act as a transmembrane domain. In addition, the DNA sequence of the MAN #1 fragment appears to be related to a single gene and is expressed ubiquitously in a variety of mouse tissues and cell types.

3.2 MATERIALS AND METHODS

Cell culture and Western blotting analysis

P19 embryonal carcinoma (EC) cells were provided by Dr. M. McBurney (Ottawa Cancer Research Group, University of Ottawa, Can.). Cells were maintained at 1 x 10^6 cells/ml in Eagle's MEM (Flow Laboratories Inc.) at 37°C and 5% CO_2. The medium was supplemented with 10% HI FCS (Gibco BRL) as well as antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Gibco BRL). Proteins were harvested from cells and run on a SDS-PAGE gel, transferred to nitrocellulose and processed for Western blot analysis as mentioned in materials and methods in section 2.2 of chapter 2. The MAN antiserum was used at a dilution of 1:30,000 in PBS/Tween-20.

cDNA library construction and screening

Total RNA was isolated by lithium chloride (LiCl) extraction (Auffray and Rougeon, 1980) of day 6 DMSO treated P19 cells, which contained abundant cardiac muscle. Poly A $^+$ mRNA was selected by standard protocols (Sambrook *et al.*, 1989). cDNA was synthesized from poly A $^+$ mRNA using an oligo dT Not1 primer-adapter and the superscript lambda system kit (Gibco BRL). Sal1 adapters were ligated to the ends. The cDNA was subsequently digested with Not1 and size fractionated by column chromatography, yielding 300 ng of size-selected cDNA. Part of this cDNA (20 ng) was ligated into λgt22a phage arms, which had been digested with Not1 and Sal1, and packaged using the BRL λ packaging system (Gibco BRL). A total of 2 × 10^6 plaque forming units were obtained.

Standard procedures (Sambrook *et al.*, 1989) were used to plate

500,000 plaque forming units onto 10 separate 150 mm plates. After 4 hr at 42°C, Hybond-N filters impregnated with 10 mM isopropyl-thiogalactoside (IPTG) were placed over the forming plaques. The plates and filters were incubated for an additional 7 hr at 37°C and then the filters were removed and analyzed for reactivity to the MAN antiserum. The filters were processed for Western blot analysis as outlined in materials and methods section 2.2, except that the MAN antiserum was used at a dilution of 1:20,000 in PBS/Tween-20 for 1 hr, followed by an incubation for 1 hr with a HRP-conjugated goat anti-human IgG (1:2,000; Amersham Canada Inc.).

One positive plaque was identified from the 500,000 plaques screened and isolated after three rounds of plaque purification. Standard procedures (Sambrook *et al.*, 1989) were used to polyetheylene glycol (PEG) precipitate and purify phage DNA and ligate the cDNA insert into the Not1/Sal1 sites of pBluescript (KS orientation, Stratagene Cloning Systems, La Jolla, Ca, USA). This cDNA insert was termed MAN #1 and was used to rescreen the original 500,000 plaques as well as an additional 500,000 plaques which were freshly plated as described (Sambrook *et al.*, 1989). Plaques were transferred to Hybond-N and denatured by autoclaving for 2 min at 100°C. DNA was cross-linked by ultraviolet (UV) irradiation and the filters were hybridized for 16 hr at 42°C with the radiolabeled MAN #1 cDNA. The 1 kilobase (kb) Not/Sal fragment of MAN #1 was radiolabeled to over 10^9 counts per min (cpm)/μg with [α^{32}P] - 2'-deoxycytidine 5'-triphosphate (dCTP) using a multiprime labelling kit (Amersham Canada Inc.). The probe was purified on a spin column

of Sephadex G-50 (Pharmacia Biotech Inc., Baie d'Urfé, Québec, Can.). Washing was performed for 30 min at RT in 2 × SSC (3 M NaCl and 0.34 M sodium citrate, pH 7.0), 0.2% SDS and for 15 min at 65°C in 0.2 × SSC, 0.2% SDS. Hybridization was visualized by autoradiography. One positive plaque was identified and purified from the freshly plated 500,000 plaques. This cDNA was termed MAN # 2. This λgt22a cDNA expression library was prepared and screened by Dr. Ilona Skerjanc.

Two additional clones were retrieved from a commercially made P19 EC undifferentiated Uni-Zap XR cDNA library (Stratagene Cloning Systems, La Jolla, Ca, USA). cDNA synthesis was performed using an oligo dT Xho1 primer. EcoR1 adapters were added to the ends and the cDNA was digested with Xho1. cDNAs were ligated into the polylinker of pBluescript, within λZap phage arms, creating a uni-directional library (Stratagene).

Established protocols (Stratagene) were used to screen 1 × 10^6 plaque forming units on 20 separate 150 mm plates. Plaques were transferred to Hybond-N filters and denatured for 2 min in 1.5 M NaCl, 0.5 M NaOH; neutralized for 5 min in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0); and rinsed for 30 seconds (sec) in 0.2 M Tris-HCl (pH 7.5). DNA was cross-linked by UV irradiation and the filters were hybridized for 16 hr at 42°C with the MAN #1 cDNA fragment which was radiolabeled with [α^{32}P] - dCTP and purified over a spin column, as described above. The filters were washed for 30 min at RT in 3 × SSC, 0.1% SDS and again for 15 min at 65° C in 2 × SSC, 0.1% SDS. Two positive plaques, designated MAN #3 and #4, were identified by

autoradiography. The MAN #3 cDNA was recovered within the pBluescript phagmid using the Stratagene *in vivo* excision protocol (Stratagene).

MAN sequence analysis

Sequencing was performed with the T_7 sequencing kit with Deaza-guanosine 5'-triphosphate (GTP) from Pharmacia (Pharmacia Biotech Inc.). To resolve gel compressions, the DNA products from the sequencing reactions were separated by electrophoresis in a 6% polyacrylamide gel containing 7 M urea and 40% formamide. Rsa1 and Alu1 fragments of MAN #1 were subcloned and the miniprep DNA sequenced (Kraft *et al.*, 1988). Gaps in the sequence were completed by using synthetic oligonucleotides, 5'CAACAAGACGCGGAACAG3'; 5'CAGTACCGCGGGCTCAGA3';5'GGAAGCCGCACTCGTGGT3';5'ACGGTGAGGACCGCGACC3'; 5'AGCACTACTCGGACTCG3'. Both MAN #1 and #2 were completely sequenced on both strands. The MAN #3 cDNA was sequenced using an ABI Prism DNA sequencer (Dr. McBurney, Ottawa Cancer Research Group, Ottawa, Can.). Both strands of MAN #3 were sequenced using Universal forward and reverse primers (Pharmacia Biotech Inc.).

Southern blot analysis

Genomic DNA was phenol extracted and ethanol precipitated from P19 cells, using a standard method (Sambrook *et al.*, 1989). Genomic DNA (10 µg) was digested with EcoR1 or with EcoR1 and Not1 overnight at 37°C and then separated by electrophoresis in a 1% agarose gel. The DNA in the agarose gel was denatured for 30 min in 1.5 M NaCl, 0.5 N NaOH and neutralized for 30 min in 1.5 M NaCl, 1 M Tris(pH 7.4). The DNA was transferred to Hybond-N (Amersham

Canada Inc.) by capillary blotting and was cross-linked by UV irradiation. The membrane was hybridized to DNA probes of the 1kb Not1/Sal1 MAN #1 fragment labelled to over 10^9 cpm/μg with [α ^{32}P]-dCTP using a multiprime labelling kit (Amersham Canada Inc.). The probes were purified on a spin column of Sephadex G-50 (Pharmacia Biotech Inc.) and hybridized for 16 hr at 42°C. Washing was performed for 30 min at RT in 2 × SSC, 0.2% SDS. Hybridization was visualized by autoradiography.

RNase protection

To produce the desired anti-sense RNA for RNase protection, the first 763 bp of MAN #1 were removed, creating a plasmid termed MAN (763bp-997bp). This construct was made by digesting MAN #1 in pBluescript with Sal1, filling in the 5′ overhang with Klenow activity, digesting with Pvu11, and re-ligating the plasmid. MAN(763bp-997bp) was linearized in the multiple cloning site of pBluescript with Hinc11 so that the resulting riboprobe was complementary to nucleotides 763-997 of MAN and carried some vector sequences. Anti-sense RNA was synthesized from linearized MAN (763bp-997bp) and from pTRI-β-actin-mouse plasmid DNA using T_7 RNA polymerase, as described in the MAXIscript *in vitro* transcription kit protocol (Ambion Inc., Austin, Texas, USA). The MAN and actin probes migrated on the acrylamide/urea gel as single bands of 250 bp and 227 bp, respectively, and incorporated [α^{32}P]-dCTP to a specific activity of about 5 × 10^8 cpm/μg RNA. The RNA probes were purified from a 5% acrylamide/8 M urea gel as described in RPA11 Ribonuclease Protection Assay Kit (Ambion Inc.).

The RNase protection was performed as described in the RPA11 kit (Ambion Inc.) by hybridizing 2.2 fm of MAN riboprobe with 10 μg of total sample RNA or yeast tRNA. The control actin riboprobe was hybridized to 5 μg of total sample RNA or yeast tRNA. Total RNA was isolated (Auffray and Rougeon, 1980) from P19 stem cells treated with either DMSO or retinoic acid (RA), from mouse brain, liver, kidney and spleen. After RNase digestion of the hybridized probe and sample RNAs, protected fragments were separated on a 5% polyacrylamide/8 M urea gel and visualized by autoradiography.

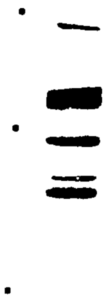
To estimate the size of the bands protected from RNase digestion, the 1 kb ladder was labelled with [γ-^{32}P]ATP using the exchange reaction with bacteriophage T_4 polynucleotide kinase, as described in Sambrook *et al.* (1989). The labelled 1 kb ladder was subjected to electrophoresis alongside the RNase protection samples. A graph of log molecular weight versus distance from the origin was used to determine the sizes of the protected bands. The calculated molecular weights agreed with the predicted molecular weights within an error of 10%. This RNase protection assay was performed in collaboration with Dr. Ilona Skerjanc.

3.3 RESULTS

To further study the MAN antigens, I chose to screen a P19 EC cDNA expression library because, as illustrated in figure 8, differentiated P19 cells express all three MAN polypeptides. A library was constructed from day 6 DMSO treated P19 poly(A)$^+$ mRNA and the MAN antiserum was used to retrieve clones which encoded MAN-reactive antigens. Initially, a 1.0 kb positive clone (MAN #1) was isolated from screening 5 × 10^5 plaques. This cDNA was subsequently used to reprobe the original 5 × 10^5 plaques as well as an additional 5 × 10^5 which were freshly plated. A single positive clone (MAN #2) was identified from the freshly plated 5 × 10^5 plaques. By sequencing, the MAN #2 clone was found to be identical to the MAN #1 clone with the exception of an additional two nucleotides at the 5′ end. DNA sequencing analysis of the MAN #1 (997 nucleotides long) cDNA revealed that it contained a large open reading frame (ORF) with a potential initiator methionine at the 5′ end but no polyadenylation tail or stop codons due to an internal Not1 restriction site (Fig.9). The MAN #1 cDNA is predicted to encode a protein of 331 amino acids with a calculated molecular weight of 35 kDa. There does not exist an in-frame stop codon upstream of the DNA sequence encoding the potential initiator methionine, indicating that the MAN #1 cDNA encodes only a fragment of the full length transcript. Using the Kyte and Doolittle hydrophobicity plot (Kyte and Doolittle, 1982), the translated MAN #1 polypeptide is shown to be greater than 90% hydrophilic with a short 18 amino acid hydrophobic stretch (Fig.9). DNA sequencing of

Figure 8. Detection of MAN antigens in homogenates from undifferentiated and DMSO-differentiated P19 cells

Proteins were harvested from undifferentiated (A) and differentiated (B) P19 cells and prepared for immunoblotting with the MAN antiserum. As illustrated, both cell types express all three major MAN antigens, and additional minor species. The black squares represent pre-stained molecular weight markers: bovine serum albumin, 84 kDa, ovalbumin, 47 kDa and carbonic anhydrase, 33 kDa.


A

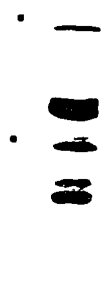

B

Figure 9. Nucleotide sequence of the MAN #1 cDNA fragment

The sequence of the MAN #1 clone is shown with the deduced MAN #1 polypeptide sequence indicated by one-letter amino acids (A). Bold italics represents the potential NLS sequence, amino acids 41-48. The underlined DNA sequence encodes for the eleven amino acid glutamine stretch. Bold letters from amino acids 112-129 is the hydrophobic domain. Panel B is the Kyte and Doolittle (1982) hydrophobicity plot of the deduced MAN #1 polypeptide.

A

```
gaaaATGGCGGCGGCGACGGCGGCGGCGGCGCCTCAGCAGCTCTCGGATG   50
    m  a  a  a  t  a  a  a  a  p  q  q  l  s  d  e
AGGAGCTTTTCTCTCAGCTCCGCCGTTACGGCTTATCTCCGGGTCCCGTG   100
  e  l  f  s  q  l  r  r  y  g  l  s  p  g  p  v
ACGGAGAGCACCCGGCCGGTCTACCTCAAGAAGCTGAAGAAGCTTCGCGA   150
t  e  s  t  r  p  v  y  l  k  k  l  k  k  l  r  e
GGAAGAGCAGCAGCAACAGCAGCAACAGCAGCAGCAGCAGCACCGGGCGG   200
 e  e  q  q  q  q  q  q  q  q  q  q  q  h  r  a  g
GGGGCCGCGGCAACAAGACGCGGAACAGTAATAACAATAACACGGCGACG   250
  g  r  g  n  k  t  r  n  s  n  n  n  n  t  a  t
GCGATGGGGGGCCGGCCGGGCTCCGGGGACCTCGCGTACTTACGGAGCCC   300
a  m  g  g  r  p  g  s  g  d  l  a  y  l  r  s  p
CGCGGGCCTCGGCCGCCTGTCGGCCTCGGCTGCCGAGAGCCCCGTGGCAG   350
  a  g  l  g  r  l  s  a  s  a  a  e  s  p  v  a  g
GAGGCTCCGGGGGCGCGGCGGCCGTCCCCGCGGCGGGCAGCAAAGTGCTG   400
  g  s  g  g  a  a  a  v  p  a  a  g  s  k  v  l
CTGGGCTTCAGCTCGGACGAGTCGGACGTGGAGGCCAGCCCGCGGGAGCA   450
l  g  f  s  s  d  e  s  d  v  e  a  s  p  r  e  q
GGCGGGCGGCGGCGGCGGTGGCGGCGCGCGGAGGGACCGGGCTGCGCTCC   500
 a  g  g  g  g  g  g  a  r  r  d  r  a  a  l  q
AGTACCGCGGGCTCAGAGCCCCGCCGGCGCCCCCGGCCGCGGGCGAGGTG   550
 y  r  g  l  r  a  p  p  a  p  p  a  a  g  e  v  t
ACGGGCGGCCACCCGGGCGAGCGGAGGAAGCCGCACTCGTGGTGGGGGGC   600
  g  g  h  p  g  e  r  r  k  p  h  s  w  w  g  a
GCGGAGGCCGGCGGGCCCCGAGCCGCAACCCCCGGCGGCGGGGAGCGACG   650
r  r  p  a  g  p  e  p  q  p  p  a  a  g  s  d  g
GGGCCGCGGAGGACGCGGACGAGGAGCTGGCGGACGGTGAGGACCGCGAC   700
 a  a  e  d  a  d  e  e  l  a  d  g  e  d  r  d  p
CCCGAGGCCGAGGAGCCGCTGTGGGCCAGCCGAGCGGTGAACGGCAGCCG   750
  e  a  e  e  p  l  w  a  s  r  a  v  n  g  s  r
GCTTCTCCCCTACAGCAGCTGCCGGGAGCACTACTCGGACTCGGAGGAGG   800
l  l  p  y  s  s  c  r  e  h  y  s  d  s  e  e  e
AGGAGGAGGAGGGGGAGGAGGACGGCGACGTGGCCCCGGCCAGACAGGTA   850
 e  e  e  g  e  e  d  g  d  v  a  p  a  r  q  v  l
TTAAAGGACGACTCCCTCGCCCGGCATCGACCCAGACGGAGCCATAGTAA   900
 k  d  d  s  l  a  r  h  r  p  r  r  s  h  s  k  p
GCCGTTCTCGGCGCTGACTGCTAAATCTGGCGGCAGCCGGCAGGAGACTT   950
  f  s  a  l  t  a  k  s  g  g  s  r  q  e  t  s
CGGTTCAGGGAGGGGGAGCACTCGCGATGAATGACAGGGCGGCGGCG      997
v  q  g  g  g  a  l  a  m  n  d  r  a  a  a
```

B

MAN #1 revealed an unusually high GC content (72%), but searches with Genbank sequences did not indicate any obvious homology. However, at the amino acid level, the polypeptide encoded by MAN #1 has potential glycosylation, myristoylation and phosphorylation consensus sites (Fig.10). Apart from this, the only other noticeable features of the sequence are a potential NLS and an eleven amino acid glutamine stretch found at the 5′ end of the sequence, an element which is often characteristic of transactivation domains within transcription factors (Fig.9).

Attempts were made to isolate clones encoding sequences further 5′ and/or 3′ to the MAN #1 cDNA. A P19 EC undifferentiated cDNA expression library was screened with a MAN #1 radiolabeled probe. From 1×10^6 primary plaques plated, two potential positive clones (MAN #3 and MAN #4) were plaque purified through a secondary and tertiary screening. Following isolation and DNA sequence analysis, it was discovered that both MAN #3 and #4 show little sequence homology with the original MAN #1 cDNA.

To examine whether the DNA sequence of the MAN #1 fragment is related to a single gene or a family of genes, I probed genomic southerns. P19 genomic DNA was isolated and digested with either EcoR1 or EcoR1 and Not1. These enzymes were chosen because neither of these sites are found within the MAN #1 clone. As shown in figure 11, probing the southern with the radiolabeled MAN #1 cDNA resulted in a single band at 2.5 kb in the lane which had been digested with EcoR1 alone and another single band at 1.3 kb in the lane which was digested with both EcoR1 and Not1. The shift in size

Figure 10. Schematic diagram of the post-translational modification consensus sites contained in the MAN #1 polypeptide

Deduced amino acid sequence of the polypeptide encoded by the MAN #1 cDNA. Double underlined (_) residues correspond to casein kinase II phosphorylation consensus sites. The dots (°) represent protein kinase C phosphorylation consensus sites. Arrows (▾) designate glycosylation consensus sites and the bold lettering are myristoylation consensus sites.

M A A A T A A A A P Q Q L S D E E L F S 20
Q L R R Y G L S P G P V T E S T R P V Y 40
L K K L K K L R E E E Q Q Q Q Q Q Q Q Q 60
Q Q H R A G G R G N K T R N S N N N N T 80
A T A M G G R P G S G D L A Y L R S P A 100
G L G R L S A S A A E S P V A G G S G G 120
A A A V P A A G S K V L L G F S S D E S 140
D V E A S P R E Q A G G G G G G G A R R 160
D R A A L Q Y R G L R A P P A P P A A G 180
E V T G G H P G E R R K P H S W W G A R 200
R P A G P E P Q P P A A G S D G A A E D 220
A D E E L A D G E D R D P E A E E P L W 240
A S R A V N G S R L L P Y S S C R E H Y 260
S D S E E E E E E G E E D G D V A P A R 280
Q V L K D D S L A R H R P R R S H S K P 300
F S A L T A K S G G S R Q E T S V Q G G 320
G A L A M N D R A A A 331

Figure 11. Genomic Southern analysis of the MAN #1 gene

P19 genomic DNA digested with either EcoR1 (lane 1) or EcoR1 and Not1 (lane 2) restriction enzymes was probed with the MAN #1 cDNA. A single hybridizing 2.5 kb fragment and 1.3 kb fragment were detected in lanes 1 and 2, respectively.

1 2

2.5 →

1.3 →

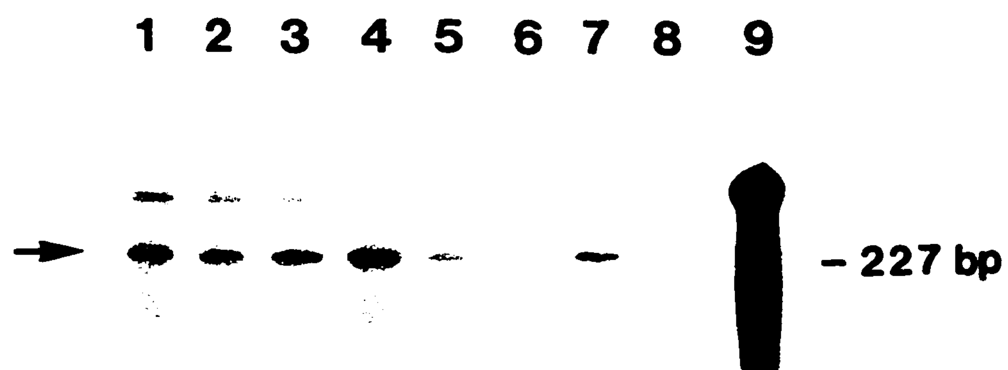
of the hybridizing band in the double digested sample confirms the existence of the internal Not1 site within the gene. Thus, the single band found in both lanes suggests that within P19 cells the DNA sequence of the MAN #1 fragment is related to a single gene.

Attempts to visualize the size of the MAN #1 mRNA transcript was unsuccessful by Northern blot analysis. However, the relative abundance of the mRNA in a variety of mouse tissues could be analyzed (Fig.12). By RNase protection, a riboprobe derived from the 3′ end of the MAN #1 clone can be seen to protect a predicted 227 bp fragment in each lane except the yeast tRNA control sample. In addition, a second protected band can be seen migrating slower than the 227 bp fragment in every lane. This is due to the residual template DNA which was not completely digested after DNase treatment and is present in the lane with just the probe alone. Using an actin riboprobe on aliquots of the same samples, it can be seen that each lane was equally loaded for total mRNA. The results indicate that there does not appear to be any significant difference in the level of expression of the MAN #1 mRNA in any of the RNA samples from either liver, kidney or spleen, although there appears to be slightly more in brain tissues. As well, no change in the expression levels is detected between undifferentiated P19 cells and P19 cells which have been induced to differentiate with either DMSO or RA. Together, these data suggest that the MAN #1 mRNA is ubiquitously expressed in all samples tested and that its regulation is not dependent on the phenotype nor the state of differentiation of the cells.

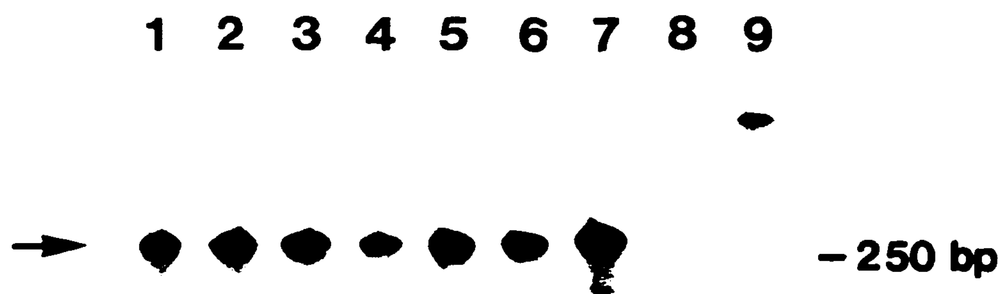
Figure 12. Analysis of MAN #1 mRNAs in a variety of mouse cell types by RNase protection

Total RNA was isolated from undifferentiated P19 cells (lane 1), P19 cells treated with DMSO (lane 2) or RA (lane 3), from mouse brain (lane 4), liver (lane 5), kidney (lane 6) and spleen (lane 7). Each of the samples, including yeast tRNA (lane 8), were hybridized with either a MAN #1(227 bp) (A) or actin (250 bp) (B) riboprobe. The MAN #1(227 bp) riboprobe can be seen to protect a predicted 227 bp fragment in all lanes with the exception of the yeast tRNA sample. The level of MAN #1 mRNA expression appears the same in all samples, with possibly a slight increase in expression in mouse brain tissues. Lane 9 represents loading of the MAN #1 (A) and actin (B) riboprobes alone.

A



B



3.4 DISCUSSION

In this study, the MAN antiserum was used to isolate two encoding cDNAs, termed MAN #1 and MAN #2. DNA sequencing of both clones revealed that they differed by only two extra nucleotides at the 5′ end of MAN #2. Analysis of the MAN #1 (997 nucleotides long) clone indicated that the cDNA encoded a potential initiator methionine in-frame with the deduced ORF. This methionine conforms to the standard Kozak consensus sequence (Kozak, 1986) and thus suggests that the amino terminus of the MAN mRNA transcript may have been cloned. However, there does not exist an in-frame stop codon upstream of the proposed encoded initiator methionine. Confirmation of this start site requires additional upstream sequences.

The λgt22a expression library was designed with an oligo dT Not1 primer-adapter. Isolation of the insert from the phage arms required digestion with both Sal1 and Not1 enzymes. Examination of the 3′ end of the clone showed that it lacks a polyadenylation tail and stop codon due to an internal Not1 restriction site, indicating that the MAN #1 cDNA encodes only a fragment of the full length transcript.

Comparing the DNA sequence of MAN #1 with that of the nuclear lamins revealed no homology, thus ruling out the possibility of having cloned a minor lamin isotype. In fact, homology searches of Genbank sequences only showed significant alignment with cDNAs which possessed stretches of trinucleotide repeats, such as the CAG repeat encoding the 11 amino acid glutamine stretch, within MAN #1

(Fig.9). Many transcription factors, such as the androgen receptor (Chang *et. al.*, 1988), have trinucleotide repeat sequences within their transactivation domains. The CAG repeat within MAN #1 is quite short, but experiments using sequences ranging in size from 10-30 glutamines were sufficient to activate transcription when fused to the DNA binding domain of GAL4 (Gerber *et al.*, 1994).

Several human diseases have been reported to be associated with the expansion of trinucleotide repeats (reviewed by Green, 1993). In many of these genes, the normal range varies between 5-60 repeats. Although often found within the untranslated regions of mRNAs, such as with the transcript associated with myotonic dystrophy (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). The trinucleotide repeat can be found in the coding region (La Spada *et al.*, 1991; Orr *et al.*, 1993). The MAN antiserum used to isolate the MAN #1 cDNA was collected from a patient who had been diagnosed with an unusual case of collagenosis (Paulin-Levasseur *et al.*, 1996). I was interested to know if the length of the MAN #1 repeat was polymorphic. PCR primers which flanked the repeat were used to screen human genomic libraries. However, no polymorphic trinucleotide repeat sequences were identified (in collaboration with Dr. R. Korneluk, Dept. of Genetics, Children's Hospital of Eastern Ontario, Canada). Therefore, in our case it is unlikely that such a mechanism of trinucleotide expansion can be used to explain the patient's disorder.

According to the Kyte and Doolittle hydrophobicity plot (Fig.9; Kyte and Doolittle, 1982), the MAN #1 polypeptide is

hydrophilic throughout most of its sequence. However, there is a short (18 amino acids) hydrophobic region at position 112-129. This stretch of non-polar residues could represent a hydrophobic pocket within the protein or it could signify a transmembrane domain. This region is long enough to form a single α-helix transmembrane domain (Popot *et al.*, 1990; Wistow *et al.*, 1991), which is typical of type 2 integral membrane proteins. Recent cloning of the LAP2 cDNA showed that it encoded a strongly hydrophilic polypeptide with a single 22 amino acid transmembrane domain at its carboxyl terminus (Furukawa *et al.*, 1995). There is no significant sequence similarity between these two proteins, other than a similar percentage of hydrophilic residues. A large nucleoplasmic domain, as found in LAP2, could be characteristic of integral membrane proteins involved in binding structures (i.e. nuclear lamina) to the inner nuclear membrane (Furukawa *et al.*, 1995). The confirmation of the MAN #1 polypeptide as an integral membrane protein will require not only cell fractionation experiments, but the cloning of the full length cDNA.

By searching the MAN #1 polypeptide for conserved motifs (SwissProt database), it was discovered that the polypeptide encoded multiple myristoylation consensus sites. This is a post-translational modification where a 14 carbon fatty acid chain is attached to the amino terminus of proteins likely to associate with the lipid membrane (Towler and Glaser, 1986). Within the family of nuclear lamin proteins, both pre-lamin A and lamin B are isoprenylated (Beck *et al.*, 1988; Farnsworth *et al.*, 1989;

Vorburger *et al.*, 1989). Isoprenylation is believed to facilitate the incorporation of pre-lamin A and lamin B into the nuclear lamina (Krohne *et al.*, 1989). Once incorporated, the isoprenylated carboxyl terminus of pre-lamin A, but not lamin B, is proteolytically cleaved (Beck *et al.*, 1988). As a consequence, at mitosis mature lamin A is solubilized, whereas lamin B remains attached to the nuclear vesicles (Gerace and Blobel, 1980), presumably aided through the isoprenyl group (Hennekes and Nigg, 1994). During mitosis, the MAN antigens temporally disassemble and reassemble with lamin B but not lamins A and C (Paulin-Levasseur *et al.*, 1996). This observation would suggest that the MAN antigens, like lamin B, could associate with the nuclear vesicles. Thus far, sequence analysis of the MAN #1 polypeptide has revealed two possible mechanisms through which association with the nuclear vesicles could be possible, either post-translational modification (myristoylation) or the presence of a transmembrane domain.

The MAN antiserum recognizes three major polypeptides in a number of different vertebrate species (Paulin-Levasseur *et al.*, 1996). These proteins could be the alternatively spliced products of a single transcript. By northern blot analysis, it was not possible to detect the size of the MAN #1 mRNA. This could be a result of the high turnover rate or the low abundance of the message. As a consequence, it is not possible to determine one way or another if alternative splicing is occurring. However, in a recent experiment, isolated MAN polypeptides were subjected to partial protease digestion followed by immunoblotting with the MAN

antiserum. Each one of the 78, 58 and 40 kDa proteins produced a similar proteolytic profile (see appendix). This confirms that the three major MAN antigens are related structurally. Genomic southerns probed with the MAN #1 cDNA produced a single hybridizing band, indicating the DNA sequence of the MAN #1 fragment is related to one gene and not a family of genes (Fig.11). Although highly suggestive, further evidence will be required to establish that the three MAN antigens are the alternative spliced products of a single transcript.

In order to study the expression pattern of the MAN mRNA transcript, it was necessary to perform an RNase protection assay. A riboprobe, derived from the 3′ end of the MAN #1 cDNA, was able to protect a 227 bp mRNA fragment in every lane (Fig.12). The transcript was present in mouse tissue (brain, liver, kidney and spleen) and P19 differentiated and undifferentiated cells. The level of MAN mRNA expression appeared constant across all samples. In contrast, studies on the expression of lamins within many cell and tissue types have indicated that B-type lamins are ubiquitously expressed, whereas lamins A and C are under developmental control (Stewart and Burke, 1987; Rober *et al.*, 1989). In this experiment, there was no detectable change in the amount of MAN mRNA found in P19 cells (Fig.12, lane 1) versus P19 cells induced to differentiate with either RA or DMSO (Fig.12, lanes 2 and 3). Therefore, unlike lamins A and C (Stewart and Burke, 1987), MAN mRNA is not under transcriptional regulation associated with cellular differentiation.

4.0 THE PARTIAL MAN #1 cDNA ENCODES FOR A NUCLEAR POLYPEPTIDE THAT IS IMMUNOLOGICALLY RELATED TO ALL THREE MAJOR MAN ANTIGENS

4.1 INTRODUCTION

Autoantibodies have been used as diagnostic markers for autoimmune disorders, such as rheumatoid arthritis (Stollar, 1991). These antibodies have also served as useful tools for examining both the organization and function of specific antigens. The MAN antiserum has been characterized because it recognizes antigens localized at the NE (Paulin-Levasseur *et al.*, 1996). To study these antigens in more detail, the antiserum was used to screen a cDNA expression library for clones encoding MAN-reactive polypeptides. The identification of a positive clone using such a strategy presents inherent difficulties because the immunological reaction between the antibody and its antigen occurs against a background of endogenous bacterial proteins. Furthermore, as only a third of the cDNA clones would usually be expressed in the correct reading frame in any given library, the chances of isolating a false positive are not negligible. Therefore, it was essential to validate that the MAN #1 cDNA encodes the desired epitope(s).

I have performed a series of experiments which provide evidence that the polypeptide encoded by the MAN #1 cDNA bears at least two epitopes in common with the three endogenous MAN polypeptides. I demonstrate also that one of these epitopes maps to the amino terminus of the predicted amino acid sequence, encompassing the eleven amino acid glutamine stretch.

The presence of a MAN epitope within the MAN #1 polypeptide does not guarantee that the corresponding cDNA encodes one of the

three endogenous MAN antigens. In an attempt to gain insight into the characteristics of the MAN #1 polypeptide, I have transiently transfected P19 cells with a 6-mycMAN #1 expression construct. My results show that the MAN #1 polypeptide contains sequences which facilitate its translocation into the nucleus. These data suggest that the putative NLS located in the amino terminus of the predicted amino acid sequence may be functional and that the MAN #1 cDNA encodes a potential nuclear protein.

4.2 MATERIALS AND METHODS

Cell cultures

HeLa (CCL 2) cells and P19 EC cells were maintained in culture under the conditions described in chapter 2 (section 2.2) and chapter 3 (section 3.2), respectively.

Transient transfection of P19 cells

P19 EC cells were transiently transfected with either a DNA expression construct containing the Pgk promoter (Adra *et al.*, 1987) driving the expression of 6-mycMAN #1 transcript or a vector lacking the Pgk promoter and containing unrelated sequences in its multiple cloning site (pKJ53). Cells were plated at a density of 5 x 10^5 cells/60 mm dish and left to grow overnight. Cells were then transfected with a total of 10 μg of DNA/dish using a calcium phosphate ($CaPo_4$) method (Chen and Okayama, 1987). The DNA precipitate was left on the cells for 6 hr and then removed with two washes with PBS. The dishes of cells were supplemented with αMEM (Gibco BRL) medium containing FCS (Gibco BRL) and grown overnight. The cells were prepared 16 hr later for either immunofluorescence microscopy or harvested for Western blot analysis.

Expression of β-gal/MAN fusion proteins and adsorption/elution of MAN antibodies

To express high levels of β-gal/MAN #1 fusion protein from the λgt22A-cDNA MAN clone, 100 μl of a high titre stock of this clone was incubated for 10 min at 37°C with 1 ml of an overnight culture of Y1090 bacteria. The cultures were diluted to 50 ml with Luria-Bertani (LB) medium, containing ampicillin and maltose. After

shaking for 1 hr at 37°C, IPTG was added (1 mM final concentration). The cultures were incubated a further 5 hr with shaking at 37°C and then centrifuged at 5,000 rpm for 10 min. The pellet (P1) was solubilized in 1 ml of sample buffer (SB) for SDS-PAGE (Laemmli, 1970). Proteins from the supernatant (S1) were precipitated with TCA, as previously described (Paulin-Levasseur *et al.*, 1996), and solubilized in 1 ml of SB. P1 and S1 were then stored at -80°C. A parallel culture of Y1090 bacteria was infected with an unrelated λgt22A-cDNA clone and processed exactly in the same fashion to obtain control pellet (P2) and supernatant (S2).

Aliquots of 100 µl of P1, S1, P2 and S2 were applied across the width of 5% stacking gels and were separated through 10% resolving gels (Laemmli, 1970). Proteins were then electrotransferred to nitrocellulose membranes (Towbin *et al.*, 1979). A vertical strip was cut from each of the blots, blocked for 1 hr with 5% nonfat dried milk and 0.05% Tween 20 in PBS and then processed for immunodetection of MAN fusion proteins according to the protocol described in chapter 3, section "cDNA Library Construction and Screening". These strips were used as a reference to position the fusion products on blots. Reactive bands on P1 and S1 blots as well as the corresponding regions on P2 and S2 blots were excised and blocked for 1 hr with 5% nonfat dried milk and 0.05% Tween 20. They were then incubated with the MAN antibody (1:20,000 in PBS) for 3 hr at RT and 16 hr at 4°C. After this step, the supernatant was collected, corresponding to the non-adsorbed (NA) fractions of the antibody: NAP1, NAS1, NAP2 and NAS2. To

recover the adsorbed (A) fractions of the antibody, the reacted nitrocellulose bands were cut in small pieces and incubated for 10 min with 3.5 ml of 100 mM glycine (pH 2.5). The reaction was neutralized with 0.35 ml of 1 M Tris (pH 8.0), bringing the pH to 7.0, and the eluted antibodies were recovered in the aqueous phase: AP1, AS1, AP2 and AS2.

Construction and expression of 6-His-tagged MAN #1 deletion mutants

The MAN #1 clone in pBluescript was used to generate a 6-HisMAN #1 DNA construct and a series of His-tagged MAN #1 cDNA deletion constructs. The MAN #1 and MAN(144) DNA fragments were produced by digesting the MAN #1 clone with either Not1 or Hind III, respectively. Using the large fragment (klenow) of DNA polymerase 1 (Gibco BRL), the resulting 5′ overhangs were filled in with dNTPs. This was followed by digestion with Sal1, releasing the MAN #1 and MAN(144) from pBluescript. The MAN(564) and MAN(287) fragments were created by first digesting the MAN #1 clone with Sal1, followed by either Sma 1 (MAN(564)) or Rsa 1 (MAN(287)). The different pieces of DNA were resolved on a 1% agarose gel, and the individual MAN #1 cDNA fragments were excised and eluted from the gel (Sambrook *et al.*, 1989).

Each of the MAN #1 cDNA fragments were ligated directionally into a bacterial expression vector (pQE31) driven by the *E.coli* T_5 promoter with two lac operator sequences (Qiagen Inc., Chatsworth, Ca, USA). In brief, the expression vector was digested with HindIII. The 5′ overhang was filled in with dNTPs, followed by digestion with Sal1. T_4 DNA ligase was used to ligate the MAN #1

cDNA fragments (5′ Sal1-blunt 3′) directionally into the expression vector (5′ Sal1- blunt 3′), downstream of a sequence encoding for six consecutive histidine residues (Sambrook *et al.*, 1989).

The different His-tagged MAN #1 fusion proteins were expressed within M15[pREP4] bacteria by induction with 2mM IPTG at 37°C for 3 hr (small-scale cultures) or 5 hr (large-scale cultures). Proteins were harvested under denaturing conditions (8M urea). The bacterial lysates were either used directly in Western blot analysis or affinity purified over a Nickel column (QIAexpress protocol manual, Qiagen Inc.). Collected samples were stored at 4°C.

Western blot analysis

Cellular proteins, from cycling HeLa and P19 EC (transfected and untransfected) cells, were harvested according to the method outlined in section 2.2. Lysates from HeLa, P19 EC and M15 bacteria (transformed and untransformed) were solubilized in 1x SDS-sample buffer (Laemmli, 1970) and run on 5% stacking and 12% resolving SDS-PAGE gels (Laemmli, 1970). The proteins were then electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Immunoblotting was performed as described in the general protocol in section 2.2.

Expressed 6-His-tagged MAN #1 fusion proteins were detected using the MAN antiserum at a dilution of 1:10,000. The secondary antibody was a HRP-conjugated goat anti-human IgG (Amersham Canada Inc.) used at 1:2,000 (Fig.19). Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Canada Inc.) on

Dupont Reflection film (Mandel Scientific Company LTD., Guelph, Ont.).

In transfected and untransfected P19 EC cells, the 6-mycMAN #1 fusion protein was detected with an undiluted monoclonal anti-human myc antibody supernatant (9E10; American Type Culture Collection), while the MAN antigens were recognized with the MAN antiserum diluted 1:20,000. Secondary antibodies include a biotinylated sheep anti-mouse Ig (1:5,000; Amersham Canada Inc.) and a biotinylated sheep anti-human IgG (whole antibody; Amersham Canada Inc.) used at 1:4,000. The signal was amplified using HRP-streptavidin (Amersham Canada Inc.) at 1:2,000. All first and secondary antibodies, as well as streptavidin-HRP were diluted in PBS/Tween-20. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Canada Inc.) on Hyperfilm ECL (Amersham Canada Inc.).

Peptide competition experiment

Cellular proteins from HeLa cells were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis (see chapter 2, section 2.2). Primary antibodies were preincubated with one of 50 µg/ml of poly-L-glutamine (Sigma Canada, Mississauga, Ontario, CAN.), 50 µg/ml poly-L-glutamate (Sigma Canada) or 100 µg/ml of 6-HisMAN #1 competitor peptide overnight at 4°C. All antibody mixtures and streptavidin-HRP, were incubated with the nitrocellulose membrane for 1 hr at RT. The MAN antiserum was used at a dilution of either 1:50,000 PBS/Tween-20 or 1:200,000 PBS. A biotinylated sheep anti-human Ig secondary antibody (Amersham Canada Inc.) was used at 1:4,000. To amplify the signal

streptavidin-HRP (Amersham Canada Inc.) was used at a dilution of 1:3,000. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Canada, Inc.) on Hyperfilm ECL (Amersham Canada, Inc.).

Far-Western experiment

The protocol used for far-Western blot analysis was identical to the procedure outlined in section 2.2. To summarize, 100 µg/ml of the 6-HisMAN #1 fusion protein was used to probe HeLa homogenates for 1 hr. This was followed by incubation with a mouse anti-histidine IgG (1:2,000; Qiagen Inc.) and then incubation with a biotinylated sheep anti-mouse Ig secondary antibody (1:4,000; Amersham Canada Inc.). Streptavidin-HRP was used at a dilution of 1:3,000 (Amersham Canada Inc.). Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Canada, Inc.) on Hyperfilm ECL (Amersham Canada, Inc.).

Indirect immunofluorescence

P19 cells were plated onto coverslips and subsequently fixed and stained 24 hr later as described in chapter 2 (materials and methods). A primary mouse monoclonal anti-human myc antibody supernatant was used undiluted (American Type Culture Collection). A secondary CY3 conjugated donkey anti-mouse IgG (Amersham Canada Inc.) was used at 1: 400 PBS/Tween-20.

4.3 RESULTS

4.3.1 Antibody adsorption/elution, competition and far-Western experiments demonstrating that the MAN #1 polypeptide contains a MAN epitope

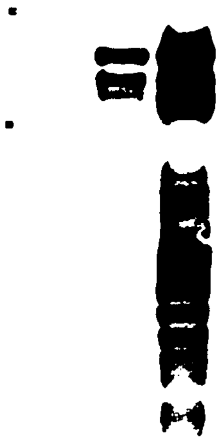
The MAN antiserum recognizes at least three major polypeptides in a variety of vertebrate species (Paulin-Levasseur *et al.*, 1996). To validate that the MAN #1 cDNA encoded a MAN related polypeptide, I performed an antibody adsorption/elution experiment. The β-gal/MAN #1 fusion protein was expressed from the λgt22A-cDNA clone (described in materials and methods). A bacterial lysate (P1) and a protein precipitate from the culture supernatant (S1) were run on a SDS-PAGE gel and transferred to nitrocellulose. As illustrated in figure 13, the MAN antiserum specifically recognizes the β-gal/MAN #1 fusion protein, including lower molecular weight bands, in both the S1 and P1 fractions (Fig.13A lanes 1 and 2 respectively). In contrast, the serum did not react with similar fractions isolated from a clone expressing an unrelated β-gal/fusion protein (Fig.13B). Antibody fractions which reacted (AP1 and AS1) or did not react (NAP1 and NAS1) with β-gal/MAN #1 were collected and used to analyze lysates from either HeLa, differentiated P19 or undifferentiated P19 cells (Fig.14). Figure 14A shows detection of the MAN antigens with the AP1 antibody fraction. The protein profile for all three samples in panel A is similar to that detected with the whole MAN antiserum (Fig.14C). Residual MAN antibodies were still present in the NAP1 fraction and reacted with MAN antigens in all three protein samples as illustrated in figure 14B. This experiment was repeated for a control β-gal/fusion

Figure 13. Western blot analysis of bacterially expressed β-gal/MAN #1 fusion protein with the MAN antiserum

Both the β-gal/MAN #1 (A) and an unrelated β-gal fusion protein (B) were expressed within bacteria. A bacterial lysate (lane 1) and protein precipitate from the culture supernatant (lane 2) were run on SDS-PAGE gels and prepared for immunoblotting with the MAN antiserum. The serum specifically recognizes protein bands in panel A. A 146 kDa protein and two lower molecular weight protein bands (140 kDa and 134 kDa) are detected in the lysate (lane 1), while the same major bands and a smear of lower molecular weight bands are present in the supernatant (lane 2). The black squares correspond to pre-stained molecular weight markers: myosin, 200 kDa and β-galactosidase, 116.25 kDa.

A

1 2



B

1 2

Figure 14. MAN antiserum adsorption/elution experiment demonstrating the immunological relatedness of the MAN #1 polypeptide to all three major MAN antigens

Adsorbed and non-adsorbed antibody fractions were collected from β-gal/MAN #1 and an unrelated β-gal fusion protein probed with the MAN antiserum. HeLa (lane 1), P19 undifferentiated (lane 2), and P19 differentiated (lane 3) protein extracts were resolved on SDS-PAGE gels and prepared for immunoblotting with each of these antibody fractions. Panel A shows detection of all three major MAN antigens with the adsorbed β-gal/MAN antibody fraction, while panel B represents the blot probed with the non-adsorbed antibodies. Panel D shows that none of the MAN antibodies were adsorbed by the unrelated β-gal fusion protein, while panel E illustrates that the non-adsorbed fraction reacts with the MAN antigens. Panel C represents probing with the unfractionated antiserum. Panel F is the control blot probed with secondary antibodies alone. The black squares correspond to pre-stained molecular weight markers: bovine serum albumin, 84 kDa and ovalbumin, 47 kDa.

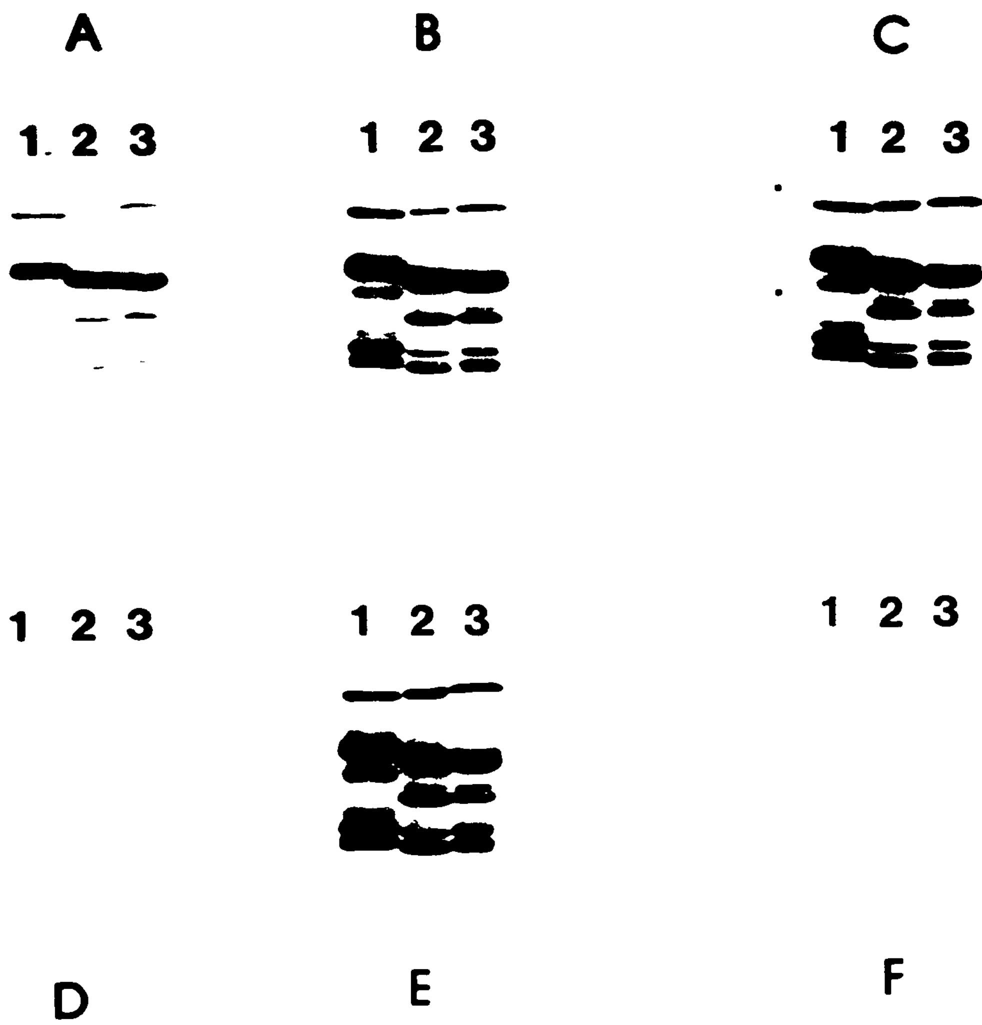
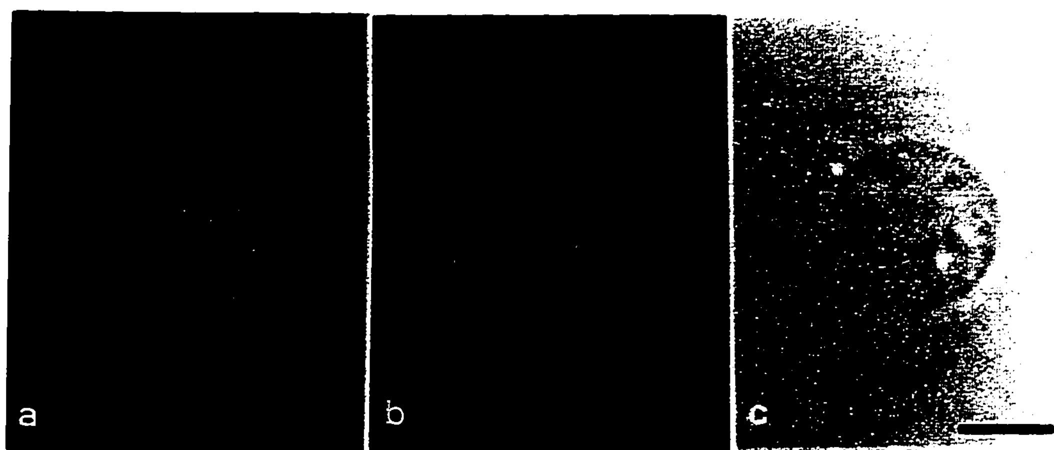

A
B
C
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
D
E
F

Figure 15. Immunofluorescence microscopic observation of an interphase HeLa cell stained with the β-gal/MAN #1 adsorbed antibody fraction

MAN antibodies which bound to the β-gal/MAN #1 fusion protein on Western blots were collected and used to stain HeLa cells. Similar to the staining pattern observed for the whole antiserum (Fig.4), the adsorbed antibody fraction shows a smooth staining of the nuclear periphery (a). Cells were counterstained with Hoechst 33258 to visualize the DNA (b). The general morphology of the cell was observed by phase contrast (c). Bar, 10 μm.


a
b
c

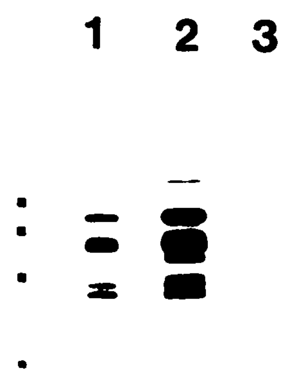
protein unrelated to the MAN #1 cDNA. As shown in figure 14D, the AP2 antibody fraction did not recognize the MAN antigens. Panels E and F of the same figure show the absence of signal on blots probed with NAP2 fraction and the secondary antibody, respectively. By immunofluorescence, the AP1 antibodies, collected from the β-gal/MAN #1 fusion protein, still produced a strong peripheral staining of the nucleus (Fig.15). Taken together, these data suggest that I have isolated a cDNA fragment encoding a polypeptide which shares common epitopes with all three major MAN antigens and that these same epitopes are also detected at the nuclear periphery.

To further support these results, I designed a peptide competition experiment using a purified 6-HisMAN #1 fusion protein as a competitor in Western blot analysis with the MAN antiserum (Fig.16). HeLa lysates were run on SDS-PAGE gels, transferred to nitrocellulose and probed with either the antiserum alone (A and B, lane 1) or the antiserum and 100 μg/ml of 6-HisMAN #1 (A and B, lane 2). At an antibody dilution of 1:50,000, it was found that addition of the 6-HisMAN #1 peptide did not produce a decrease in the signal (A, lane 2), as compared to a control blot probed with the MAN antiserum alone (lane 1). The antibody concentration was believed to be in excess of the amount of competitor peptide. Consequently, the experiment was repeated, but with the antibody diluted 1:200,000 (Fig. 16B). In this case, the antiserum has been diluted to the limit of ECL detection since only two of the three MAN antigens are recognized in the positive control (lane 1). In

Figure 16. Competition assay between the MAN antigens and the 6-HisMAN #1 fusion protein in Western blot analysis with the MAN antiserum

Immunoblots of HeLa lysates were probed with the MAN antiserum at a dilution of 1: 50,000 (panel A) and 1: 200,000 (panel B), either alone (lane 1) or preincubated with 100 µg/ml of 6-HisMAN #1 fusion protein (lane 2). The experiment demonstrates that, while the dilution of the antiserum reduces the number of detectable MAN antigens in the control (B, lane 1), preincubation of the antiserum with 6-HisMAN #1 amplifies the signal many fold (B, lane 2). Lane 3 (A and B) represents HeLa lysates probed with only the secondary antibody. Black squares represent biotinylated molecular weight markers: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa and carbonic anhydrase, 31 kDa.

A



B

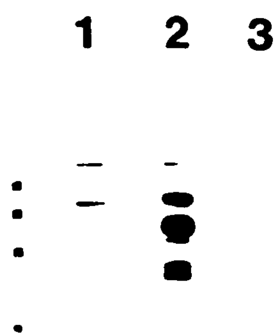
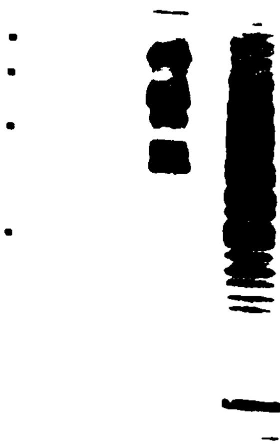


Figure 17. Far Western experiment with the 6-HisMAN # 1 fusion protein

Immunoblots of Hela lysates were probed with a 1: 200,000 dilution of the MAN antiserum (lane 1), Man antiserum (1:200,000) preincubated with 100 µg/ml of 6-HisMAN #1 fusion protein (lane 2), 100 µg/ml of 6-HisMAN #1 alone, followed by detection with an anti-histidine antibody (lane 3) and an anti-histidine antibody (lane 4). As compared to the control (lane 2), the 6-HisMAN #1 fusion protein (lane 3) does not bind to any of the cellular MAN antigens, but appears to interact non-specifically with a large subset of endogenous proteins. The squares to the left of the figure represent the position of biotinylated molecular weight markers: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa and carbonic anhydrase, 31 kDa.


1 2 3 4

this experiment, there was an increase in the signal detected on the blot which was incubated with the 6-HisMAN #1 peptide and the antiserum (lane 2). There are two explanations for this result, either the peptide is acting as a bridge between MAN antibody molecules or the 6-HisMAN #1 peptide is capable of directly binding to the endogenous MAN proteins.

To determine if the 6-HisMAN #1 peptide is capable of binding to the MAN antigens, the fusion protein was used as a probe in a far-Western experiment (Fig.17). Lane 3 shows HeLa lysates which were incubated with 100 μg/ml of 6-HisMAN #1 peptide and subsequently probed with a monoclonal anti-histidine antibody. The 6-HisMAN #1 peptide is clearly binding to a large subset of proteins (lane 3). However, when aligned with a duplicate blot probed with the MAN antiserum preincubated with 6-HisMAN #1 (lane 2), none of the endogenous MAN antigens are bound by the 6-HisMAN #1 peptide alone (lane 3). In fact, the majority of reactive bands detected in lane 3 appear to be a result of the peptide binding to endogenous proteins non-specifically. Therefore, the cause of the amplified signal, detected in figure 16B, is most likely due to the 6-HisMAN #1 peptide acting as a bridge between antibody molecules.

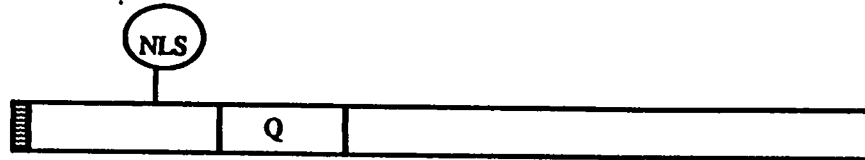
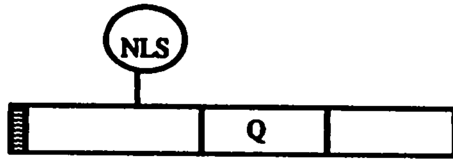
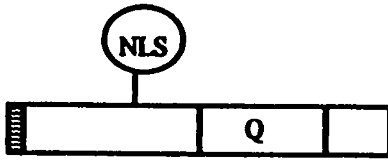
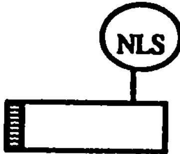
4.3.2 Bacterial expression and immunodetection of a series of histidine-tagged MAN #1 fusion proteins illustrate that a MAN epitope is contained within a region encompassing the glutamine stretch of the MAN #1 polypeptide

I have demonstrated that the polypeptide encoded by the MAN #1 cDNA shares an epitope with all three major MAN antigens. In order to map this epitope within the MAN #1 amino acid sequence, a

commercial system was used for the expression and purification of recombinant proteins (see materials and methods). The first step involved creating a series of 6-His-tagged MAN #1 deletion constructs (Fig.18). The recombinant vectors were subsequently used to transform an *E.coli* strain (M15[pREP4]; carrying a lac repressor plasmid), designed for inducible expression of fusion proteins. After transformed cells were induced with 2 mM IPTG for three hr, proteins were extracted under denaturing conditions (8M urea) and used for Western blot analysis with the MAN antiserum (Fig.19). Figure 19A is a blot of protein lysates taken from bacterial cultures which were either not induced (Fig. 19A, lane 1:control) or induced to express the different His-tagged proteins (Fig. 19A; 6-HisMAN #1, 6-HisMAN(564), 6-HisMAN(287), 6-HisMAN(144), 6-His-dihydrofolate reductase (6-HisDHFR): lanes 2-6 respectively). Neither the uninduced lysate (Fig. 19A, lane 1) nor the expressed 6-HisDHFR (Fig. 19A, lane 6) fusion protein, were recognized by the antiserum. This indicates that the MAN antiserum does not react with the 6-His tag or with endogenous bacterial proteins. However, in lysates extracted from the induced cells (lanes 2-5), the antiserum recognized proteins migrating at 37 kDa (lane 2), 28 kDa (lane 3) and 19 kDa (lane 4), while in lane 5 no protein was detected. Figure 19B represents a duplicate blot in which bacterial lysates were first affinity purified over a Nickel column before being loaded onto an acrylamide gel. The results are the same for both experiments. However, notice the detection of high molecular weight species of HisMAN #1 present in lane 2 (Fig. 19B), most

Figure 18. Schematic diagram illustrating the series of histidine tagged MAN #1 fusion proteins

A set of MAN #1 deletion mutants were created by removing DNA sequences from the carboxy terminus of the MAN #1 cDNA fragment. A sequence encoding 6 consecutive histidine residues was ligated onto the amino terminus of each of the MAN #1 deletion fragments.

cDNAs		Fusion proteins
1.	6-HisMAN # 1	
2.	6-HisMAN (564)	
3.	6-HisMAN (287)	
4.	6-HisMAN (144)	

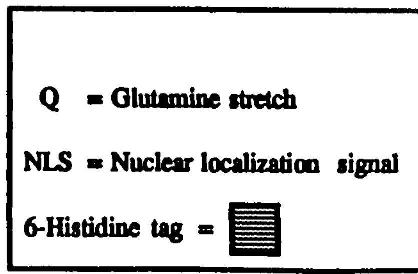
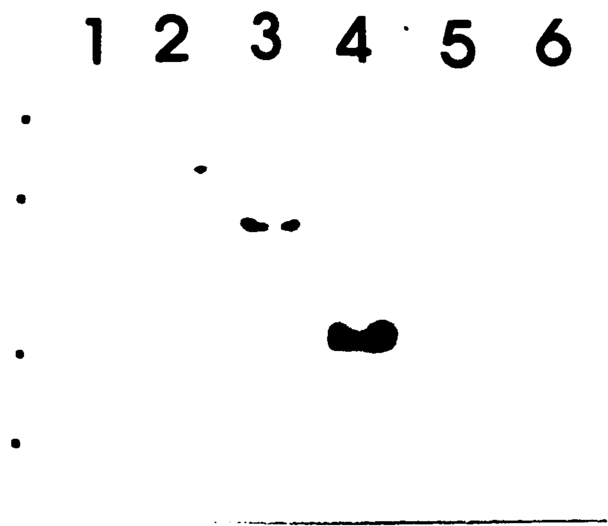
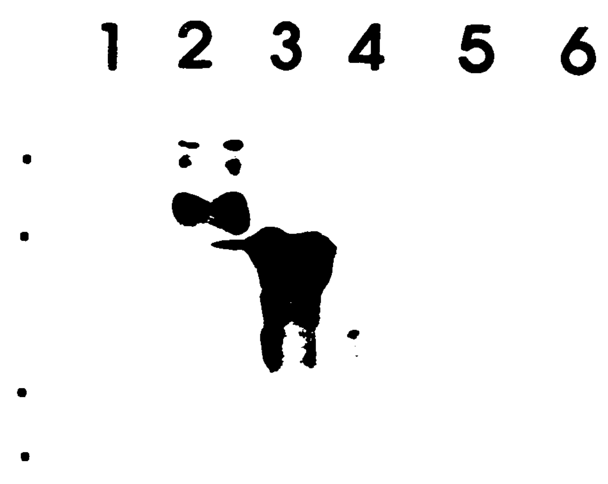


Figure 19. Mapping of a MAN epitope within bacterially expressed 6-histidine tagged MAN #1 fusion proteins

Proteins harvested from bacteria which were either not induced (lane 1) or induced to express 6-HisMAN #1 (lane 2), 6-HisMAN(564) (lane 3), 6-HisMAN(287) (lane 4), 6-HisMAN(144) (lane 5) and 6-HisDHFR (lane 6), were run directly on SDS-PAGE gels (A) or first affinity purified over nickel columns (B). The resolved polypeptides were transferred to nitrocellulose and probed with the MAN antiserum. The serum specifically recognizes 6-histidine MAN #1 fusion proteins in lanes 2-4, but not lane 5. The black squares along the left margin correspond to pre-stained molecular weight markers: ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18 kDa and lysozyme, 14 kDa.


A
1 2 3 4 5 6


B
1 2 3 4 5 6

likely the result of post-translational modification targeted to the carboxy half (564-997 bp) of the MAN #1 polypeptide. The MAN antiserum reacts specifically with His-tagged proteins in lanes 2-4, but does not recognize the 10 kDa 6-HisMAN(144) fusion protein in lane 5. The results indicate that a MAN epitope can be mapped to a region encoded by a 143 bp segment (144-287bp), found at the N-terminal of the protein.

Within the MAN #1 polypeptide, the epitope maps to a region encompassing the eleven amino acid glutamine stretch. To test whether or not the glutamine stretch represents a MAN epitope, a competition experiment was performed using either poly-L-glutamine or poly-L-glutamate (negative control) as competitors in western blot analysis with the MAN antiserum (Fig.20). Similar to the competition experiment described previously, HeLa lysates were probed with the antiserum and 50 μg/ml of competitor peptide. In figure 20A, neither poly-L-glutamine (lane 2) or poly-L-glutamate (lane 3) could compete with binding of the antibody to the endogenous MAN antigens. In fact, all three major MAN antigens, including a 130 kDa species (panel B), were detected, producing a detection profile similar to the MAN serum alone (lane 1). For this experiment, the antiserum was diluted 1:50,000. Based on the results of the competition experiments illustrated in figure 16, it was suspected that the antibody concentration was still too high for adequate competition to occur. Thus, the experiment was repeated with 50 μg/ml of peptide and the antiserum diluted 1:200,000 (Fig.20B). As predicted, under these conditions only two

Figure 20. Competition assay between the MAN antigens and either poly-L-glutamine or poly-L-glutamate in Western blot analysis with the MAN antiserum

Immunoblots of HeLa lysates were probed with the MAN antiserum at a dilution of 1: 50,000 (panel A) and 1: 200,000 (panel B), either alone (lane 1) or preincubated with 50 μg/ml of poly-L-glutamine (lane 2) or poly-L-glutamate (lane 3). Both panels A and B illustrate that neither poly-L-glutamine or poly-L-glutamate are effective competitors at either antiserum dilution. Lane 4 (A and B) represents HeLa lysates probed with the secondary antibody alone. Black squares correspond to biotinylated molecular weight markers: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa and carbonic anhydrase, 31 kDa.

A

1 2 3 4

B

1 2 3 4

of the MAN antigens were detected on the positive control (Fig.20B; lane 1). In comparison, the blots probed with the serum and poly-L-glutamine (lane 2) or poly-L-glutamate (lane 3) produced the same pattern. Therefore, neither of these peptides acted as effective competitors.

4.3.3. Transient transfection of P19 EC cells with a 6-mycMAN #1 expression construct indicates that the MAN #1 polypeptide is a nuclear protein

To establish the cellular distribution of the MAN #1 polypeptide, cultured cells were transfected with a DNA construct encoding a MAN #1 fusion protein and then prepared for immunofluorescence microscopy.

In order to distinguish the localization of the MAN #1 polypeptide from the endogenous MAN antigens, 6-myc tags were ligated onto the 5′ end of the MAN #1 cDNA. By a $CaPo_4$ method (Chen and Okayama, 1987), P19 cells were transiently transfected with this 6-mycMAN #1 construct. Figure 21 illustrates Western blots of cell lysates which were probed with either an anti-human myc antibody (Fig. 21A) or the MAN antiserum (Fig. 21B). Panel A shows that a single reactive protein with a relative mobility of 63 kDa is detected in the sample from cells transfected with the 6-mycMAN #1 construct (lane 1). Moreover, this protein was not observed in either sample of untransfected P19 cells (lane 2) or P19 cells transfected with a vector lacking the Pgk promoter and containing unrelated DNA sequences in its multiple cloning site (lane 3). The MAN #1 polypeptide contains an epitope recognized by the antiserum.

In transfected cells, the antiserum is expected to react with the 6-mycMAN #1 polypeptide in addition to the endogenous MAN antigens. Probing a duplicate blot with the MAN antiserum revealed that all three major antigens were detected in both the transfected and untransfected cell samples (panel B). However, the 63 kDa fusion protein in the transfected sample was not recognized by the MAN antiserum (panel B, lane 1).

The anti-human myc antibody was used to label P19 cells transiently transfected with the 6-mycMAN #1 construct. As observed by immunofluorescence microscopy, the transfection efficiency was approximately 5-10%. Amongst transfected cells, some exhibited nuclear localization of the 6-mycMAN #1 protein (Fig.22a). In these cases, the staining pattern was not restricted to the nuclear periphery but was diffuse throughout the nucleoplasm.

In this experiment, I observed that transfected cells displayed a variety of staining patterns. For example, there were cells in which the fusion protein was distributed equally throughout the cytoplasm and the nucleus. There were also cells which showed staining of the NE. Figure 23a illustrates a cell in which the 6-mycMAN #1 fusion protein is distributed around the nuclear periphery and throughout the cytoplasm (Fig.23a).

Often, expression of 6-mycMAN #1 coincided with gross morphological changes. Typically, undifferentiated P19 cells possess a larger nuclear versus cytoplasmic volume. However, transfected cells were often larger (Figs.23) than untransfected P19 cells (refer to Fig.22c). Figure 24 illustrates two examples of

Figure 21. Western blot analysis of P19 cells transiently transfected with the 6-mycMAN #1 DNA construct

Proteins were extracted from P19 cells which had been transfected with the 6-mycMAN #1 construct (lane 1), untransfected (lane 2) or transfected with an unrelated non-transcribed vector, designated pKJ53 (lane 3). Samples were then prepared for immunoblotting with either a anti-human myc antibody (A) or the MAN antiserum (B). Panel A shows that a single reactive band at 63 kDA is detected in the sample transfected with the 6-mycMAN #1 construct (lane 1). Probing with the serum illustrates that all three samples express the full complement of MAN antigens. Black dots correspond to biotinylated molecular weight markers: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa and soybean trypsin inhibitor, 21.5 kDA.

A

1 2 3

B

1 2 3

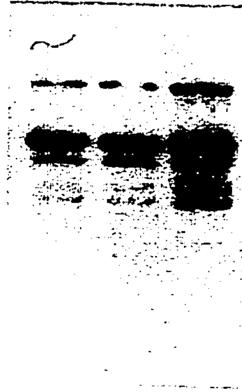


Figure 22. Cellular localization of the 6-mycMAN #1 fusion protein in transiently transfected P19 cells

Populations of P19 cells transiently transfected with the 6-mycMAN #1 construct were fixed and then labelled with an anti-human myc antibody (a). The staining pattern shows that the fusion protein is localized diffusely throughout the nucleoplasm. Cells were counterstained with Hoechst 33258 to visualize the DNA (b), while the general morphology of the cells was observed by phase contrast microscopy (c). Bar, 10 μm.

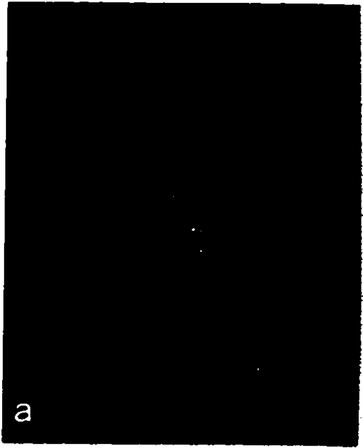
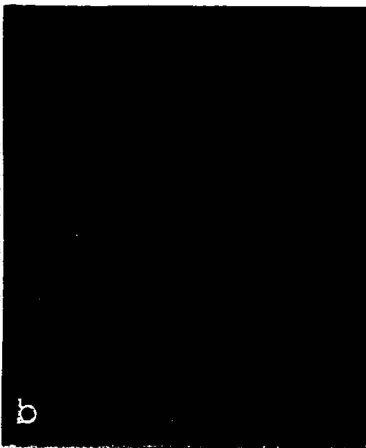
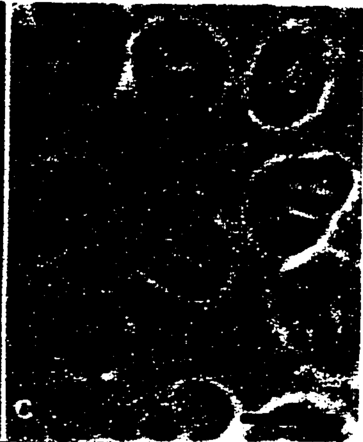
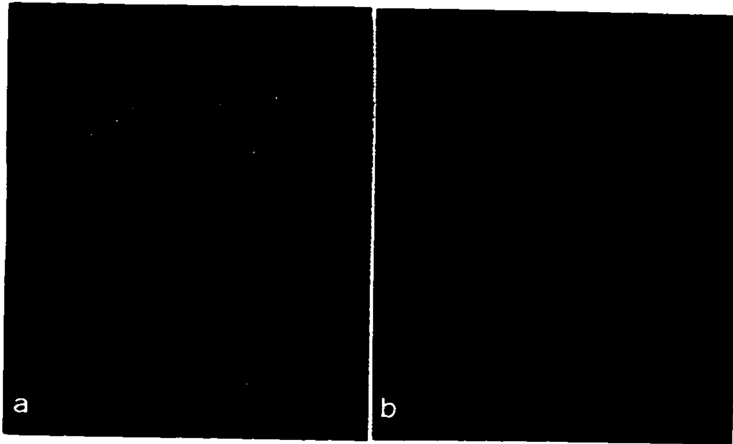

a

b

c

Figure 23. Immunofluorescence microscopic observation of the 6-mycMAN #1 fusion protein localized to the nuclear periphery in transiently transfected P19 cells

P19 cells transiently transfected with the 6-mycMAN #1 construct were fixed and then labelled with an anti-human myc antibody. Panel a shows an interphase cell displaying extensive cytoplasmic anti-human myc staining, while there is an increase in fluorescence at the nuclear periphery. Counterstaining cells with Hoechst 33258 reveals that the nucleus of this transfected cell has an unusual hook shape (b). Panel c shows a phase contrast micrograph of the same cell. Bar, 10 µm.


a
b

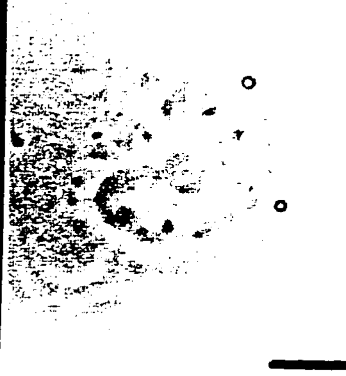
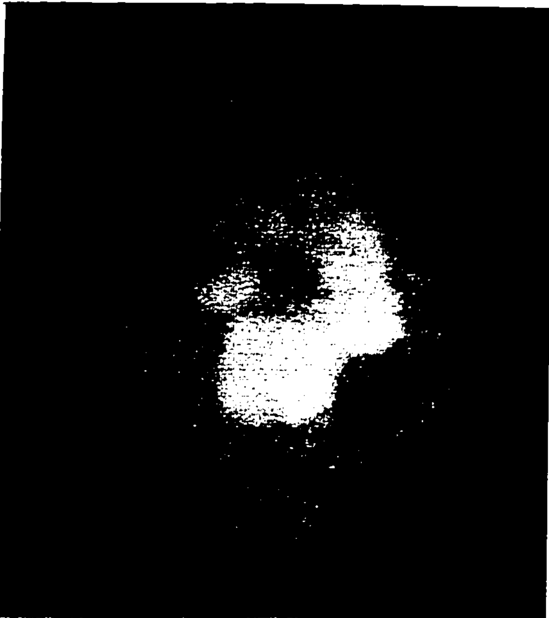
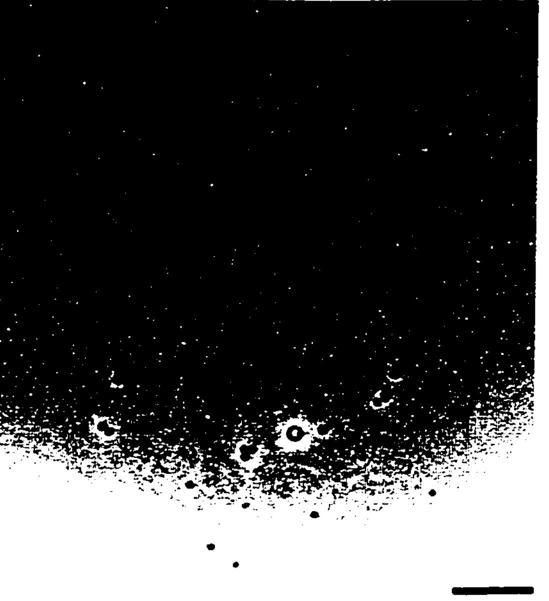


Figure 24. Effects of 6-mycMAN #1 expression on the restructuring of chromatin within transiently transfected P19 cells

P19 cells transiently transfected with the 6-mycMAN #1 construct were stained with an anti-human myc antibody (a and b). By immunofluorescence microscopy, the fusion protein is observed to be localized predominantly within the nucleus, but considerable staining is also detected within the cytoplasm. Hoechst 33258 staining shows that the chromatin in both cells has undergone extensive rearrangements (c and d). Changes to cellular morphology are also observed by phase contrast microscopy (e and f). Bar, 10 µm.





transfected cells displaying unusual morphology. In panels a and b, 6-mycMAN #1 is localized both in the cytoplasm and distributed diffusely throughout the nucleus. Examining Hoechst staining of cellular DNA (Fig.24c and d) reveals that the expression of 6-mycMAN #1 has resulted in a reorganization of chromatin. In one case, not only does the nucleus appear larger but the DNA does not stain as intensely with the Hoechst dye (Fig.24c). In the second example (Fig.24d), the reorganization is more striking with the DNA rearranging itself into a "donut" configuration. The cytoplasmic staining in these two cells is not diffuse like the pattern observed in figure 23a. Instead, the anti-human myc antibody appears to label organized structures. In figure 24a, several brightly stained filaments are seen to radiate out from the nucleus towards the plasma membrane. Conversely, in panel b of this figure, the antibody labels the cytoplasm producing an intricate lattice network.

From my results, I conclude that the MAN #1 polypeptide contains a signal or partial signal which facilitates its translocation into the nucleus.

4.4 DISCUSSION

4.4.1 The polypeptide encoded by the partial MAN #1 cDNA is immunologically related to all three major MAN antigens

I have used immunological techniques to further characterize the polypeptide encoded by the MAN #1 cDNA. By a series of antibody adsorption/elution experiments, I have demonstrated that the MAN #1 cDNA encodes a polypeptide that bears epitopes in common with the three major cellular MAN antigens. Moreover, I have shown that antibodies eluted from the β-gal/MAN #1 fusion protein not only react on blots with the denatured MAN polypeptides but produce a smooth and continuous labelling of the nuclear periphery, as visualized by immunofluorescence microscopy. These data provide evidence that the polypeptide encoded by the MAN #1 cDNA is immunologically related to the MAN antigens

A peptide competition experiment was designed to test whether the protein encoded by the MAN #1 cDNA could efficiently compete with the MAN antigens for antibody binding. Surprisingly, the 6-HisMAN #1 fusion protein not only failed to compete for antibody binding but appeared to amplify the signal for the three cellular MAN antigens. The results of the far-Western experiment established that the amplified signal in the competition assay was not due to 6-HisMAN #1 binding directly to the MAN antigens. Therefore, the only alternative explanation is that the concentration of competitor peptide was not high enough to occupy both of the antigen binding sites on the MAN antibodies, and that large complexes of antibodies and peptides formed during preincubation of the competitor peptide with the MAN antiserum (see materials and

methods). These complexes are believed to be derived from a single antibody molecule interacting with two separate peptides. For this chain to be extended and the next antibody molecule to bind the complex, there must be at least two independent MAN epitopes encoded by the fusion protein.

Another piece of data gained from the competition experiment stems from the observation that all three MAN antigens were amplified apparently to the same extent. If the MAN #1 polypeptide shared one epitope with one MAN antigen but shared several with another, it might be expected that the amplified signal from the individual antigens would be different. However, this was not observed, suggesting that each of the individual MAN antigens may share the same number of epitopes with the fusion protein.

4.4.2 A conserved MAN epitope can be mapped to a region encompassing the glutamine stretch encoded by the MAN #1 cDNA

The results of the competition experiment indicated that the 6-HisMAN #1 fusion protein contained at least two independent MAN epitopes. Human autoantibodies have been reported to bind highly conserved functional domains within antigens (Tan, 1991). The predicted amino acid sequence of the polypeptide encoded by the MAN #1 cDNA contains several motifs which could represent functional domains, these include: 1) an eleven amino acid glutamine stretch (residues 52-62); 2) a potential NLS signal (residues 41-48); and 3) a potential transmembrane domain (residues 112-129). As described, a series of 6-His-tagged MAN #1 fusion proteins were expressed and used in Western blots to test whether the epitopes

recognized by the antiserum would map within these motifs. One epitope was found in the region encompassing the glutamine stretch (amino acids 46-94). To determine if the glutamine stretch contains a MAN epitope, a competition assay was performed using poly-L-glutamine as a competitor in Western blot analysis with the antiserum. My results show that poly-L-glutamine does not react with the antiserum. However, poly-L-glutamine may not compete because it lacks the proper context (i.e. flanking sequences within the MAN #1 polypeptide). Thus, I can only conclude that the glutamine stretch within MAN #1 does not constitute by itself an epitope.

Immunoblotting protein samples containing the full length 6-His-tagged MAN #1 peptide revealed the presence of higher migrating MAN-reactive protein species. These protein bands had a relative mobility of approximately 43-47 kDa, and were not detected in the other samples. These results would suggest that the 6-HisMAN #1 fusion protein undergoes post-translational modification in bacteria. The amino acid sequence of the MAN #1 polypeptide contains glycosylation, myristoylation and phosphorylation consensus sites. However, bacteria lack the specific eukaryotic machinery which are necessary for efficient glycosylation and myristoylation (reviewed by Marston, 1986). Dadssi and colleagues (1989) have demonstrated that exogenous peptides can be phosphorylated by *E. coli* protein kinases. Furthermore, a calcium and phospholipid-dependent PKC activity was identified in bacterial extracts (Norris *et al.*, 1991). Amino acid sequence analysis of the

predicted MAN #1 polypeptide has revealed several PKC consensus sites. I propose that the higher molecular weight 6-HisMAN #1 isoforms are the result of either protein phosphorylation or a bacterial specific modification. The fact that only the full length fusion protein was modified implies that the targeted sites are encoded within the carboxy half of the cDNA, between bps 564-997.

4.4.3 Transient transfection of P19 cells with a 6-mycMAN #1 expression construct provides evidence that the MAN #1 polypeptide contains a functional NLS sequence

In the predicted open reading frame, the MAN #1 cDNA encodes a potential NLS sequence. More similar to the basic stretch of residues found in nuclear proteins such as the SV40 large T-antigen (Kalderon *et al.*, 1984), the putative NLS found in MAN #1 is composed of lys leu leu lys leu leu lys arg (Fig.9). To determine the cellular localization of the MAN #1 protein, P19 cells were transiently transfected with the 6-mycMAN #1 expression construct.

By immunoblotting, the myc antibody specifically recognized a single protein in the transfected cell sample. The relative mobility of the reactive protein was calculated to be 16 kDa greater than the predicted value of the fusion protein (6-myc epitope = 12 kDa; MAN #1 polypeptide = 35 kDa). Under these denaturing conditions, the observed shift in relative mobility is likely the result of post-translational modification (e.g. glycosylation, myristoylation and phosphorylation). Probing a duplicate blot with the antiserum revealed that all three major MAN antigens are detected in the sample from cells transfected with the

6-mycMAN #1 cDNA. However, the 63 kDa 6-mycMAN #1 fusion protein was not recognized (21B, lane 1). This result was unexpected since, within transfected cells, the antiserum should react with both the fusion protein and the endogenous MAN antigens. The recombinant 6-mycMAN #1 construct was sequenced across the myc/MAN DNA junction, therefore I can disregard the possibility that the DNA ligation reaction caused a shift in the open reading frame (i.e. through the addition or deletion of nucleotides), producing a polypeptide lacking the MAN epitope. The alternative explanation is that due to the transfection efficiency (5-10%), the expression levels of the fusion protein are low relative to the endogenous MAN antigens. This would imply that, at the protein level, the two (or more) MAN epitopes present on 6-mycMAN #1 may not produce a signal strong enough to be detected by ECL, as compared to the signal produced by the 6 myc tags on the fusion protein.

It has been shown that the MAN #1 cDNA encodes epitopes which are shared amongst the three major MAN antigens. Furthermore, all three antigens have been found to co-partition with the nuclear lamina during nuclear matrix extraction (Paulin-Levasseur *et al.*, 1996). From this data, it might be expected that the MAN #1 polypeptide could also be localized to the nuclear periphery. By immunofluorescence microscopy, the 6-mycMAN #1 fusion protein was found to localize diffusely throughout the nucleus with some fluorescence staining also present in the cytoplasm.

The results can be explained two different ways. The 6-mycMAN #1 protein may contain a motif which allows it to be targeted to

the NE. However, if overexpressing the fusion protein results in the saturation of binding sites at the nuclear periphery, there could be an accumulation of fusion protein in the nucleoplasm. For example, exogenous lamin C is stored in the nucleoplasm prior to its incorporation into the lamina following cell division (Horton *et al.*, 1992). Alternatively, the 6-mycMAN #1 may not be targeted to the nuclear periphery because it lacks the required elements. Due to an internal Not1 restriction site, the MAN #1 cDNA does not encode a poly A tail. Thus, the clone is considered only a partial fragment of the full length cDNA (refer to chapter 3, section 3.4). The possibility remains that there are uncloned sequences encoding for motifs responsible for association of the full length MAN #1 protein with the NE. In transfection experiments, it was found that lamin cDNAs with mutated CaaX motifs cause the expressed proteins to accumulate within the nucleoplasm, suggesting that isoprenylation is required for lamin incorporation into the nuclear lamina (Holtz *et al.*, 1989; Kitten and Nigg, 1991; Lutz *et al.*, 1992). Furthermore, transfecting cells with DNA using $CaPo_4$ (Chen and Okayama, 1987) can often result in a cell possessing multiple copies of the construct. This does not allow for control of protein expression levels between cells. In consideration that nuclear import is saturable, involving specific NLS receptors (Roberts *et al.*, 1987; Nelson and Silver, 1989), the observed cytoplasmic staining could be the consequence of over taxing the protein import pathway.

5.0 CONCLUSION

I have focused my research towards further characterizing the MAN antigens. This family of immunologically related proteins has the unusual feature of being non-lamin components of the nuclear lamina (Paulin-Levasseur *et al.*, 1996). With the exception of perichromin (McKeon *et al.*, 1984) and P1 (Chaly *et al.*, 1984), few other proteins besides the nuclear lamins (Gerace *et al.*, 1978; Krohne *et al.*, 1978; Gerace and Blobel, 1980) had been localized to the lamina. The current interest surrounding this cellular compartment has stemmed from experiments which have linked the lamina with such important nuclear functions as NE assembly-disassembly, DNA replication, cell differentiation and chromatin organization (reviewed by Moir *et al.*, 1995). Undoubtedly, the characterization of nuclear lamina proteins will provide insight into the mechanisms which allow the lamina to exert its function. For this reason, I have chosen to study the MAN antigens immunologically, by documenting both their interphase and mitotic fate relative to the nuclear lamins. In addition, I have used the MAN antiserum to clone a cDNA fragment encoding a MAN reactive polypeptide. In this section, I will summarize my data and relate possible functions of the MAN antigens, including the MAN #1 polypeptide, to proposed functions of the nuclear lamina.

During interphase, the nuclear lamina is thought to be involved in the maintenance of nuclear integrity by providing structural support. For instance, biochemical data have shown that components of the nuclear lamina form an insoluble polymer which,

apposed to the inner membrane, would likely act to maintain the shape of the NE (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976; Gerace *et al.*, 1978). In addition, it was found that immunodepleting lamin B_3 from *Xenopus* nuclear extracts results in mechanically fragile nuclei (Newport *et al.*, 1990; Meier *et al.*, 1991). These data, along with the discovery that lamin cDNAs are structurally similar to the cDNAs encoding cytoplasmic IF proteins (McKeon *et al.*, 1986; Fisher *et al.*, 1986; Höger *et al.*, 1988, 1990), have provided the evidence supporting an architectural role for the lamina. In comparison, the MAN antigens have been shown to co-partition with the nuclear lamins during nuclear matrix extraction (Paulin-Levasseur *et al.*, 1996). This demonstrates that the MAN antigens are a component of the insoluble polymer which has been used to describe the nuclear lamina in various cell types (Aaronson and Blobel, 1975; Gerace *et al.*, 1978; Gerace and Blobel, 1980; Aebi *et al.*, 1986). Therefore, it is possible to argue that the MAN antigens, like the lamins, aid the nuclear lamina in maintaining the structural integrity of the NE. How this might be accomplished is unclear. Based on the sequence of the MAN #1 cDNA, there does not appear to be any obvious encoded domains which would facilitate protein dimerization, such as the heptad repeat in the central rod domain of nuclear lamins (Steinert and Roop, 1988).

The other potential functions attributed to the nuclear lamina during interphase include DNA replication and DNA organization. Already mentioned are the series of experiments which have shown that lamin B intranuclear foci co-localize with PCNA at sites of

BrDU incorporation, implying that lamin B is involved in DNA replication (Moir *et al.*, 1994). Complementing these results are experiments performed with *Xenopus* nuclear extracts. In this case, immunodepleting lamin B_3 blocks DNA replication in reconstituted nuclei (Newport *et al.*, 1990; Meier *et al.*, 1991; Jenkin *et al.*, 1993). Being a component of the nuclear matrix, lamin B could be acting to bridge the replication machinery to the nuclear scaffold. As a consequence of sharing similar biochemical properties, it might be expected that the MAN antigens would co-localize with lamin B foci. However, by confocal microscopy it was discovered that, while the MAN antigens co-localize with lamin B at the nuclear periphery, they were absent from intranuclear foci. Hence, it suggests that the MAN antigens are not directly involved in DNA replication.

As a result of being apposed to both the inner nuclear membrane and peripheral chromatin, one of the first proposed functions of the nuclear lamina has been its involvement in higher order chromatin structure (Coggeshall and Fawcett, 1964). Recent experiments have illustrated that a proportion of peripheral chromatin lies in close enough proximity to the NE to form direct contact with the nuclear lamina (Paddy *et al.*, 1990; Belmont *et al.*, 1993). Moreover, *in vitro* studies have reported that pure lamins can directly bind to both telomeric DNA sequences (Shoeman and Traub, 1990) and A-T rich MAR/SAR regions (Ludérus *et al.*, 1992, 1994). In the transient transfection experiments with the 6-mycMAN #1 cDNA, a proportion of the transfected cells displayed

gross alterations in chromatin structure. In several examples, the chromatin mass changed from the typical spherical shape characteristic of untransfected P19 cells to an almond shape in transfected cells. This unusual morphology was also reported in somatic cells which ectopically expressed germ cell specific lamin B_3 (Furukawa and Hotta, 1993). In this case, lamin B_3 was successfully targeted to the NE, but nuclei had an unusual hook shape. It is clear that expression of lamin B_3 caused a remodelling of the NE as well as a reorganization of chromatin. From the transfection experiments with 6-mycMAN #1, only a few cells showed apparent staining of the NE. However, even in the illustrated example (figure 22a), the nuclei appeared hooked instead of spherical. These experiments suggest that the 6-mycMAN #1 protein may be capable of interacting either directly or indirectly with the chromatin and the NE.

The composition of the nuclear lamina undergoes a significant change as a consequence of cellular differentiation. Undifferentiated cells have been found to express only lamin B whereas most other mammalian somatic cells carry a full complement of lamins A/C and B (Stewart and Burke, 1987; Höger *et al.*, 1988, 1990). The induction of lamin A/C expression has been correlated with the onset of differentiation. This has led researchers to believe that lamins A/C are required for maintaining a differentiated phenotype (Rober *et al.*, 1989; Peter and Nigg, 1991). By RNase protection, the MAN #1 mRNA transcript was found to be ubiquitously expressed in all samples examined. The detection of

the MAN #1 mRNA within both differentiated and undifferentiated P19 cells indicates that, like lamin B, its expression is not dependent on the state of cellular differentiation. Considering also that the MAN antigens are also expressed across vertebrate cells (Paulin-Levasseur *et al.*, 1996), it is possible to conclude that the MAN antigens are likely involved in conserved housekeeping functions.

In recent years, experiments using mammalian and *Xenopus* cell-free systems have been invaluable in studies addressing the role of nuclear lamins in NE reformation. The importance of nuclear lamins in NE reassembly was first illustrated in antibody inhibition experiments (Burke and Gerace, 1986). From these studies a model developed in which lamins A/C were believed to attach to segregating chromosomes prior to lamin B (Burke and Gerace, 1986). Here, lamins A/C are proposed to act as attachment sites for lamin B-associated membrane vesicles. This model does not explain NE reassembly in cells expressing only lamin B, and is in direct conflict with my own results as well as other published reports (Meier and Georgatos, 1994). Yet, it is clear that nuclear lamins are essential for NE reformation. For example, immunodepleting lamins B_2 and B_3 from *Xenopus* cell extracts prevents NE reassembly around sperm chromatin (Dabauvalle *et al.*, 1991). By immunofluorescence microscopy, I have observed that the MAN antigens are spatially and temporally associated with lamin B during mitosis. Both lamin B and the MAN antigens disassembled after and reformed prior to lamins A/C. Both LAPs 1 and 2 have been reported to associate with segregating chromosomes in anaphase

(Foisner and Gerace, 1993). As integral membrane proteins, LAPs 1 and 2 were proposed to target membrane vesicles to chromosomes (Foisner and Gerace, 1993). In future, it would be of interest to determine the relative fates of the MAN antigens and the LAP proteins during mitosis. From my results it can be proposed that the MAN antigens may play a role in some of the early events of NE reformation. The apparent association between the MAN antigens and lamin B could imply that the antigens are either directly or indirectly associated with membrane vesicles. The MAN antigens could bind to an integral protein or associates with the lipid membrane through a post-translational modification, such as myristoylation (refer to chapter 3, section 3.4). As suggested for the LAP proteins, the MAN antigens could target membrane vesicles to chromatin.

There are still many questions concerning the function of the MAN antigens which remain to be addressed. Some of these issues have developed as a direct result of the experiments discussed in previous chapters. Presently, the tools available to study these questions include the MAN antiserum and the MAN #1 cDNA fragment.

If an immunological approach is to be used in future experiments, it would be advantageous to produce specific antibodies against each of the three major MAN antigens. This would allow us to detect differences in their respective spatial and/or temporal distribution, which may reflect specific functions of the proteins during the cell cycle. For instance, the MAN antiserum labels the NE of interphase cells in a smooth continuous pattern.

However, the serum recognizes all three major MAN antigens. There remains the possibility that one or more of the antigens are distributed unevenly around the nuclear periphery, possibly in a punctate pattern similar to what is observed with anti-nuclear pore antibodies. In addition, documenting the temporal reassembly of MAN proteins at the end of mitosis may reveal that the individual antigens reform around decondensing chromosomes sequentially. This could imply that only certain antigens are involved in the early steps of NE reassembly.

A future priority will be the cloning of a full length MAN #1 cDNA clone. Currently, expression of the partial DNA fragment within P19 cells reveals that the encoded polypeptide is localized to the nucleus but not targeted to the NE. It will be of great interest to determine if the expressed full length protein will be directed to the NE. Moreover, it would be important to note if overexpression of the protein affects the organization of chromatin and the NE in the same manner as the ectopic expression of lamin B_3 within somatic cells (Furukawa and Hotta, 1993).

APPENDIX

Part I: WESTERN BLOT ANALYSIS OF PROTEOLYTICALLY DIGESTED MAN ANTIGENS.

INTRODUCTION

The family of nuclear lamin proteins were initially characterized immunologically (Gerace *et al.*, 1978; Krohne *et al.*, 1978). However, their structural similarities were only confirmed once the first lamin cDNAs were cloned (McKeon *et al.*, 1986; Fisher *et al.*, 1986). The MAN antiserum recognizes three major polypeptides by Western blot analysis (Paulin-Levasseur *et al.*, 1996). In addition, all three antigens co-partition with the nuclear lamins during nuclear matrix extraction (Paulin-Levasseur *et al.*, 1996), indicating that they qualify operationally as constituents of the nuclear lamina and that they may be structurally related. However, to confirm the structural relatedness of these proteins will require either: 1) cloning the individual cDNAs or 2) using biochemical methods to examine the properties of the individual polypeptides.

To address this issue, the three major MAN antigens were isolated and subjected individually to partial proteolytic digestion with V_8 protease. Each of the three digested samples were then run on SDS-PAGE gels and probed with the MAN antiserum. From this experiment it was demonstrated that all three antigens share similar proteolytic fragments. Thus, it can be concluded that these polypeptides are both immunologically and structurally related.

MATERIALS AND METHODS

Cell culture

HeLa cells were maintained in culture as outlined in chapter 2, section 2.2.

Isolation of the MAN antigens

Cellular proteins were harvested from populations of cycling HeLa cells according to the method described in chapter 2, section 2.2. The proteins were separated on a 5% stacking and 12% resolving SDS-PAGE gel (Laemmli, 1970). Subsequently, each of the MAN antigens (78, 58 and 40 kDA) were excised directly from the gel by estimating the position of the proteins relative to Coomassie pre-stained standards (Biorad). These gel slices were loaded on a second SDS-PAGE gel. Samples were either: 1) separated electrophoretically and silver stained (Harlow and Lane, 1988); 2) subjected to partial proteolytic digestion, separated on a SDS-PAGE gel and silver stained; or 3) subjected to partial proteolytic digestion, resolved on a SDS-PAGE gel and analyzed by Western blot with the MAN antiserum.

Western blot analysis of proteolytically digested polypeptides

Each of the gel slices containing either the 78, 58 or 40 kDa MAN antigens were first soaked in transfer buffer prior to being loaded into the wells of a 5% stacking and 12% running SDS-PAGE gel (Laemmli, 1970). A solution of sample buffer with V_8 protease (0.05 mg/ml) was then added to each of the wells. The proteins were run through the stacking gel. The gel was then stopped for 30 min, allowing the samples to continue their incubation in V_8 protease.

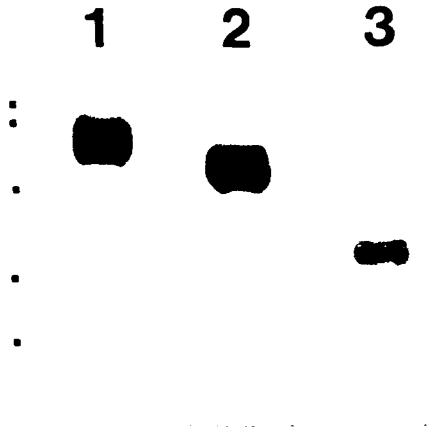
After the electrophoretic separation was completed, the samples were electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979) and processed for Western blotting analysis with the MAN antiserum (refer to chapter 2, section 2.2). The MAN antiserum was used at a dilution of 1:15,000 in PBS/Tween-20. A biotinylated sheep anti-human secondary antibody (Amersham Canada Inc.) was used at 1:1,000 in PBS/Tween-20. Streptavidin-HRP (Amersham Canada Inc.) was diluted 1:2,000 in PBS/Tween-20. Immunoreactive bands were detected using a chemiluminescence kit and visualized on Hyperfilm ECL (Amersham Canada Inc.).

Proteolytic digestion of the MAN antigens was performed by Guoxiang Chen.

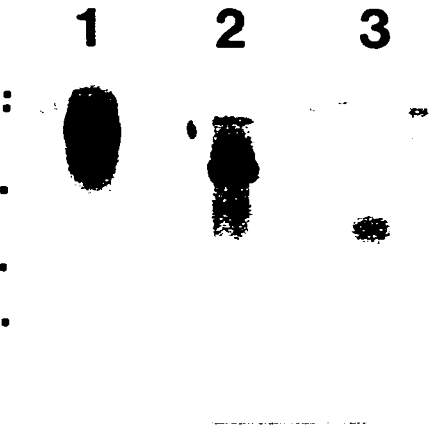
Figure 25. Partial proteolytic digestion of the MAN antigens

Isolated MAN antigens (78 kDa, lane 1; 58 kDa lane 2 and 40 kDa lane 3) were silver stained (A) or partially digested with V_8 protease (B and C) and either silver stained (B) or probed with the MAN antiserum (C). Panel C illustrates that each of the digested antigens share similar proteolytic fragments. The black squares to the left of the panels correspond to pre-stained molecular weight markers: phosphorylase b, 101 kDa; bovine serum albumin, 83 kDa; ovalbumin, 50.6 kDa; carbonic anhydrase, 35.5 kDa and soybean trypsin inhibitor, 29.1 kDa.

A



B



C

Discussion

The results of the proteolytic digestion experiment confirm that, at the protein level, the three major MAN antigens are structurally related. Hence, the three polypeptides must share motifs which are larger than what could be encompassed by a single shared epitope. In this thesis, I discuss the isolation of a partial cDNA fragment (MAN #1) encoding for a MAN-reactive polypeptide (refer to chapters 3 and 4). The MAN #1 cDNA has not been shown to encode for one of the three major endogenous MAN proteins. However, the MAN #1 polypeptide fragment does contain two independent MAN epitopes which are shared with all three endogenous antigens. Based on the results of a genomic southern probed with the MAN #1 cDNA, it appears that the DNA sequence of the MAN #1 fragment is related to a single gene. If in the future the MAN #1 polypeptide is confirmed to be one of the three major MAN antigens, then the results of the genomic southern would support a mechanism involving alternative splicing.

Part II: Western blot analysis and immunofluorescence microscopy of the stably transfected M1 cell line

Introduction

It has been reported that compositional changes in the nuclear lamina are often correlated with alterations in cell function (Moir *et al.*, 1995). For example, expression of A-type lamins is observed only within cells which have undergone differentiation (Stewart and Burke, 1987; Rober *et al.*, 1989; 1990). This change in lamina composition is believed to influence interactions between the NE and chromatin, ultimately affecting gene expression (Rober *et al.*, 1989; Peter and Nigg, 1991). However, the issue remains controversial. For instance, Peter and Nigg (1991) demonstrated that premature expression of lamins A within P19 cells did not result in an up regulation of differentiation-specific genes. Conversely, transfecting a muscle cell line with lamin A caused a transient increase in several muscle specific genes (Lourim and Lin, 1992).

Transient transfection of P19 cells with a 6-mycMAN #1 DNA construct resulted in morphological changes and a reorganization of DNA. To determine whether or not the observed changes to chromatin structure affected gene expression, a P19 cell line (M1) stably transfected with the 6-mycMAN #1 construct was induced to differentiate into muscle upon treatment with DMSO. By immunofluorescence microscopy, the 6-mycMAN #1 fusion protein was seen to be localized diffusely throughout the nucleoplasm. Surprisingly, Hoechst staining of M1 nuclei did not show the characteristic hook shape observed in the transient transfectants.

However, after exposure to DMSO, it was discovered that the M1 clone was incapable of differentiating into cardiac muscle. Therefore, stable integration of a DNA expression construct into P19 cells appears to interfere with normal muscle development.

Materials and Methods

Cell Culture and Stable transfection of P19 cells

P19 cells were grown in culture as outlined in chapter 3, section 3.2. The equivalent of 2 x 10^6 P19 cells were transfected with both the 6-mycMAN #1 construct and a construct containing the puromycin resistance gene driven by the Pgk promoter, according to the general method described in section 4.2. Following removal of the DNA precipitate, cells were supplemented with αMEM medium containing FCS and 100 μg/ml of puromycin (Gibco BRL). Cells were maintained in selection medium for 2 weeks, at which point isolated colonies resistant to puromycin were picked and grown for subsequent use in Western blot analysis.

Western blot analysis of resistant colonies

Protein samples were harvested from cycling populations of stably transfected P19 cells according to standard protocols (refer to chapter 2, section 2.2). Samples were then run on a 5% stacking and 12% resolving SDS-PAGE gel (Laemmli, 1970) and electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979). The blots were probed with an undiluted mouse monoclonal anti-human myc antibody supernatant (9E10; American Type Culture Collection). This was followed by incubation with a biotinylated sheep anti-mouse IgG (whole antibody; Amersham Canada Inc.) diluted 1:2,000 in PBS/Tween-20. The signal was then amplified using a streptavidin-HRP (Amersham Canada Inc.) at 1:2,000 in PBS/Tween-20. The reactive proteins were detected using a chemiluminescence kit and visualized on Hyperfilm ECL (Amersham Canada Inc.).

Indirect immunofluorescence microscopy

The detailed outline describing the protocol for cell fixation and staining is given in section 2.2. The monoclonal mouse anti-human myc antibody was used undiluted (American Type Culture Collection). The secondary antibody was a CY3 conjugated donkey anti-mouse IgG (Amersham Canada Inc.) diluted 1:4,000 in PBS. Observations were performed on a Zeiss Axiophot and recorded using Kodax color film. The images were printed on Kodak Colorease.

Figure 26. Screening of puromycin resistant P19 colonies for the expression of the 6-mycMAN #1 fusion protein

Western blot analysis of protein samples harvested from puromycin resistant P19 colonies co-transfected with the Pgk-puromycin resistance gene and 6-mycMAN #1 DNA constructs. Protein samples from M1 (lane 1), M2 (lane 2), M3 (lane 3) and M4 (lane 4) resistant colonies were probed with an anti-human myc antibody. As illustrated, only the M1 clone expresses a myc tagged protein, migrating at 54 kDA. Black squares to the left of the figure correspond to biotinylated molecular weight markers: bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa and carbonic anhydrase, 31 kDa.

1 2 3 4

Figure 27. Comparison of the relative mobilities of the 6-mycMAN #1 fusion protein expressed within transient versus stable transfectants

Proteins were extracted from P19 cells which had been either transiently (lane 1) or stably (lane 2) transfected with the 6-mycMAN #1 construct, untransfected (lane 3) or transfected with pKJ53 (lane 4), an unrelated non-transcribed vector. The samples were run on a SDS-PAGE gel, transferred to nitrocellulose and probed with the an anti-human myc antibody. Two reactive protein bands are detected on the blot, a 63 kDa species in lane 1 and a smaller 54 kDa protein in lane 2. The black squares correspond to biotinylated molecular weight markers: phosphorylase b, 97.4; bovine serum albumin, 66.2; ovalbumin, 45; carbonic anhydrase, 31 kDa and soybean trypsin inhibitor, 21.5 kDa.

1 2 3 4

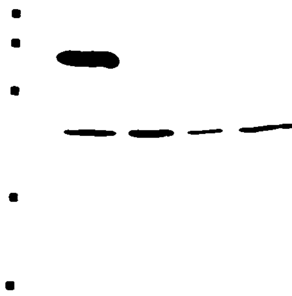
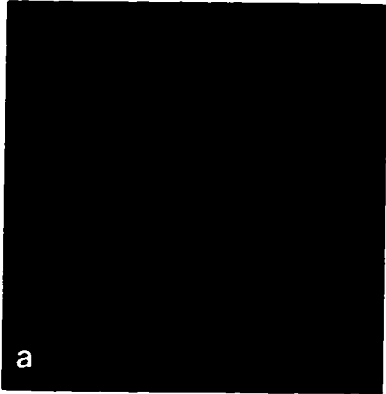


Figure 28. Cellular distribution of the 6-mycMAN #1 fusion protein within stably transfected P19 cells

Immunofluorescence localization of 6-mycMAN #1 and cellular MAN antigens in P19 cells stably transfected with either the 6-mycMAN #1 construct (M1) or with a vector lacking the Pgk promoter and containing unrelated sequences in its multiple cloning site (pKJ53). The M1 (a-f) and pKJ53 (g-l) cells were labelled with a mouse anti-human myc antibody (a,g). As illustrated, only the M1 cells show the diffuse nuclear distribution of 6-mycMAN #1 (a). Labelling both M1 and pKJ53 with the MAN antiserum (d and j respectively) reveals that both cell types show the MAN antigens localized to the nuclear periphery. Cells were counterstained with Hoechst 33258 to visualize the DNA (b, e, h and k) and observed by phase contrast (c, f, i and l). Bar, 10.3 µm.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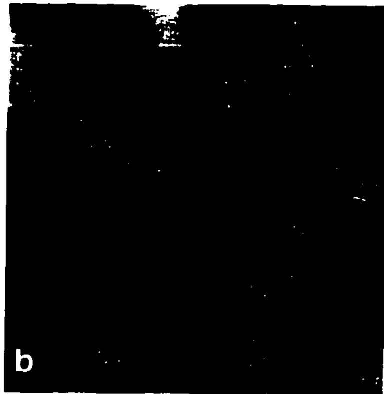
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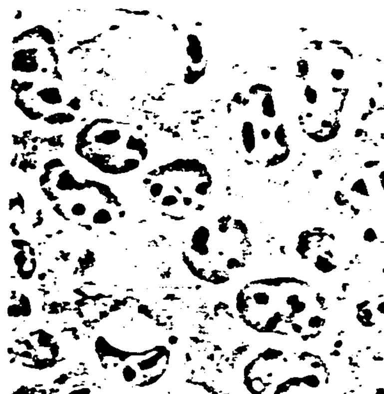
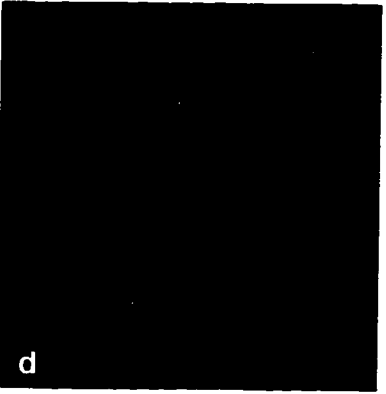
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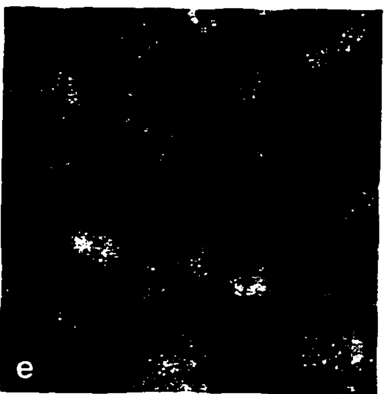
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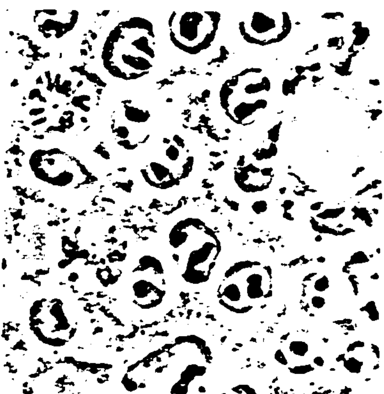

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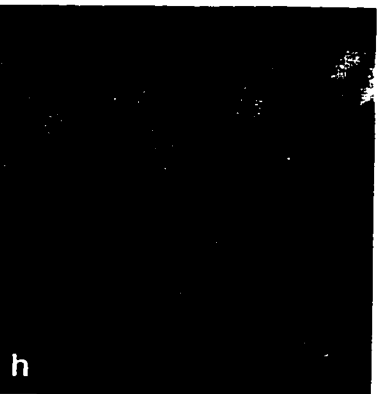
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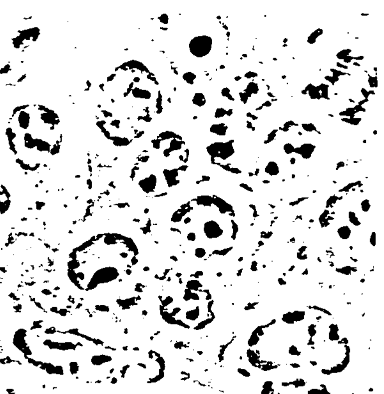

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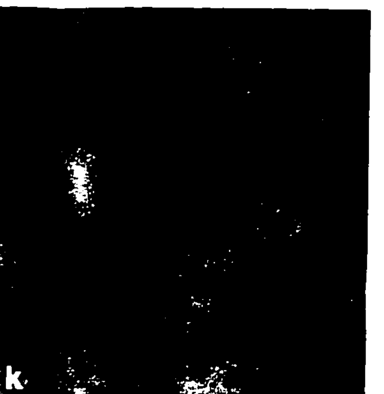
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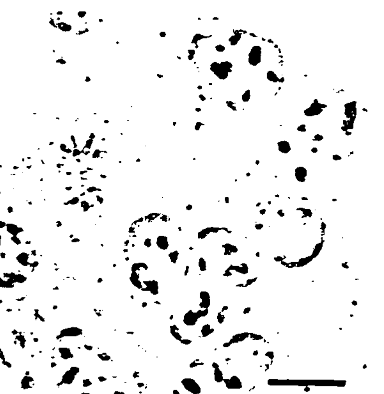

Discussion

From these experiments, it can be concluded that genomic integration of the 6-mycMAN #1 and puromycin resistance gene constructs, while not producing gross morphological changes to chromatin, does influence gene expression (inhibition of cellular differentiation).

Unlike the experiments by Peter and Nigg (1991) and Lourim and Lin (1992), the 6-mycMAN #1 fusion protein was not targeted to the nuclear periphery, but was distributed throughout the nucleoplasm. As described in chapter 4 of this thesis, the transient transfectants often displayed a hook shaped nuclei. Therefore, it was questioned whether or not these gross morphological changes to the DNA had an affect on gene expression. To address this issue, a P19 cell (M1) with a stably integrated 6-mycMAN #1 construct was isolated. Surprisingly, the M1 clones did not display the typical hook shaped nuclei of the transient transfectants. In fact, staining cells with the MAN antiserum demonstrated that the organization of the NE is indistinguishable from controls. These results may reflect the relatively low expression levels of the fusion protein within the M1 clones, as compared to the transient transfectants. In conclusion, my results indicate that genomic integration of a DNA construct can affect gene expression, causing inhibition of cellular differentiation of P19 cells into muscle.

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