1987

The Nuclear Pore Complex: Novel Glycoprotein Constituents

Laura Isabelle Davis

THE NUCLEAR PORE COMPLEX: NOVEL GLYCOPROTEIN CONSTITUENTS

A thesis submitted to the faculty of The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

by
Laura Isabelle Davis

March 31, 1987
The Rockefeller University
New York, New York

Abstract

Nuclear pore complexes are large, uniform organelles with a mass estimated to be on the order of 10^8 daltons. They are ubiquitous components of the nuclear envelope, providing a channel through which macromolecular transport between the nucleus and cytoplasm occurs. Thus far, almost nothing is known about the proteins that comprise this organelle.

In this thesis, I describe the production of anti-nuclear monoclonal antibodies, and their use in identifying some of the constituents of the nuclear pore complex. In particular, I describe studies using an antibody that recognizes predominantly a 62 kD protein (p62) of rat liver nuclei. This protein remains associated with the nuclear pore complex-lamina fraction that results from treatment of nuclei with DNase, RNase and nonionic detergent. Immunofluorescence microscopy revealed a strikingly punctate pattern of nuclear rim staining. Using immunogold electron microscopy, p62 was specifically localized to the pore complex. This indicated that pore complexes could be resolved by fluorescence microscopy, and therefore that this assay can be used to screen for antibodies against the pore complex.

Pulse chase analysis of labelled tissue culture cells showed that p62 is synthesized as a soluble cytoplasmic precursor, which is incorporated into the nuclear fraction with an unusually long half time of about six hours. Additionally, p62 is posttranslationally modified by the addition of GlcNAc residues. In fact, it is a member of a family of novel glycoproteins that are labelled *in vitro* when nuclei are incubated with UDP-^{3}H-galactose in the presence of galactosyl transferase. These proteins have been shown to contain O-linked monosaccharidic GlcNAc residues (Holt, G.D. and G.W. Hart 1986, *J. Biol. Chem.* 261:8049-8057). Pulse chase labelling of tissue culture cells with ^{35}S-methionine indicates that most of the sugar is added within 5 min of synthesis,

when p62 is soluble and cytosolic. Tunicamycin does not prevent glycosylation and the M_r of p62 is not affected by endoglycosidase H. Thus, the addition of GlcNAc appears to be carried out by a cytoplasmically disposed transferase and is clearly distinct from the N- and O-linked glycosylation pathways in the endoplasmic reticulum and Golgi complex.

I have also investigated the subnuclear location of some of the other GlcNAc containing proteins. Using wheat germ agglutinin, as well as another mAb with a broad specificity for nuclear GlcNAc-containing proteins, I show by immunofluorescence and protein blotting of subnuclear fractions that there are two sets of GlcNAc containing proteins, distinguished on the basis of subnuclear localization; one consists of about five polypeptides located in the interior of the nucleus, and one probably consists of as many as eight proteins in the pore complex.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Å	Angstrom
BRL	Buffalo rat liver
BSA	bovine serum albumin
Con A	Concanavalin A
D-MEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease I
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(-aminoethyl ether)-N,N,N' -tetraacetic acid
endo H	endoglycosidase H
FITC	fluorescene isothiocyanate
GalNAc	N-Acetylgalactosamine
GlcNAc	N-Acetylglucosamine
GlcNAcase	N-Acetyl-D-glucosaminidase
HAT	hypoxanthine, thymidine, aminopterin
hnRNA	heterogeneous nuclear RNA
HRP	horseradish peroxidase
kD	kilodalton
KM	Kern-matrix
mAb	monoclonal antibody
MPF	maturation promoting factor
M_r	relative mobility
NPC	nuclear pore complex
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCL	pore complex-lamina
1X PI	1ug/ml leupeptin, 1ug/ml pepstatin, 100 KIU/ml aprotinin
PMSF	phenylmethylsulfonylfluoride
PPO	2,5-diphenyloxazole

RER	rough endoplasmic reticulum
RNase	ribonuclease A
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate
snRNA	small nuclear RNA
TCA	trichloroacetic acid
TEA	triethanolamine
UDP	uridine diphospho
WGA	wheat germ agglutinin

ACKNOWLEDGEMENTS

There are many individuals to whom I am indebted for helping my in one way or another over the years. Some predate my years at The Rockefeller, and were largely responsible for my coming here. In this regard, I would especially like to thank Professor J.S. Singer, whose undergraduate seminars first introduced me to experimental cell biology. Also, Dr. Ralph Reisfeld and Dr. Soldano Ferrone, in whose laboratory I worked for three years, challenged me to work and learn to my fullest capabilities and encouraged me to continue on to graduate school. I should also like to take this opportunity to acknowledge those professors at The Rockefeller who have taken the time to teach seminars, even though it is not required of them, particularly professors Michael Young, Günter Blobel, and Anthony Cerami.

On a more practical note, I would like to thank Elena Sphicas for performing all of the sectioning for electron microscopy. Also, Warren Gallin has provided reagents, valuable advice and, most important, his time whenever it was asked for.

Within our own laboratory there are, and have been, many people who have contributed to making it a happy and stimulating place to work. Some of them require individual thanks. Greg Connor gave me enormous encouragement and much advice at the inception of this project. Emily Evans, Reid Gilmore, Nilabh Chaudhary, John Aris and Gary Greenburg have also provided unqualified help and advice. Richard Wozniak participated in producing the anti-nuclear monoclonal antibodies. More recently, Ruben Enriquez and Thomas Meier have become close collaborators on this project.

I would also like to thank the members of my thesis committee, Professors David Luck, James Darnell, and Carl Feldherr, for taking the time to provide a most thoughtful review of my work. Most importantly, I would like to thank my thesis advisor, Günter Blobel, who over the years has taught me the "art" of cell biology. I appreciate his

confidence in allowing me to undertake a project on a subject about which so little is understood, and his keen sense of when to give me advice and guidance and when to leave me to choose my own path.

Chapter 1

Introduction

1.1 The organization of the nuclear envelope

In eukaryotic cells, the chromatin is segregated from the rest of the cell by the nuclear envelope. This envelope consists of two lipid bilayers separated by a cisternal space ranging from 100 to 600 Å in thickness (Hartmann, 1953; Sjostrand and Rhodin, 1953; Palay and Palade, 1955). Thin section electron microscopy has shown that this perinuclear cisterna is contiguous with the lumen of the rough endoplasmic reticulum (RER); similarly, the outer nuclear envelope is continuous with the membranes of the RER (Watson, 1955; Gall, 1956). The lipid composition of the nuclear membrane is very similar to that of the RER (Kashnig and Kasper, 1969; Franke *et al.*, 1970; Keenan *et al.*, 1970; Kleinig, 1970), and nuclear envelope and microsomal preparations contain similar enzyme markers (Kashnig and Kasper, 1969; Zbarsky *et al.*, 1969; Berezney, 1970; Franke *et al.*, 1970; Kay *et al.*, 1972, Monneron *et al.*, 1972). The presence of ribosomes bound to the cytoplasmic surface of the outer nuclear membrane indicates that, like the RER, the nuclear envelope is active in synthesis of membrane and secretory proteins. This has also been suggested by biochemical studies showing that the perinuclear cisterna contains newly synthesized membrane and secretory proteins (Depetris *et al.*, 1963; Leduc *et al.*, 1968, 1969; Avrameas and Boutielle, 1968; Puddington *et al.*, 1985).

The inner nuclear membrane has a much more distinctive morphology. Attached to it on the nucleoplasmic side is a filamentous network called the nuclear lamina (for

review, see Gerace and Blobel, 1982; Krohne and Benavente, 1986). In mammalian somatic cells the lamina consists primarily of three proteins, which have been termed lamins A, B and C (Gerace *et al.*, 1978; Krohne *et al.*, 1978; Gerace and Blobel, 1980). Recently, molecular cloning of lamins A and C has revealed that these two polypeptides bear extensive homology to each other, as well as to intermediate filament proteins, and are capable of forming coiled coil structures typical of proteins of the intermediate filament family (Fisher *et al.*, 1986; McKeon *et al.*, 1986). In fact, Aebi *et al.* (1986) has shown that chemically dissociated lamin tetramers are capable of reassociating *in vitro* to form filamentous structures. In the same study, it was shown that the lamina of the germinal vesicle of *Xenopus laevis* oocytes is arranged as a striking tetragonal lattice of filaments with a periodicity of ~ 20 nm. Thus, although it remains to be shown that this is a general phenomenon, it is likely that the lamina forms a cage-like scaffold beneath the nuclear envelope. Since the heterochromatin is mostly localized to the periphery of the nucleus and is attached either directly or indirectly to the lamina, the lamina may also provide the structural framework for the attachment and organization of chromatin (Franke *et al.*, 1981; Gerace and Blobel, 1982; Hancock, 1982; Gerace *et al.*, 1984; Kasper, 1984; Blobel, 1985).

As early as the 1950's it was noticed that the nuclear envelope was perforated by cylindrical channels that were referred to as nuclear pore complexes (NPC's) (Callan and Tomlin, 1950; Bretschneider, 1952; Gall, 1954; Watson, 1954, 1955; Afzelius, 1955; Dawson *et al.* 1955; Palay and Palade, 1955). At these NPC's, the inner and outer membranes fuse together to form a hole that is filled with densely staining material. Immediately subjacent to the pore complex, the lamina appears to be discontinuous, and channels can be seen through the heterochromatin. NPC's are large, macromolecular structures, having an estimated molecular weight of over 100 million daltons (Blobel, 1985). They are present in all nuclei, with the possible exception of spermatozoa.

Morphological studies have demonstrated that ribonucleoproteins (RNP's)

(Stevens and Swift, 1966), as well as karyophilic proteins adsorbed to gold particles (Feldherr *et al.*, 1984) are transported through the pore complex. Indeed, because the nuclear envelope is a double membrane structure lined by a fibrous lamina, it is generally accepted that macromolecular traffic across the envelope occurs exclusively through the nuclear pore complex. This traffic must include export of newly synthesized mRNA, tRNA and ribosomal precursors, as well as import of karyophilic proteins, which are synthesized on cytoplasmic polysomes.

The pore complex has been postulated to have other functions as well. For example, Blobel (1985) has proposed that it participates in the three dimensional organization of the genome, serving to "gate" active genes such that movement of their transcripts out of the nucleus is facilitated. Whatever its function or functions might be, they are probably highly correlated with the gross structural characteristics that are described in the next chapter, since the pore complex looks essentially identical in every organism thus far examined.

The number and distribution of NPC's is highly variable (for review, see Maul, 1977). The number per nucleus ranges from approximately one hundred in yeast (Maul and Deaven, 1977) to about 40 million in *Xenopus laevis* oocyte nuclei (Scheer, 1973). Sperm nuclei have few if any pores. The number seems to be dependent on many variables, the most important of which may be the surface area to volume ratio of the nucleus, the total DNA content of the cell, and the transcriptional activity of a given cell type, an increase in any of which also increases the pore density (Maul, 1977). The distribution of NPC's is also highly variable. In most cases they seem to be randomly distributed in the envelope (Branton and Moor, 1964; Franke, 1966), however seemingly regular arrays have been demonstrated in some cell types. Furthermore, Maul (1971) has calculated that even in many cells in which they appear to be randomly distributed, the NPC's are actually spaced such that the distance between them is always a multiple of an integral length, indicating that perhaps some underlying, regular structure within the

envelope determines the spacing. Perhaps the most striking example of regularity is that seen in the germinal vesicle envelope of *Xenopus laevis* nuclei, where the NPC's are packed together in paracrystalline arrays. It may be, however, that in this case they are so organized in order to allow the maximum possible density for a nucleus that has an extremely small surface area/volume ratio.

Thus, it remains unclear whether NPC's are distributed in an ordered or random fashion and what, if anything, is the underlying signal that causes their formation in a given area. In this regard, it should be noted that NPC's could have some degree of mobility in the plane of the membrane, although the fact that they are found to be physically associated with the lamina after biochemical fractionation of the nucleus suggests that they are probably attached to the lamina *in situ*, and thus not subject to lateral movement.

Pore complexes also occur in cytoplasmic membrane systems called annulate lamellae (Swift, 1956; for review, see Kessel, 1968; Maul, 1977). These lamellae are very often juxtanuclear, and consist of stacked sheets of double membranes that are morphologically indistinguishable from nuclear envelope, and which contain numerous pore complexes. Their origin has not been established, but their apparent continuity with the RER suggests that they might be derived from RER membranes (Baca and Zamboni, 1967). In fact, it is possible that annulate lamellae represent an intermediate in the formation of nuclear envelope. Alternatively, they may represent fragments derived from the nuclear envelope itself (Hertig, 1968). Annulate lamellae are most often observed in cells with potential to undergo rapid division, such as oocytes and some tumor cells (see Kessel, 1968; Maul, 1977), an observation supporting the hypothesis that they might represent stored nuclear envelope precursors.

1.2 Structure of the pore complex

The pore complex has been the subject of numerous morphological studies (for review, see Franke, 1974), and recently its structure was determined to a resolution of 90 Å (Unwin and Milligan, 1982; see Fig 1). It consists of two coaxial rings (R), the outer and inner diameters of which are approximately 1200 Å and 600 Å respectively. Each ring is comprised of eight subunits arranged in exact eight fold symmetry about an axis perpendicular to the plane of the membrane. These rings face in opposite directions, one toward the cytoplasm and one toward the interior of the nucleus. Thus, the pore complex also demonstrates symmetry in the plane of the membrane. Spokelike structures (S) appear to emanate from each of the subunits and extend toward the middle of the NPC. At the center, there is usually a central "plug" or "granule" (C) that appears to fill most of channel. This structure is not always present, however, and it is equivocal whether it is actually a constitutive component of the pore complex. It may well represent a macromolecule that was in transit through the pore complex at the time the envelopes were isolated. This is particularly likely in a cell type such as the frog oocyte, in which the transcriptional activity is extremely high, and one might expect to see RNP's in almost every pore. On the other hand, the central granule has been seen in pore complexes of annulate lamellae, which would not be expected to be active in macromolecular transport. Thus, the identity of this structure is far from resolved.

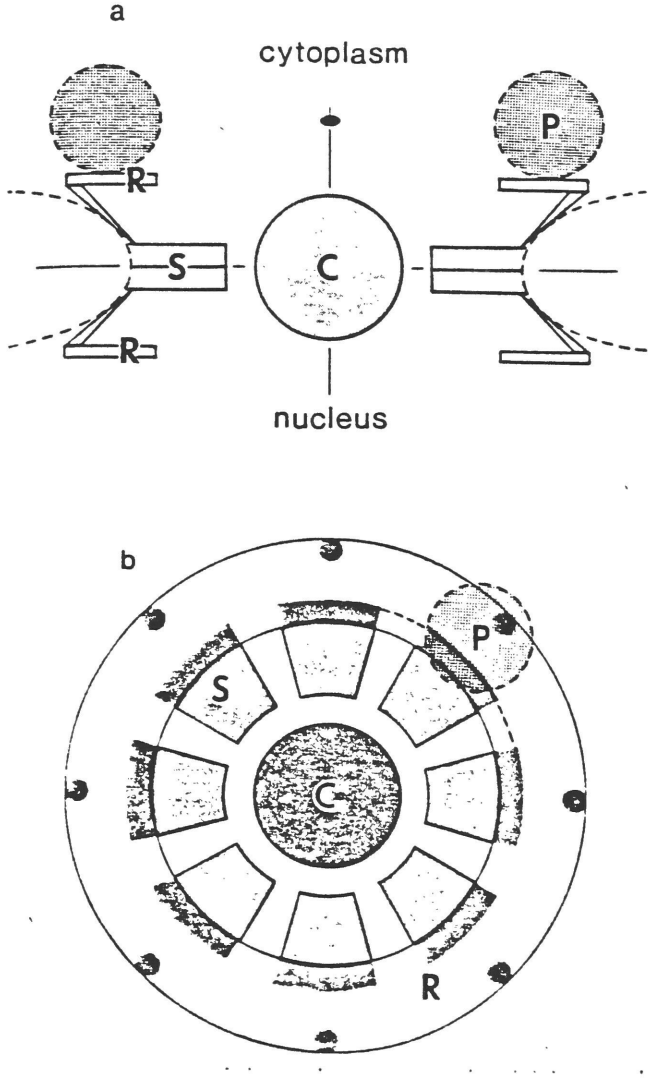
Particles (P) are associated with each of subunits on the cytoplasmic annulus. Because of their size, as well as the fact that they can be extracted by treatment with nonionic detergent, high salt or EDTA (Unwin and Milligan, 1982), it has been postulated that they are actually ribosomes bound to the pore complex (Gall, 1956, Unwin and Milligan, 1982).

Other interpretations of pore complex morphology have also been recorded, and these have been extensively reviewed by Franke (1974). For example, it has been

Figure 1

Diagram of the nuclear pore complex

A schematic of the three dimensional structure of the pore complex, as determined to a resolution of ~90 Å. Panel **a** shows a central cross section and panel **b** shows a projection down the octad axis. The major constituents are the central plug (C), the spokes (S), and the rings (R). Before detergent extraction, pore complexes also demonstrate octagonally arranged particles (P) resembling ribosomes on the cytoplasmic ring. The maximum diameter is ~1,200 Å. **Taken from**: Unwin and Milligan (1982), *J. Cell Biol.* **93**, 63-75.


a
cytoplasm
P
R
S
C
R
nucleus
b
P
S
C
R

suggested (Franke, 1974) that the pore subunits actually consist of localized coils of fibrils that extend into the cytoplasm and nucleus and that the globular nature of the subunits is an artifact of fractionation and/or fixation of nuclei. However, since the gross morphological characteristics of the pore complex are highly reproducible, even with widely divergent fixation and microscopic procedures, it is most likely that the actual structure is very similar to that described by Unwin and Milligan (1982).

1.3 Nucleocytoplasmic transport

The exclusion limit for passive diffusion of macromolecules through the NPC has been studied in detail, in order to estimate the functional dimensions of the channel. While freely permeable to small molecules and ions (Horowitz and Moore, 1974; Siebert, 1978; Paine *et al.*, 1981), the NPC does function as a barrier to the diffusion of macromolecules. Feldherr (1965) showed that particles larger than 125-145 Å in diameter were unable to traverse the nuclear envelope by microinjecting colloidal gold molecules of various sizes into the cytoplasm of amebas. Using a similar approach, Gurdon (1970) showed that ferritin (diameter 95 Å) was also excluded. Other studies have shown that the pore complex restricts the diffusion of smaller nonkaryophilic proteins to an extent roughly proportional to their molecular weight (Paine, 1975; Paine and Feldherr, 1972; Bonner, 1975a). A quantitative analysis using ^{3}H-dextrans (Paine *et al.*, 1975) concluded that the functional diameter for passive diffusion through the NPC is about 90 Å, a value that agrees with the above mentioned studies.

It is by now well established that if labelled proteins are microinjected into the cytoplasm, the nucleus accumulates specifically those proteins that are normally nuclear (Gurdon, 1970; Bonner, 1975b; Feldherr, 1975; De Robertis *et al.*, 1978; Mills *et al.*, 1980; Dabauville and Franke, 1982). Many of these molecules are larger than the functional diameter for passive diffusion of the NPC, and the rate of transport of the

others is much more rapid than can be accounted for by passive diffusion (Feldherr *et al.*, 1983). Therefore, an active process which includes some element of specificity is almost certain to play a role in transport of karyophilic proteins.

Numerous groups have looked for signals that specify nuclear entry. In one elegant study, Dingwall *et al.* (1982) iodinated the karyophilic protein nucleoplasmin and microinjected it into the cytoplasm of *Xenopus laevis* oocytes. He showed that this tetramer of 165 kD was able to migrate into the nucleus and concentrate there. He further showed that the signal for entry resided in a small N-terminal tail fragment that could be cleaved away from the core domain, such that the latter could no longer enter the nucleus. Importantly, by injecting the core fragment into the nucleus and showing that it also could not exit, he was able to show that this signal was indeed involved in transport, and was not merely a binding domain for nucleoplasmin within the nucleus. Subsequently, Feldherr *et al.* (1984) were able to show that if nucleoplasmin was adsorbed to colloidal gold particles of various sizes before microinjection into the oocyte, all of the particles were transported with equal efficiency, even though the largest was twice size of the diffusion limit of the NPC. Again, if the core fragments lacking the putative signal were used to coat the gold particles, no transport to the nucleus occurred.

Many groups have attempted to define the sequence requirements for nuclear localization of proteins using the techniques of molecular genetics, (for review, see Smith *et al.*, 1985). Kalderon and coworkers (Kalderon *et al.*, 1984a; Kalderon and Smith, 1984) as well as Lanford *et al.* (1984), isolated mutants of SV40 large T antigen that were defective for nuclear localization and showed that deletion of a small lysine rich sequence was sufficient to cause this phenotype. It was subsequently shown (Kalderon *et al.*, 1984b) that this sequence could act as a signal to direct nuclear accumulation of the cytoplasmic protein pyruvate kinase. Similar approaches have shown that such sequences also exist in influenza virus nucleoprotein (Davey *et al.*, 1985), the yeast nuclear protein GAL4 (Silver *et al.*, 1984), and polyoma virus large-T

antigen (Richardson *et al.*, 1986), the latter of which appears to contain two such sequences. However, a search of protein sequence data banks (Smith *et al.*, 1985) failed to show a convincing correlation between nuclear localization and possession of sequences homologous to known nuclear localization sequences. One caveat in the interpretation of all of these studies is the inability to rigorously differentiate sequences that mediate transport from those that confer an affinity for internal structures of the nucleus that would allow intranuclear concentration of these proteins.

Still less is known about the transport of RNA out of the nucleus. RNA transport presumably also has some element of specificity, since the RNA content of the nucleus and cytoplasm is very different (Getz *et al.*, 1975; Hough *et al.*, 1975; Levy *et al.*, 1976; Herman *et al.*, 1976: Bantle and Hahn, 1976; Wold *et al.*, 1978; Berget *et al.*, 1977; Chow *et al.*, 1977; Klessig, 1977). The majority of nuclear hnRNA is not transported, but is instead degraded within the nucleus (Soeiro *et al.*, 1968; Salditt-Georgieff and Darnell, 1982). Likewise, immature mRNA is generally not found in the cytoplasm, but is instead constrained to the nucleus. Whether these phenomena are the result of differential transport, or are due to other factors such as binding within the nucleus or degradation before transport, is still unclear. Numerous groups have also shown that microinjection of RNA-protein moieties that are normally found in the nucleus (snRNAs, 5S RNA) concentrate there after injection into the cytoplasm, whereas those that are normally cytoplasmic (tRNAs, 7S RNA and 42S RNP) remain in the cytoplasm (DeRobertis *et al.*, 1982; Mattaj, *et al.*, 1983; De Robertis, 1983; Zeller *et al.*, 1983).

The specificity of RNA transport has also been investigated using genetic techniques similar to those used in identifying the nuclear localization sequences of proteins. Zasloff (1983) microinjected tRNA into *Xenopus* oocyte nuclei and assayed for its appearance in the cytoplasm by microdissection. He found that transport was saturable, and that two different tRNA molecules could compete with each other for export. Furthermore, Tobian *et al.* (1985) showed that mutant tRNAs could be isolated

that were defective for transport, and that those most affected contained mutations in the D- and T-stem loop regions.

Much of the mechanistic work that has been done has utilized *in vitro* transport systems in which cells are usually labelled with ^{3}H-uridine, lysed, and their nuclei isolated. The rate of efflux of labeled RNA is then assayed under various conditions. Agutter *et al.* (1976, 1977, 1979) and Clawson *et al.* (1978, 1980) have shown that RNA transport appears to be coupled to activity of a nucleoside triphosphatase, and that hydrolysis of high energy phosphate bonds is necessary for transport (Clawsen *et al.*, 1978). This activity has been localized biochemically and electron microscopically to the nuclear envelope.

Thus, although little is yet certain concerning the mechanisms by which compartmentalization of macromolecules between the nucleus and cytoplasm is effected, most of the available evidence supports the hypothesis that transport itself is highly specific and is probably mediated, at least in part, at the level of the pore complex.

1.4 The behavior of the nuclear envelope at mitosis

During mitosis in higher eukaryotes, the nucleus undergoes drastic rearrangements resulting in the condensation of the chromatin into discrete chromosomes, and the disassembly of the nuclear envelope into membrane vesicles morphologically indistinguishable from RER. During this process, the lamina is depolymerized such that lamins A and C are solubilized, while lamin B remains associated with membranes (Gerace and Blobel, 1980). NPC's disappear abruptly upon vesiculation of the envelope. Nothing is known concerning the fate of the NPC constituents during mitosis, except that morphologically distinguishable NPC's or subunits thereof are not discernible. While it is possible that it becomes completely disassembled into monomeric proteins, it is perhaps more likely that the NPC dissociates

into discrete macromolecular structures, such as annular subunits, spokes, central granule *etc.* Some components might even bind to the chromosomes, serving as markers for those regions of the chromatin that were near the pore, so that these can be reoriented properly when the interphase structure of the nucleus is restored. It is also possible that some NPC components might remain associated with the membrane vesicles, as does lamin B (Gerace and Blobel, 1980). One candidate for such behavior would be the 190 kD integral membrane glycoprotein that is located specifically in the region of membrane forming the wall of the pore complex, and may serve to anchor it to the nuclear envelope (Gerace *et al.*, 1982).

During metaphase, the mitotic spindle is formed and the chromosomes line up along the metaphase plate. The chromosomes migrate to opposite poles during anaphase. For the duration of these two phases, flattened membrane vesicles that presumably derive from the nuclear envelope lie largely adjacent to the spindle. At telophase, the daughter chromosomes decondense, membrane vesicles begin to bind to the chromatin and flatten out, pore complexes reappear and the lamina is reassembled to form a complete nuclear envelope.

Only recently has significant progress been made in our understanding of how the entry into mitosis is regulated. A partially purified activity termed Maturation Promoting Factor (MPF) is capable of inducing nuclear envelope breakdown when microinjected into oocytes or embryos (Masui and Markert, 1971; Reynhout and Smith, 1974; Drury, 1978; Wasserman and Smith, 1978; Wu and Gerhart, 1980; Kishimoto *et al.*, 1982; Adlakha *et al.*, 1985). MPF is present in an inactive form in interphase cells, and is rapidly activated just before the onset of mitosis (Wasserman and Masui, 1975; Miake-Lye *et al.*, 1983; Gerhart *et al.*, 1984; Newport and Kirschner, 1984). It has recently been shown that the cell cycle can be regulated entirely by repeated injection of MPF followed by its subsequent inactivation after mitosis (Newport and Kirschner, 1984).

It is not known whether MPF is part of a fundamental cell cycle oscillator or

rather responds to one, and neither an activator of MPF nor its substrate have yet been identified. It has been suggested that it might be part of a cascade that results in phosphorylation of numerous proteins involved in mitosis (Maller *et al.*, 1977). One of the most obvious possible substrates would be the lamins, since the disassociation of the lamina occurs concomitant with, and is probably triggered by, hyperphosphorylation of all three lamins (Gerace and Blobel, 1980; Ottaviano and Gerace, 1984; Miake-Lye and Kirschner, 1985; Suprynowicz and Gerace, 1986).

Structural rearrangement and assembly of nuclear envelope must also occur to some extent during interphase, since the surface area of the nucleus is roughly doubled at this time, as is the total number of NPC's (Maul *et al.*, 1971, 1972). In fact, the bulk of incorporation of newly synthesized pore complex proteins probably occurs at interphase; the appearance of NPC's after mitosis most likely represents reassembly from preexisting constituents. Thus, there must be a mechanism whereby the cell can insert nuclear envelope material into the intact nucleus. Virtually nothing is known, however, about the dynamics of interphase pore complex biosynthesis and assembly.

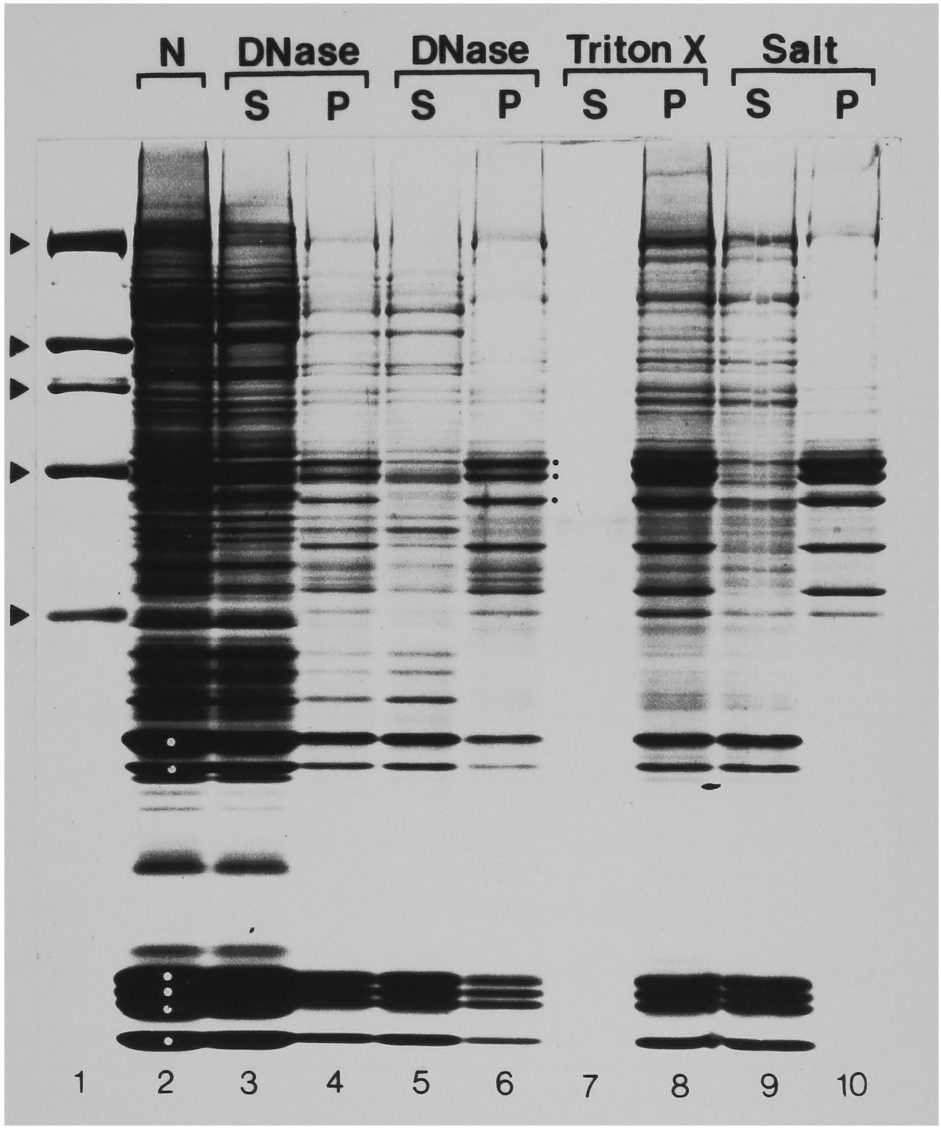
1.5 Biochemical characterization of the nuclear envelope

Nuclei and nuclear envelope preparations have been isolated from numerous sources using many different conditions (Blobel and Potter, 1966; Zbarsky *et al.*, 1969; Kashnig and Kasper, 1969; Berezney *et al.*, 1970; Franke *et al.*, 1970; Zentgraf *et al.*, 1971; Kay *et al.*, 1972; Bornens, 1973; Bornens and Courvalin, 1973; Franke and Scheer, 1974, Philipp *et al.*, 1976; for review see Franke, 1974). Our laboratory has primarily used nuclei isolated from rat liver by a method that yields large numbers of nuclei essentially free from contamination by other cellular organelles (Blobel and Potter, 1966). The polypeptide profile of these nuclei is very complex, with the major protein constituents being the histones (Fig 2, lane 2, see also Aaronson and Blobel, 1974, 1975;

Figure 2

Subfractionation of rat liver nuclei

Isolated rat liver nuclei were subfractionated as described in section ***2.2***, and aliquots of 20,000 xg_{av} supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE on 10-15% gradient gels. The gel was stained with Coomassie Blue. Whole nuclei (2 eq); lane 2. First DNase digestion (2 eq); lanes 3 and 4. Second DNase digestion (4 eq); lanes 5 and 6. Triton X-100 extraction (8 eq); lane 7 and 8. 500 m*M* NaCl extraction of material from lane 8 (8 eq); lanes 9 and 10. Lane 1 contains molecular weight markers as follows: Myosin, 200 kD;β-Galactosidase, 116.25 kD; Phosphorylase B, 92.5 kD; Bovine serum albumin, 68 kD; and Ovalbumin, 43 kD. white dots indicate histones, black dots indicate the lamins.


N
DNase
DNase
Triton X
Salt
S
P
S
P
S
P
S
P
1
2
3
4
5
6
7
8
9
10

Jackson, 1976; Dwyer and Blobel, 1976; Gerace *et al.*, 1978). Nuclease digestion (Kay *et al.*, 1972) yields nuclear envelope "ghosts" which consist of largely intact double membrane structures with associated lamina and pore complexes, but devoid of intranuclear material (Dwyer and Blobel, 1976). The polypeptide profile of the remaining material (Fig 2, lane 6 and Aaronson and Blobel, 1975; Dwyer and Blobel, 1976)) shows that most of the polypeptides have been removed, including almost all of the histones. While many minor proteins are left, the major proteins remaining are the three lamins (black dots). Treatment of these envelopes with 2% Triton X-100 delipidates both membranes, thus solubilizing the outer nuclear membrane and the contents of the perinuclear cisterna (Aaronson and Blobel, 1974, 1975; Dwyer and Blobel, 1976). The remaining material consists of pore complexes connected to one another through attachment to the lamina, and is referred to as the crude pore complex-lamina (PCL) fraction (Fig 2, lane 8 and Dwyer and Blobel, 1976). Further extraction of this fraction using high concentrations of monovalent cations releases a number of minor proteins (Fig 2, lane 9), leaving the lamins and some other polypeptides (Fig 2, lane 10, Dwyer and Blobel, 1976), but does not change the overall morphology of the PCL fraction (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976). This fraction is composed of 95% protein, 3% DNA and 2% RNA, and does not contain any measurable phospholipid (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976).

Gerace *et al.* (1978) have shown that the three lamin proteins are localized exclusively within the lamina and are not found in the NPC. Therefore, the pore complex must be composed of some of the numerous minor proteins that are contained in the PCL fraction. In fact, it is likely that some of the NPC proteins are represented only once in each of the various subunit structures of the pore. If the two rings are identical and each contain eight subunits, then these pore complex proteins would be present in as few as 48,000 copies per cell in rat liver nuclei, which have ~3,000 pores per nucleus.

In part because of the small amounts of these proteins present, very little progress

has been made in identifying the constituents of the pore. Gerace *et al.* (1982) have identified a 190 kD glycoprotein that is localized at the junction of the inner and outer nuclear envelope membranes. Most of the mass of this integral membrane protein lies within the lumen of the envelope, and only a small portion is exposed at the nuclear pore side. Thus, gp190 may form an anchoring site for the pore complex.

So far no constitutive proteins of the nuclear pore complex have been identified. The major obstacle has been the fact that attempts to obtain purified pore complexes or subunits thereof through further biochemical perturbation of the PCL fraction have resulted in the loss of morphologically identifiable NPC subunits. Since copurification with a morphologically homogeneous preparation of NPC's would be the only assay for pore complex proteins, conventional biochemical techniques have proven inadequate at dissecting out the components of the NPC.

To circumvent these difficulties, we decided to approach the problem by generating antibody probes that are specific for proteins of the nuclear pore complex. To do this, we made monoclonal antibody producing hybrids from spleen cells of mice immunized with nuclear material, screened for nuclear envelope specificity using protein blotting and immunofluorescence, and sublocalized the antigens recognized by one envelope specific antibody using immunoelectron microscopy.

In this thesis, I describe the use of this monoclonal antibody, as well as one other one, to identify a family of nuclear proteins, some of which are constitutive proteins of the NPC, and others of which are inside the nucleus. My studies have concentrated primarily on one NPC protein, p62. Surprisingly, p62 is synthesized as a soluble, cytoplasmic polypeptide, and is modified soon after synthesis by the addition of monosaccharidic GlcNAc residues through an O-linkage to serine and threonine. Since this addition occurs in the cytoplasm, we believe that it represents a novel cellular pathway for glycosylation.

Chapter 2

Materials and Methods

2.1 Materials and reagents

Anti-lamin B and anti-lamins A and C antisera were a generous gift of L. Gerace (Johns Hopkins). The myeloma cell line, NS-1 was kindly provided by C. Russo (Cornell University Medical College). Aprotinin (Trasylol) was obtained from Mobay chemicals. *Staph aureus* V8 protease, affinity purified alkaline phosphatase-conjugated goat anti-mouse IgG and affinity purified goat anti-mouse IgG were from Cooper Biomedical. FITC-conjugated rabbit anti-mouse IgG was obtained from Miles Scientific. The Vectastain-ABP kit used to detect biotin-labelled WGA was from Vector Labs. WGA-Sepharose, CNBr-activated Sepharose and Protein A-Sepharose were purchased from Pharmacia Fine Chemicals. ^{35}S-methionine (20-30 Ci/m*M*) and D-[6-^{3}H(N)]-glucosamine dihydrochloride (20-30 ci/m*M*) were obtained from New England Nuclear. Uridine diphospho-[6-^{3}H]-galactose (5-15 Ci/m*M*) was obtained from Amersham. Leupeptin, endoglycosidase H, tunicamycin, goat anti-mouse IgG adsorbed to 5 nm gold, and N-Acetyl-D-glucosaminidase were from Boehringer Mannheim biochemicals. N-Acetylglucosamine was obtained from Aldrich. All reagents for cell culture were obtained from GIBCO. All other reagents were purchased from Sigma Chemical Company.

2.2 Isolation and subfractionation of rat liver nuclei

Rat liver nuclei were prepared from 200-250 gram male Sprague Dawley rats that had been starved for 16 hr before sacrifice. Nuclei were isolated as described (Blobel and Potter, 1966), except that all solutions were buffered with 10 m*M* triethanolamine-HCl (TEA-HCl), pH 7.4 instead of Tris-HCl, and contained 0.5 m*M* phenylmethylsulphonylfluoride (PMSF). In addition, the homogenization buffer was made 5 m*M* in iodoacetamide and aprotinin was added to 100 KIU/ml. After isolation the absorbance at 260 nm was measured, aliquots of nuclei were centrifuged at 1,000 xg_{av} for 10 min and the pellets frozen at -70° C. In all subsequent steps, the amount of material derived from 1 A_{260} unit of isolated nuclei (approximately 3 x 10^6 nuclei) is referred to as 1 equivalent (eq).

DNase and RNase treatment of nuclei was carried out essentially according to Dwyer and Blobel (1976), with some modifications. For DNase treatment, nuclei were resuspended, by vortexing briefly, to a concentration of 100 eq/ml in 0.1 m*M* $MgCl_2$ and then immediately diluted with four volumes of 10% (w/v) sucrose, 10 m*M* TEA-HCl pH 8.5, 0.1 m*M* $MgCl_2$, 0.5 mM PMSF and 1 mM dithiothreitol (DTT). Five micrograms of DNase I and 1 ug of RNase A were added per ml and the sample incubated for 15 min at room temperature. After underlayering with a fifth of the sample volume of ice cold 30% sucrose, 10 m*M* TEA-HCl pH 7.4, 1 m*M* $MgCl_2$, 0.5 m*M* PMSF, and 1 m*M* DTT in Corex tubes, the sample was centrifuged at 20,000 xg_{av} for 10 min at 4° C. The pellet was resuspended to 100 eq/ml by vortexing in 10% sucrose, 10 m*M* TEA-HCl pH 7.4, 0.1 m*M* $MgCl_2$, 0.5 m*M* PMSF, and 1 m*M* DTT and then 25 ug DNase and 5 ug RNase were added per ml. The sample was incubated and centrifuged as before except that the sucrose cushion used to underlayer was 5 m*M* in $MgCl_2$. The pellet, representing crude nuclear envelopes, was resuspended to 100 eq/ml by vortexing in 10% sucrose, 10 m*M* TEA-HCl pH 7.4, 5 m*M* $MgCl_2$, 0.1 m*M* PMSF, and 1 m*M* DTT. In some cases, Triton

X-100 was then added from a stock solution (20% w/v) to a final concentration of 2%. In others, the pellet was instead directly extracted with salt by addition of 4 *M* NaCl to a final concentration of either 140 m*M*, 0.5 *M*, or 1 *M*. All samples were incubated at 4°C for 5 min prior to centrifugation as before. The Triton X-100 treated sample (crude PCL) was sometimes subsequently extracted with NaCl, as described above. Aliquots of supernatant and resuspended pellet fractions from each step were precipitated with 10% trichloroacetic acid (TCA), and the precipitates washed with acetone before being subjected to SDS-PAGE.

2.3 Monoclonal antibody production

Seven equivalents of rat liver nuclei isolated as described above were resuspended in 1 ml of 250 m*M* sucrose, 50 m*M* TEA-HCl pH 7.4, 25 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* PMSF, and 1 m*M* DTT. One hundred microliters of 20% Triton X-100 were added, and the sample incubated on ice for 15 min, before centrifuging at 4° C for 10 min at 20,000 xg_{av}. The pellets were resuspended in 200 ul of sterile phosphate buffered saline (PBS), and injected intraperitoneally into a six week old BALB/ c female mouse. This procedure was repeated every three to four weeks for a total of four injections and a final boost was given one week later.

Three days after the final boost the mouse was sacrificed, its spleen removed and cells allowed to fuse for 8 min with myeloma cells (NS-1) at a ratio of 5 X 10^7 /1 x 10^7 spleen cells/ myeloma cells in 300 ul of D-MEM without added serum, containing 50% polyethylene glycol-1500. To stop fusion, 5 ml of D-MEM containing 10% fetal calf serum and 50 ug/ml gentamycin (complete D-MEM) were added dropwise and cells were pelleted by centrifugation at 1,000 xg_{av} for 5 min. They were resuspended in 20 ml of complete D-MEM containing 15% fetal calf serum and incubated for 12-18 hr before plating into five 96-well microtiter dishes at 100 ul/well in HAT medium,

(complete D-MEM containing 1 x 10^{-4} *M* hypoxanthine, 4 x 10^{-7} *M* aminopterin, 1.6 x 10^{-5} *M* thymidine and 15% fetal calf serum). Two drops of HAT medium were added to each well after five days, and wells were vacuumed and refed three days later. Supernatants were harvested when the clones began to spend their medium, and screened using protein blots.

Hybridomas which showed reactivity by protein blotting (see section ***2.4***) were expanded and screened by fluorescence (see section ***2.5***). Interesting cell lines were then subcloned by limiting dilution and frozen at -90° C.

2.4 Immunoblotting

All incubations were performed at room temperature for 4 hr, and all washes were done in three changes of excess PBS containing 0.5% Tween 20, for 30 min each wash.

For screening hybridomas, nitrocellulose blots were prepared by passive transfer from preparative SDS-polyacrylamide gels of DNase treated rat liver nuclei (10 eq/cm gel width), followed by blocking in PBS containing 0.5% Tween 20 and 4% bovine serum albumin (BSA). Nitrocellulose strips (20 cm x 0.5 cm) were incubated with 100 ul of culture supernatant diluted into 3 ml PBS containing 0.5% Tween 20 and 2% BSA. They were then washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:500 in the same buffer used to dilute the culture supernatant. After washing again, strips were assayed for alkaline phosphatase activity, essentially according to Mc Gadey (1970). Blots were rinsed twice in 50 m*M* glycinate pH 9.6, and then incubated in 50 m*M* glycinate pH 9.6 containing 0.1 mg/ml p-nitro tetrazolium chloride, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 4 mM $MgCl_2$, made immediately before use. The reaction was stopped by rinsing with water, and strips were dried under vacuum.

Protein blots of rat liver nuclear subfractions were obtained by electrophoretic transfer from 10-15% gradient SDS-polyacrylamide gels to nitrocellulose sheets, and were treated as specified above, except that 25 ml volumes were used for antibody incubations. Also, mAb 414 and 457 supernatants were diluted 1:3 instead of 1:30.

2.5 Immunofluorescence microscopy

Unless otherwise noted, all incubations were carried out at room temperature on a slowly rotating orbital shaker, and all washes were done in PBS for about 1 min each. Buffalo Rat Liver (BRL) cells grown on coverslips for 48 hr were washed 3 times, and fixed for 20 min with a fresh solution of 2% formaldehyde in PBS. After washing 3 times, cells were permeabilized with methanol at -20°C for 5 min. They were then washed 3 times and incubated for 1 hr with PBS containing 2% BSA, to block nonspecific binding sites. Undiluted mAb 414 culture supernatant was then added and the cells again incubated for 1 hr, after which they were washed 5 times over a 30 min period. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at a dilution of 1:200 in PBS containing 2% BSA was then added and the cells incubated in the dark for 1 hr, after which they were washed 5 times over a 30 min period. Cover slips were mounted in a solution of p-phenylene diamine at 1 mg/ml in 90% glycerol, pH 8.0 (Johnson and Noqueire Araujo, 1981), sealed and viewed under a Zeiss photomicroscope III. Photographs were taken using Kodak Tri-X pan film at ASA 800.

Alternatively, cells were first extracted *in situ* essentially as described (Staufenbiel and Deppert, 1984). Briefly, BRL cells grown as above were washed 3 times with Kern-matrix buffer (KM buffer): 10 m*M* N-morpholinoethanesulphonic acid, pH 6.2, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10% glycerol, 100 KIU/ml aprotinin. Cells were then extracted with KM buffer containing 1% Triton X-100 and 1 m*M* EGTA for 3 min and again for 30 min, both incubations on ice. After washing 3 times with KM buffer,

cells were incubated for 15 min at 37° C with KM buffer containing 50 ug/ml DNase I. Cells were then either rinsed 3 times with KM buffer and treated as above, beginning with the BSA blocking step, or further extracted by incubating on ice for 30 min with KM buffer containing 1 *M* NaCl and 1 m*M* EGTA, before processing for fluorescence.

2.6 Immunoelectron microscopy

Rat liver nuclei were treated for immunoelectron microscopy as described (Gerace *et al.*, 1982), with the following modifications. As primary antibody, we used ascitis fluid of mAb 414, diluted 1:10 in NB3 (8.5% sucrose, 10 m*M* sodium phosphate, pH 7.4, 140 m*M* NaCl, 0.002 m*M* $MgCl_2$, 0.2 m*M* EGTA, 0.5 m*M* PMSF) containing 2% BSA. Incubation was carried out for 1 hr, after which dishes were washed as described. Then goat anti-mouse IgG adsorbed to 5 nm colloidal gold diluted 1:10 in NB3 containing 2% BSA was added and incubated for 60 min at room temperature. Subsequent manipulations were performed exactly as described. Samples were examined with a JEOL 100C electron microscope at 80 kV.

2.7 Cell culture labelling and immunoprecipitation

Steady state labelling and immunoprecipitation were done essentially as described by Gerace and Blobel (1980), with some modifications. For steady-state ^{35}S-methionine labelling experiments, BRL cells were grown to subconfluency on 100 cm^2 Petri dishes before labelling for 12-16 hr with 40 uCi/ml ^{35}S-methionine (~1,000 ci/m*M*) in complete D-MEM without methionine. Dishes were chilled on ice and rinsed 3 times with PBS, after which cells were scraped into 10 ml PBS and centrifuged at 1,000 xg_{av} for 3 min. All subsequent manipulations were done at 0° C. Cells were resuspended in 1 ml homogenization buffer (10 m*M* TEA-HCl, pH 7.4; 10 m*M* NaCl; 1.5

m*M* $MgCl_2$; 1 m*M* PMSF; and 1X PI. The cells were incubated for 15 min and then homogenized with 15-20 strokes in a glass-Teflon homogenizer. One milliliter of compensating buffer, (10 m*M* TEA-HCl, pH 7.4; 270 m*M* NaCl; 1.5 m*M* $MgCl_2$; 1 m*M* PMSF; and 1X PI) was then added and the homogenate was centrifuged at 15,000 xg_{av} for 15 min. Supernatants were adjusted to 2 m*M* EDTA and 0.4% SDS. Pellets were resuspended in 2 ml of 0.4% SDS; 10 m*M* TEA-HCl, pH 7.4; 140 m*M* NaCl; 2 m*M* EDTA; 1 m*M* PMSF and sonicated briefly. All samples were boiled for 5 min and allowed to cool before being adjusted to a volume of 4 ml and final concentrations of 0.2% SDS; 1% Triton X-100; 50 m*M* TEA-HCl, pH 7.4; 100 m*M* NaCl; 2 m*M* EDTA; 2% BSA; 1 m*M* PMSF; 1X PI. Samples derived from pellets were further centrifuged at 15,000 xg_{av} for 15 min to clear them of particulate matter.

One milliliter of sample was used for each immunoprecipitation. Ten microliters packed Protein A-Sepharose were incubated with 1 ml mAb 414 culture supernatant, or 10 ul of anti-lamin serum diluted to 500 ul with wash buffer, for 3 hr at 25° C, washed 3 times with wash buffer (0.2% SDS; 1% Triton X-100; 50 m*M* TEA-HCl, pH 7.4; 100 m*M* NaCl; 2 m*M* EDTA; 2% BSA), and then incubated with 1 ml aliquots of cell extracts (see above) overnight at 4° C. The beads were then washed 8 times with wash buffer and 2 times with PBS. Fifty microliters of SDS-PAGE sample buffer (4% SDS; 330 m*M* Tris base; 2 m*M* EDTA; 100 mM DTT; 0.01% bromphenol blue) were then added to the beads and the samples were boiled for 5 min, immediately prior to performing SDS-PAGE.

For labelling with ^{3}H-glucosamine, BRL cells were grown to subconfluency on two 60 mm^2 Petri dishes in complete medium. The cells were labelled for 12 hr with 2 ml/dish of complete medium (minus sugar) containing 100 uCi/ml ^{3}H-glucosamine. They were then processed exactly as were cells labelled with ^{35}S-methionine.

2.8 Pulse-chase labelling

For the long term pulse-chase experiment, BRL cells were grown on 60 mm^2 Petri dishes to subconfluency and then starved with complete D-MEM without methionine for 90 min before labelling for 30 min with 80 uCi/ml of ^{35}S-methionine in complete D-MEM without methionine. Plates were rinsed once and then complete D-MEM was added to chase. At appropriate time points one dish was removed, washed on ice with cold PBS and processed as above, except that all volumes were reduced by a factor of four. The same protocol was followed for the short term pule-chase experiment except that a concentration of 100 uCi/ml ^{35}S-methionine was used and the cells were labelled for 5 min instead of 90 min.

2.9 Sucrose gradient centrifugation

BRL cells on one 100 mm^2 Petri dish were labelled to steady state and the cells subjected to hypotonic lysis as described above, except that the volumes were reduced by a factor of four. After the initial 15,000 xg_{av} centrifugation step, 500 ul of supernatant were loaded directly onto each of two 13 ml 5-20% sucrose gradients containing 10 m*M* TEA-HCl, pH 7.4; 140 m*M* NaCl; 1.5 m*M* $MgCl_2$; 1 m*M* PMSF; 1X PI. Gradients were centrifuged in an SW40 rotor at 40,000 rpm (280,000 xg_{av}) for 6 hr at 4^o C, after which 400 ul fractions were collected. The pellet from one tube was resuspended in 400 ul gradient buffer. Fractions were brought to 4% SDS and 2 mM EDTA and processed for immunoprecipitation as described above, yielding final volumes of 800 ul/sample.

2.10 Isolation of p62 by immunoaffinity chromatography

Ascitic fluid was treated with caprylic acid (Russo *et al.*, 1983), which

precipitates the bulk of the serum proteins except for immunoglobulins. It was then dialyzed extensively against Mc Ilvaine's sodium citrate buffer pH 6.0, diluted with this buffer to a total protein concentration of 5 mg/ml, and incubated with CNBr-activated Sepharose for 4 hr at 4° C.

A crude pore complex-lamina fraction (see above) derived from 2,000 eq of rat liver nuclei was resuspended in 2 ml of 50 m*M* TEA-HCl, pH 7.4; 100 m*M* NaCl; 2 m*M* EDTA; and 1 m*M* PMSF by homogenization in a 6 ml glass-Teflon homogenizer. After adjusting to 2% SDS and boiling for 5 min, the sample was centrifuged for 15 min at 15,000 xg_{av} in an Eppendorf centrifuge to remove particulate material. The supernatant was adjusted to 0.4% SDS; 2% Triton X-100; 50 m*M* TEA-HCl, pH 7.4; 0.5 *M* NaCl; 2 m*M* EDTA and 1 m*M* PMSF (SDS/Triton buffer), in a final volume of 10 ml. After the addition of nonionic detergent, the sample was maintained at 4°C.

The solubilized material was then loaded onto a column packed with 5 ml of mAb 414-conjugated Sepharose which had been preequilibrated with 20 column volumes of SDS/Triton buffer. The load was cycled through the column at 5 ml/hr for 12 hr. Ten milliliters of flowthrough were collected into a single fraction and the column washed for 24 hr with 50 column volumes of SDS/Triton buffer. Elution was carried out by adding 5 ml 50% ethylene glycol, pH 11.5 and incubating for 15 min; this elution step was then repeated. The eluted material was precipitated with TCA and the precipitate extracted with acetone before resolving by SDS-PAGE. The column was immediately washed with PBS and stored in PBS containing 0.02% sodium azide. The column could be reused up to three times.

2.11 Probing of protein blots with lectins

Unless otherwise mentioned, all washes were done with 3 changes of excess PBS containing 0.5% Tween 20 (wash buffer) for 30 min each wash, and all incubations were

for 4 hr at room temperature. SDS-polyacrylamide gels were electroblotted onto nitrocellulose at 40 volts for 8-12 hr, after which the nitrocellulose sheets were blocked by incubation in wash buffer. Blots were probed with biotinylated wheat germ agglutinin (WGA) diluted 1:200 in wash buffer. After incubation and washing, blots were incubated with a preformed avidin-biotin-HRP conjugate made in wash buffer according to the manufacturers' instructions, and incubated for 1 hr. Blots were then washed and assayed for peroxidase activity by rinsing with 10 m*M* Tris-HCl pH 7.4, and incubating briefly in this buffer containing 0.25 mg/ml 3,3'-dimethoxybenzidine dihydrochloride and 0.5% H_2O_2. Reaction was stopped by rinsing with water, and strips were dried under vacuum.

2.12 In vitro galactosylation of nuclear proteins

Galactosyl transferase-mediated galactosylation of terminal GlcNAc residues was carried out essentially as described by Torres and Hart (1984), with some modifications. A total of 60 equivalents of rat liver nuclei (~1.8 x 10^8 nuclei) prepared as described above (see section ***2.2***), were resuspended in 60 ul of STEAKM [250 m*M* sucrose; 10 m*M* TEA-HCl, pH 7.4; 25 m*M* KCl; 1.5 m*M* $MgCl_2$; 0.3 m*M* PMSF]. Ten microliters of 100 m*M* galactose, 2 ul of 125 m*M* 5'-AMP, 2 ul of 250 m*M* $MnCl_2$, and 4 ul of galactosyl transferase were then added in that order. To start the reaction, 20 ul of Uridine diphospho-[6-^{3}H]-galactose were added and the sample was then incubated at 37^{o}C for 30 min. After incubation, 1 ml of ice cold STEAKM was added and the sample was divided into 3 equal aliquots, which were centrifuged at 15,000 x g_{av} at 4^{o}C for 10 min. Two of the resulting pellets were each sonicated briefly in 250 ul of a buffer containing 0.4% SDS; 50 m*M* TEA-HCl, pH 7.4; 100 m*M* NaCl; 2 m*M* EDTA and 0.3 m*M* PMSF. After incubation in a boiling water bath for 5 min, samples were centrifuged at 15,000 x g_{av} for 10 min to clear them of particulate matter. The samples were brought

to a final volume of 500 ul and final concentrations of 0.2% SDS; 1% Triton X-100; 50 m*M* TEA-HCl, pH 7.4; 100 m*M* NaCl; 2 m*M* EDTA; 2% BSA; and 0.3 m*M* PMSF. These samples were then immunoprecipitated as described (see section ***2.7***), with either mAb 414 or mAb 457. In the latter case, the affinity resin was prepared by first incubating 10 ul Protein A-Sepharose with 40 ug affinity purified rabbit anti-mouse IgG, before adding 1 ml of culture supernatant containing mAb 457. The other pellet was treated in the same manner , except that it was solubilized in 250 ul of buffer containing 0.4% SDS; 50 m*M* TEA-HCL, pH 7; 100 m*M* NaCl, 1 m*M* $MgCl_2$; and 0.3 m*M* PMSF. The final buffer conditions for precipitation of this sample were 0.2% SDS; 1% Triton X-100; 50 m*M* TEA-HCl, pH 7.4; 100 m*M* NaCl, 2 m*M* $MgCl_2$ and 0.3 m*M* PMSF. This sample was precipitated with 10 ul of WGA-Sepharose and washed with the same buffer that was used for precipitation. All samples were subjected to SDS-PAGE. The gel was impregnated with 20% PPO in DMSO, dried and fluorographed for 24 hr.

2.13 Glycosidase digestion of immunoprecipitates

N-Acetyl-D-glucosaminidase digestion: Fifty microliters of a solution containing 50 m*M* citrate buffer, pH 4.5; 2 m*M* EDTA; 6 ug/ml leupeptin; 6 ug/ml pepstatin; 200 KIU/ml Trasylol; 0.5 m*M* PMSF, with or without 1 mg/ml N-Acetyl-D-glucosaminidase (GlcNAcase) and 400 m*M* GlcNAc, were added to 10 ul of Protein A-Sepharose beads containing immunoadsorbed p62 (see section ***2.7***). Samples were incubated 15 hr at 37°C, after which the supernatant was removed and precipitated with 10% TCA. TCA pellets were dissolved in SDS-PAGE buffer and the contents transferred back to the corresponding Sepharose pellet to elute any p62 which may have remained bound to the Sepharose. The samples were boiled for 5 min and subjected to SDS-PAGE. The gel was impregnated with 20% PPO in DMSO, dried and fluorographed for 5 days on Kodak X-AR film.

For GlcNAcase digestion of whole rat liver nuclei, 100 eq of nuclei were resuspended in 200 ul of buffer containing 250 m*M* sucrose; 10 m*M* TEA-HCl, pH 7.4; 25 mM KCl; 1.5 mM $MgCl_2$; 6 ug/ml leupeptin; 6 ug/ml pepstatin; 200 KIU/ml Trasylol; 0.5 m*M* PMSF, with or with out 1 mg/ml GlcNAcase. The sample was incubated 24 hr at 37°C and was then precipitated with 10% TCA. The TCA precipitate was dissolved in sample buffer and subjected to SDS-PAGE.

Endo H digestion: Twenty-five microliters of a solution containing 160 m*M* citrate buffer, pH 5.5; 0.3% SDS; 0.4 m*M* PMSF and 0.05 U endo H were added to 10 ul of Protein A-Sepharose containing immunoadsorbed p62. The samples were incubated and processed as described for those digested with GlcNAcase.

2.14 Tunicamycin treatment

BRL cells were grown to subconfluency on 60 mm^2 Petri dishes in complete D-MEM. Tunicamycin was added to a final concentration of 1 ug/ul, 4 hr prior to replacement of the growth medium with complete D-MEM (minus methionine), containing 40 uCi/ml ^{35}S-methionine and 1 ug/ml tunicamycin. A control plate was labelled in the absence of tunicamycin. Cells were labelled for 12 hr, after which the monolayers were washed three times with PBS and the cells were scraped off with a rubber policeman. One fifth of the cell suspension was centrifuged at 1,000 x g_{av} for 3 min, and then 1 ml of 50 m*M* TEA-HCl, pH 7.4; 0.5 *M* NaCl; 0.5% Tween 20; 1 m*M* $MnCl_2$; 1 m*M* $CaCl_2$ was added to the pellet. The cells were vortexed gently and incubated on ice for 15 min to lyse. The lysate was centrifuged at 15,000 x g_{av} for 15 min and the resultant supernatant was incubated with 20 ul Concanavalin A-Sepharose to control for the effectiveness of the inhibition by tunicamycin. The other 4/5 of the cell suspension was centrifuged at 1,000 x g_{av} for 5 min and processed as described above

(see section ***2.7***) except that 1 ml instead of 250 ul of homogenization buffer was used.

2.15 Peptide mapping of galactosylated proteins

An immunoprecipitate from ^{3}H-galactosylated rat liver nuclei using mAb 414 was prepared exactly as described above (see section ***2.12***), except that 60 equivalents of labelled nuclei were used for a single immunoprecipitation. The sample was electrophoresed on a 1 mm thick 7% SDS-polyacrylamide gel, after which the stacking gel was removed and the lane containing the sample excised from the gel. The excised gel strip was laid horizontally across the top of the stacking gel of a 1.2 mm thick 10-15% gradient SDS-polyacrylamide gel. Proteolytic digestion with V8 protease was carried out essentially as described (Cleveland *et al.*, 1977), with some modifications. The gel strip was overlayed with buffer containing 250 m*M* Tris base; 0.1% SDS; 1 m*M* EDTA; 10% glycerol; 20 m*M* dithiothreitol and 2ug/ml *Staph aureus* V8 protease. Electrophoresis was interrupted for a period of 30 min after stacking had been achieved, to allow digestion. The gel was impregnated with 20 % PPO in DMSO, dried, and fluorographed for 2 weeks on Kodak X-AR film.

2.16 Wheat germ agglutinin affinity chromatography

Two thousand equivalents (~6 x 10^9 nuclei) of Triton X-100 treated rat liver nuclear envelopes were prepared as described above (see section ***2.2***). Envelopes pelleted in a 30 ml glass Corex tube were further extracted by resuspension in 20 ml of 10% w/v sucrose; 10 m*M* TEA-HCl, pH 7.4; 5 m*M* EDTA; 0.3 m*M* PMSF and 1 m*M* dithiothreitol (DTT) and incubation on ice for 5 min. The sample was underlayered with 30% sucrose; 10 m*M* TEA-HCl, pH 7.4; 0.1 m*M* $MgCl_2$; 0.3 m*M* PMSF and 1 m*M* DTT and centrifuged at 20,000 xg_{av} for 10 min. The resultant pellet was resuspended in 4 ml

of 10% sucrose; 10 m*M* TEA-HCl, pH 7.4; 5 m*M* $MgCl_2$; 2 *M* urea; 0.3 m*M* PMSF, and incubated on ice for 15 min before centrifugation for 10 min at 50,000 rpm in a TL-100.2 rotor 4°C. The supernatant was diluted to 16 ml such that the final buffer conditions were 2.5% sucrose; 10 m*M* TEA-HCl, pH 7.4; 200 m*M* NaCl; 5 m*M* $MgCl_2$; 0.5% Tween-20; 0.5 *M* urea; 0.02% NaN_3; 0.3 m*M* PMSF and 1X PI. The material was loaded onto an 8 ml WGA-Sepharose 6MB column which had been equilibrated with 100 ml of the load buffer (minus leupeptin and pepstatin), and the load was cycled through the column at 16 ml/hr for 5 hr at 4°C. Ten milliliters of flowthrough were collected and the column was washed with 200 ml 2.5% sucrose; 10 m*M* TEA-HCl, pH 7.4; 0.5 *M* NaCl; 5 m*M* $MgCl_2$; 0.5% Tween-20; 0.2% NaN_3 and 0.3 m*M* PMSF. The column was eluted at half the flow rate with wash buffer containing 100 mg/ml GlcNAc. Ten milliliter fractions of the eluate were collected. At each step during chromatography, aliquots were removed and precipitated with cold 10% TCA. TCA pellets were washed with cold acetone and dissolved in SDS-PAGE sample buffer for electrophoresis.

Chapter 3

Monoclonal antibody production and characterization of a pore complex specific antibody

3.1 Production of monoclonal antibodies

As immunogen, we used isolated rat liver nuclei that had been treated with 2% Triton X-100. This procedure yielded whole nuclei, essentially free of endoplasmic reticulum, with pore complexes completely exposed at the surface. In order to preserve as many constituents of the NPC as possible, we did not use high salt, since this could remove proteins that might be associated through ionic interactions.

As a primary screen, we used protein blots of DNase treated nuclei (nuclear envelopes). Since this fraction consists largely of nuclear envelope "ghosts" that are devoid of intranuclear material (Dwyer and Blobel, 1976), we hoped to identify those antibodies directed against nuclear envelope constituents and screen out any that were directed toward internal antigens. We screened 900 clones by this procedure, and obtained 19 stable positives.

3.2 Immunofluorescence microscopy

Antibodies showing reactivity on protein blots of nuclear envelopes were screened by immunofluorescence using Buffalo rat liver (BRL) cell monolayers that had been fixed with 2% formaldehyde and permeabilized with methanol. Fourteen clones were found to give nuclear staining (Fig 3). Three sets of antibodies gave various

patterns of intranuclear staining (Fig 3a-c). These patterns serve to illustrate the fact that intranuclear antigens tend to show a high degree of compartmentalization, and very often exclude the nucleolar regions. This is in sharp contrast to the staining pattern we obtained with antibodies directed against nuclear envelope constituents (Fig 3d and e). Antibodies against lamins A and C, for example, show a characteristic nuclear "rim" staining pattern (Fig 3d and Gerace *et al.*, 1978; Krohne *et al.*, 1978; Stick and Hausen, 1980). Staining across the nucleus is very homogeneous and is due to envelope staining above and below the plane of focus.

One set of antibodies (a representative of which is mAb 414) gave a staining pattern very similar to that that seen with anti-lamin antibodies (Fig 3e). However, unlike the continuous nuclear rim staining obtained with the latter, staining by mAb 414 was discontinuous and punctate (Fig 3e, Fig 4b). This pattern is unlikely to be an artifact of fixation because it was present regardless of the fixation procedure used. Staining of unfixed cells extracted *in situ* with Triton X-100 followed by DNase treatment, (Staufenbiel and Deppert, 1984) yielded very similar results (Fig 4c,d). Depending on the plane of focus, a punctate pattern could be discerned at the nuclear rim or at the surface of the nucleus. The pattern at the nuclear surface is shown at higher magnification in Fig 4d. Some punctate staining could also be seen in the cytoplasm, usually closely juxtaposed to the nucleus, although it was much more widely spaced and variable in extent from cell to cell. This could be due to crossreactivity with unknown components or may represent staining of annulate lamellae. Figure 4e shows cells extracted with 1 *M* NaCl in addition to Triton X-100 and DNase. While there was some loss of antigen, as judged by a diminished staining intensity, the pattern of punctate rim staining remained essentially unaltered. On the basis of the fractionation experiments presented below (see section ***3.4***), about half of p62 would be expected to be extracted by treatment with 1 *M* NaCl.

Figure 3

Indirect immunofluorescence microscopy on BRL tissue culture cells with anti-nuclear mAbs

BRL cells were fixed with formaldehyde and permeabilized with methanol as described in section ***2.5***. After incubation with various mAbs, the cells were incubated with FITC-goat anti-mouse IgG. Panels a-c show representatives of the three groups of antibodies that gave different intranuclear staining patterns. Panel d shows a mAb specific for lamins A and C. Panel e shows mAb 414.

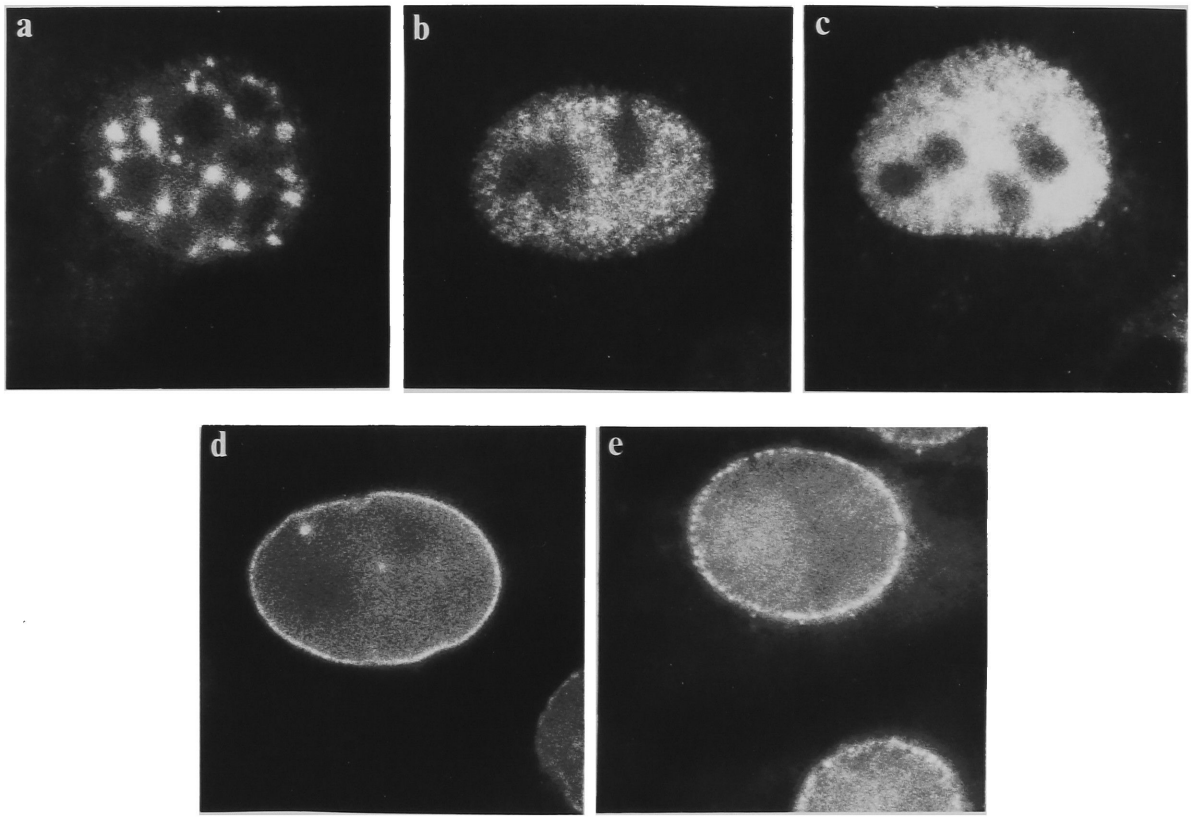
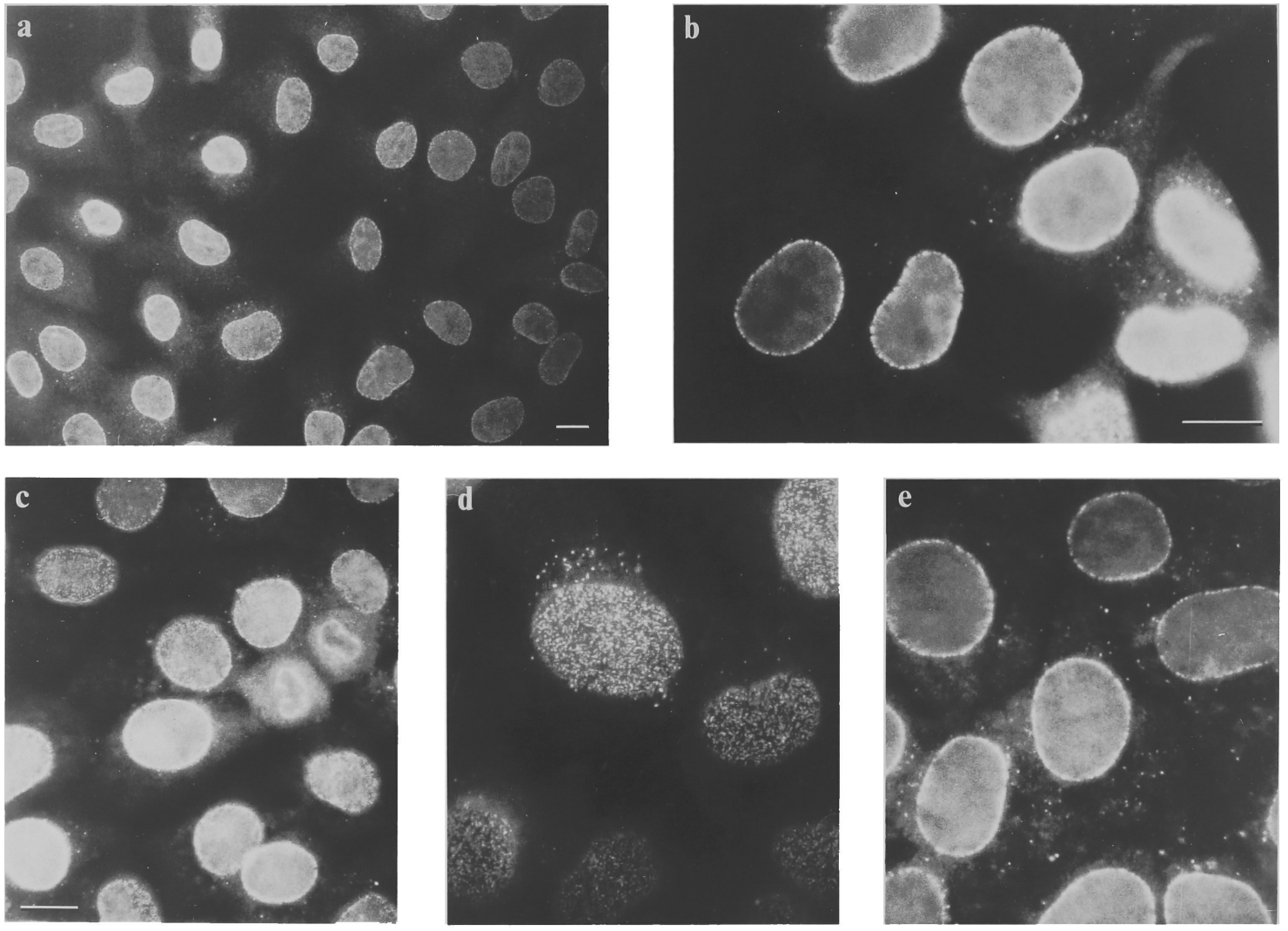

a
b
c
d
e

Figure 4

Immunofluorescence microscopy of BRL tissue culture cells using mAb 414

BRL tissue culture cell monolayers were either fixed with formaldehyde/methanol (a and b), or extracted *in situ* with Triton X-100 and DNase, without fixation (c-e, see section *2.5*). Panel e was further extracted with 1 *M* NaCl. Monolayers were then incubated with mAb 414 followed by FITC conjugated goat anti-mouse IgG. Bars = 10 um. Magnification in panels d-e are identical to that in panel b.


a
b
c
d
e

3.3 Immunoelectron microscopy

The punctate perinuclear staining led us to believe that p62 might be localized to the pore complex. To confirm this, we performed immunoelectron microscopy on thin sections of monolayers of isolated rat liver nuclei. These were first treated with 2% Triton X-100 to permeabilize the nuclei. This treatment extracts the lipids of both envelope membranes, but leaves the pore complexes attached to the nucleus (Blobel and Potter, 1966; Aaronson and Blobel, 1974). Moreover, particles observed to decorate the cytoplasmically exposed rings of the pore complex, and presumed to represent ribosomes rather than integral components of the pore complex, are removed by this treatment (Unwin and Milligan, 1982).

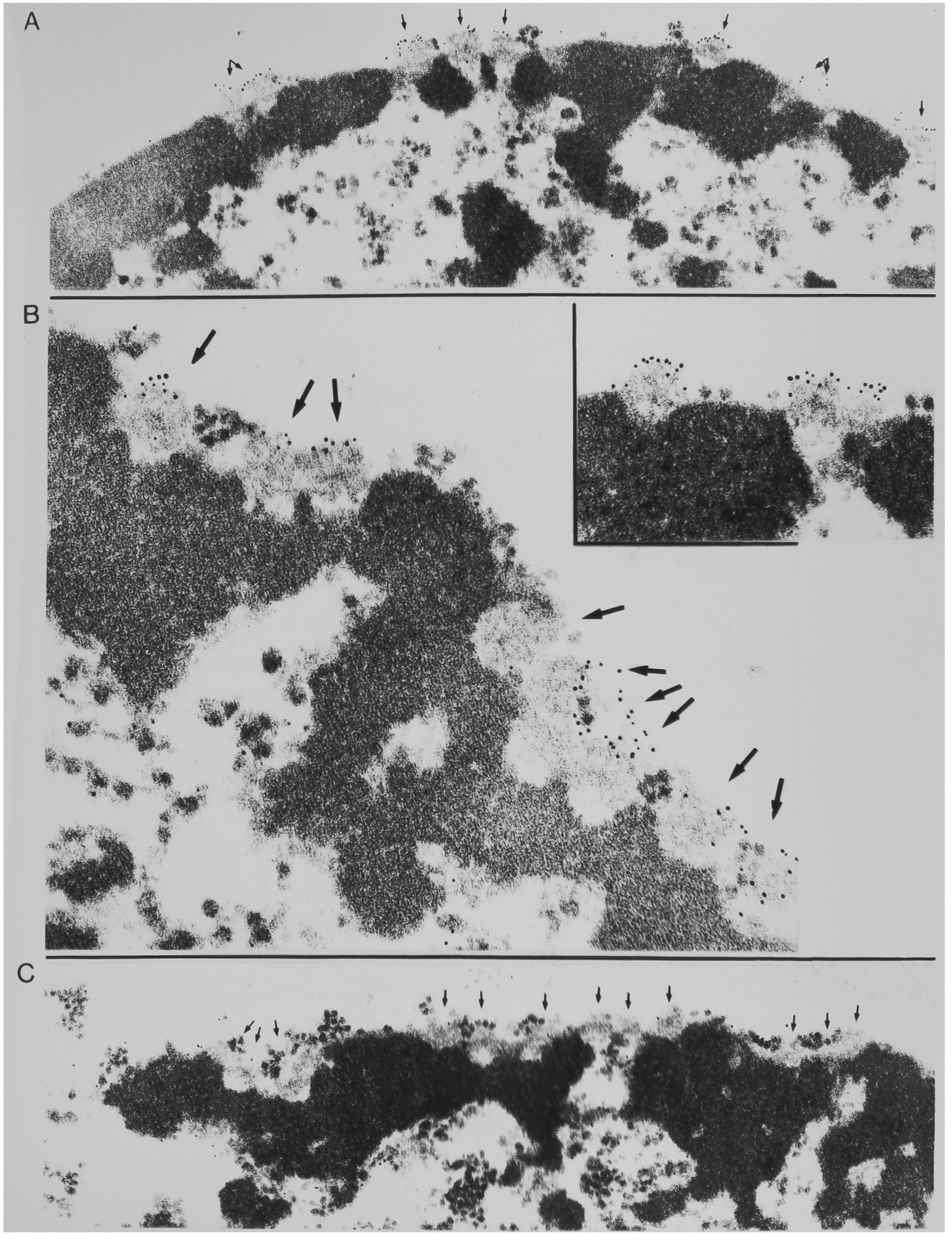
Detergent treated nuclei were lightly fixed with 0.05% glutaraldehyde and incubated, first with mAb 414, and then with a second antibody adsorbed to 5 nm colloidal gold particles. After fixation with 2.5% glutaraldehyde, nuclei were embedded, sectioned and post-stained with uranyl acetate and lead citrate (Gerace *et al.*, 1982). The gold particles were localized overwhelmingly to the pore complexes, with no staining of the interpore region representing the exposed lamina, and very few grains over the nucleoplasmic region (Fig 5a,b). Incubation with second antibody alone resulted in virtually no staining (Fig 5c). Generally, many gold particles were seen widely spaced at each pore, indicating that more than one antigen molecule is present per pore. This is consistent with the possibility that the antigen recognized by mAb 414 is found in at least one copy in each of the eight subunits, not all of which would be exposed in each section. However, definitive localization within the substructures of the pore is not possible at this resolution.

As observed with antisera raised against the 190 kD glycoprotein (Gerace *et al.*, 1982), staining appeared to be predominantly on the outer face of the pore complex; this may indicate that the antigen is localized exclusively to

Figure 5

Immunogold electron microscopy of isolated rat liver nuclei using mAb 414

Rat liver nuclei were treated with 2% Triton X-100 before fixation with 0.05% glutaraldehyde. They were then incubated either with mAb 414 (panels a and b), or without first antibody (panel c), and then with a second antibody adsorbed to 5 nm colloidal gold particles. Processing of samples is described in section ***2.6***. Arrows point to representative gold-labelled pore complexes.


A
B
C

this region, or may simply reflect a difference in accessibility between the cytoplasmic and nuclear annuli, perhaps due to the interaction of the lamina and/or chromatin with the nuclear annulus.

The antigenic site recognized by mAb 414 appears to be evolutionarily conserved. When indirect immunofluorescence microscopy was performed on cell lines from *Xenopus* (Fig 6a) or *Drosophila* (Fig 6b), mAb 414 gave a punctate nuclear rim staining pattern identical to that seen in mammalian cells. Thus, mAb 414 crossreacts with a nuclear envelope (presumably pore complex) protein in species evolutionarily quite distant from each other, indicating that the antigen may be a fundamental constituent of the pore complex.

3.4 Immunoblotting of subnuclear fractions

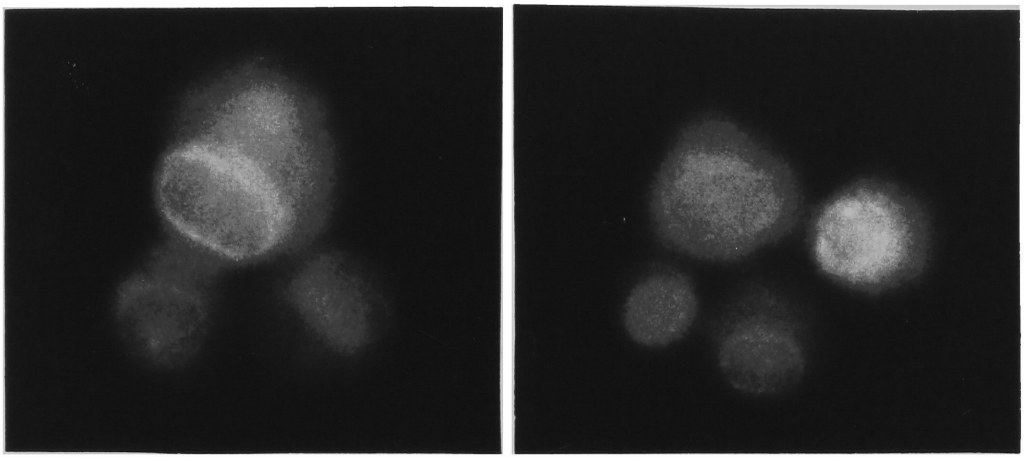
We next wanted to identify the protein(s) to which mAb 414 was binding. To this end, rat liver nuclei were fractionated by a previously described (Dwyer and Blobel, 1976) and slightly modified (see section ***2.2***) protocol that ultimately yielded an insoluble fraction containing pore complexes in association with the lamina. In this procedure, nuclei were treated with DNase and RNase two times, each at a different pH and nuclease concentration, and then either with 2% Triton X-100, 140 m*M* NaCl, or 0.5 *M* NaCl. Samples of unfractionated nuclei and of supernatant (S) and pellet (P) fractions at various steps were analyzed by SDS-PAGE. Fig 7a displays the Coomassie Blue-stained gel and Fig 7b shows the corresponding nitrocellulose blot probed with mAb 414. In whole nuclei (Fig 7b, lane 2), mAb 414 recognized a single polypeptide of molecular weight 62 kD (p62). In agreement with earlier data (Dwyer and Blobel, 1976), the bulk of nuclear protein was extracted by the two nuclease digestion steps (Fig 7a, lanes 3-6). P62, on the other hand, remained in the pellet containing the nuclear envelopes (Fig 7b, lanes 4 and 6). Interestingly, as more nuclear equivalents were loaded, another

Figure 6

Indirect immunofluorescence on Xenopus and Drosophila tissue culture cells using mAb 414

Drosophila melanogaster (panel a) or *Xenopus laevis* A6 (panel b) tissue culture cells were fixed with formaldehyde and permeabilized with methanol before incubation, first with mAb 414, and subsequently with FITC-goat anti-mouse IgG (see section ***2.5***).

a



b

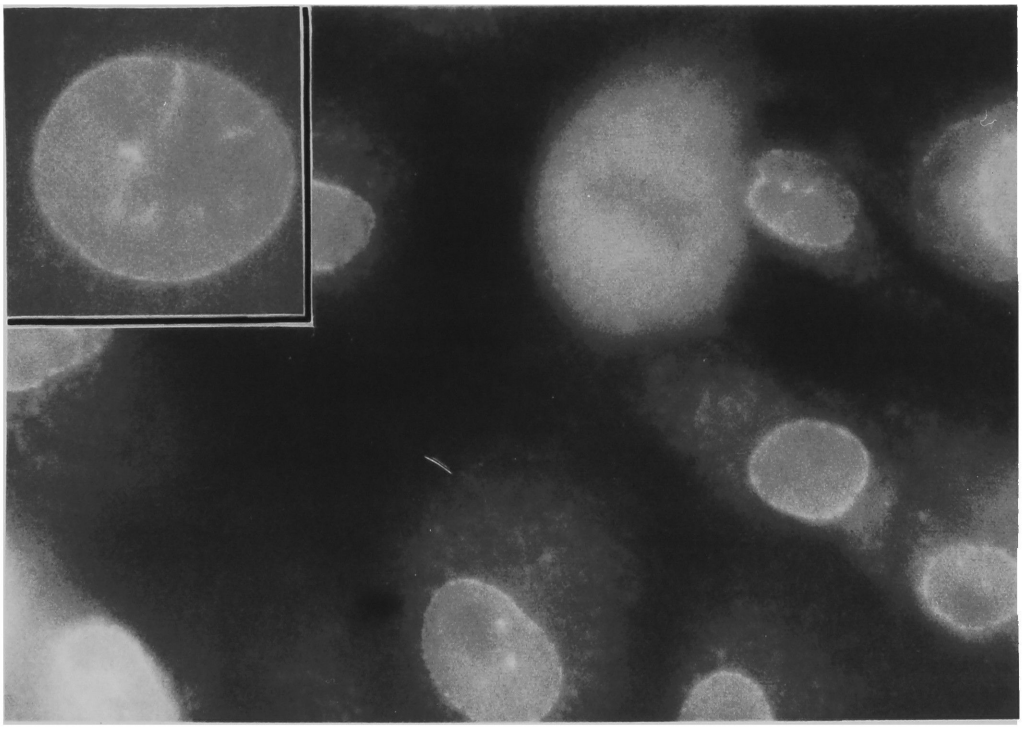
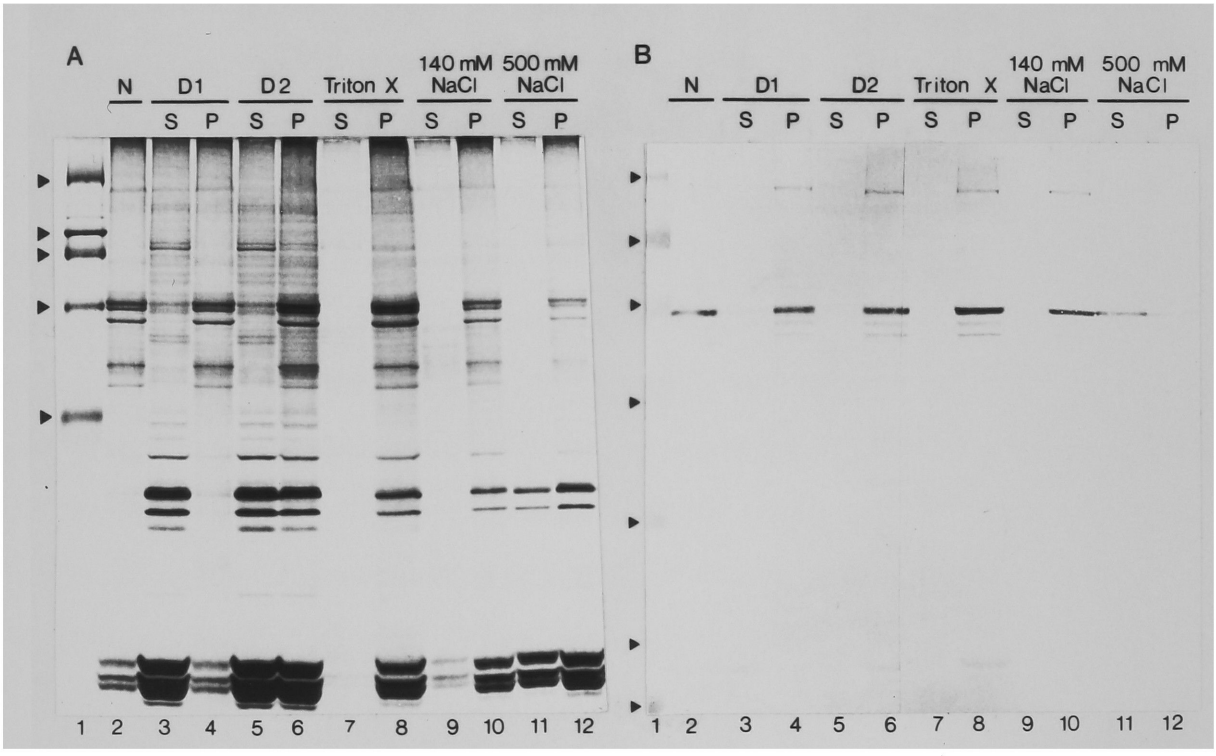


Figure 7

Localization of p62 in subfractions of isolated rat liver nuclei

Isolated rat liver nuclei were subfractionated as described in section ***2.2***, except that the $MgCl_2$ concentration was maintained at 5 m*M* throughout the fractionation, and aliquots of 20,000 xg_{av} supernatants (S) and pellets (P) subjected to SDS-PAGE on 10 -15% acrylamide gradient gels. Gels were either stained with Coomassie Blue (panel A), or blotted onto nitrocellulose and probed with mAb 414 (panel B). Lane 2; whole nuclei (2 eq). Lanes 3 and 4; first DNase/RNase digestion (2 eq). Lanes 5 and 6; second DNase/RNase digestion (4 eq). Lanes 7 and 8; extraction with Triton X-100. Lanes 9 and 10; extraction with 140 m*M* NaCl. Lanes 11 and 12; extraction with 500 m*M* NaCl, (all 8 eq). Note that salt extractions were performed on DNase treated envelopes that had not been treated with Triton X-100. Panel A, lane 1 contains molecular weight markers as follows: Myosin, 200 kD;β-Galactosidase, 116.25 kD; Phosphorylase B, 92.5 kD; Bovine serum albumin, 68 kD; and Ovalbumin, 43 kD. Panel B, lane 1 contains molecular weight markers as follows: Myosin, 200 kD; Phosphorylase B, 92.5 kD; Bovine Serum Albumin, 68 kD; Ovalbumin, 43 kD;α-Chymotrypsinogen, 25.7 kD; -Lactoglobulin, 18.4 kD; and Lysosyme, 14.3 kD


A
N
D1
D2
Triton X
140 mM NaCl
500 mM NaCl
S P S P S P S P S P
1 2 3 4 5 6 7 8 9 10 11 12
B
N
D1
D2
Triton X
140 mM NaCl
500 mM NaCl
S P S P S P S P S P
1 2 3 4 5 6 7 8 9 10 11 12

polypeptide at ~175 kD could be discerned that was also recognized by mAb 414. This apparently less abundant protein probably shares a determinant with p62, (see chapter 5). Minor bands just below p62 are variably present, and may be degradation products. P62 and p175 also remained in the pellet [the crude pore complex-lamina (PCL) fraction] after Triton X-100 extraction (Figure 7b, lane 8). However, if nuclear envelopes were instead extracted with high salt, the interaction of p62 with this fraction was destabilized. At 0.5 *M* NaCl about half of p62 was extracted (Fig 7b, lanes 11 and 12).

During the subfractionation displayed in Fig 7, the $MgCl_2$ concentration was maintained at 1 m*M*, instead of 0.1 m*M* as was used for that shown in Fig 2.. Under these conditions, chromatin folding is more stable, and the digestions with DNase are not as efficient. This is evidenced by the fact that large amounts of histone are still present after DNase digestion (Fig 7a, lane 6). We found that the association of p62 with the PCL fraction appeared to depend on whether the DNase digestions were performed at high or low magnesium concentration. If the $MgCl_2$ concentration was lowered to 0.1 m*M* throughout the fractionation, all of the p62 was extracted at 1 *M* NaCl (Fig 8, lanes 4 and 5). On the other hand, if a $MgCl_2$ concentration of 5 m*M* was used throughout, only half of the molecules were extracted at 1 *M* NaCl (Fig 8, lanes 2 and 3). Raising the $MgCl_2$ concentration after the DNase digestion could not prevent the subsequent extraction by salt of all of the p62. It is not clear whether being exposed to low $MgCl_2$ concentrations at any time has a direct effect on p62 susceptibility to subsequent salt extraction, or whether in fact the efficient removal of chromatin that occurs at low magnesium concentration leads to a loss of some component to which p62 was bound in a salt resistant manner.

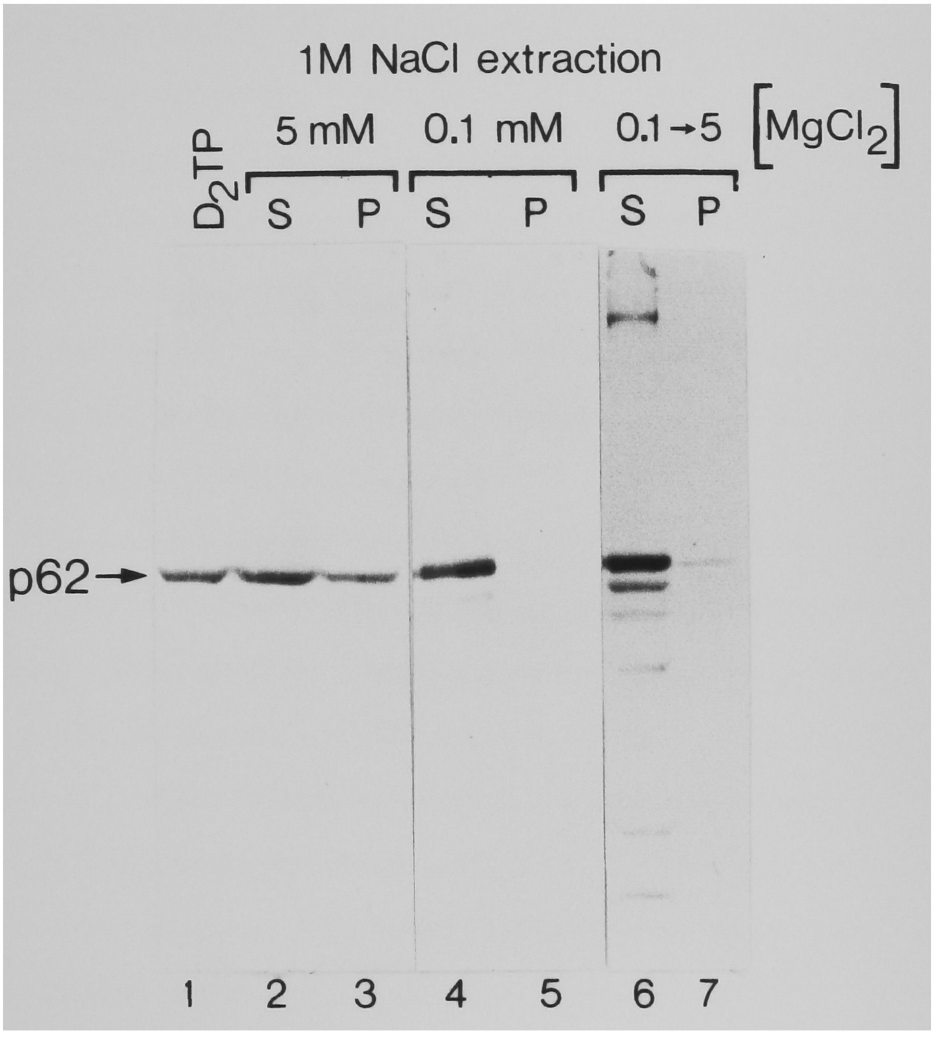
3.5 Discussion

In this chapter, I have discussed the identification of a novel protein(s) of the

Figure 8

Effect of $MgCl_2$ concentration on the sensitivity of p62 to high salt extraction

Isolated rat liver nuclei were subfractionated to yield DNase treated, Triton X-100 extracted nuclear envelopes (lane 1), and then further extracted with 1 *M* NaCl (lanes 2-7), as described in section ***2.2***. Five equivalents of each sample were subjected to SDS-PAGE, after which the gel was blotted to nitrocellulose and probed with mAb 414. The $MgCl_2$ concentration was either maintained at 5 m*M* (lanes 2 and 3) or 0.1 m*M* (lanes 4 and 5) throughout the fractionation, or increased from 0.1 m*M* to 5 m*M* after DNase digestion (lanes 6 and 7). P62 is indicated by the arrow.


1M NaCl extraction
D2TP
5 mM
0.1 mM
0.1→5
[MgCl2]
S
P
S
P
S
P
p62→
1
2
3
4
5
6
7

of the nuclear pore complex. Identification was accomplished with a monoclonal antibody raised against Triton X-100 treated rat liver nuclei. This monoclonal antibody (mAb 414) reacted predominantly with a 62 kD protein (p62) of a nuclear subfraction containing pore complexes associated with the lamina and gave rise to strikingly punctate staining of the nuclear periphery by immunofluorescence microscopy. P62 was localized specifically to the pore complexes of Triton X-100 treated nuclei by immunoelectron microscopy.

The limited resolution of immunoelectron microscopy did not allow us to localize p62 to any of the distinct substructures of the pore complex. However, the decoration of each pore complex by at least two widely spaced gold particles suggests that p62 occurs in more than one copy per pore complex. One could therefore speculate that p62 is a component of either the rings or the spokes, which each appear to be constituted of eight identical subunits (Unwin and Milligan, 1982).

The demonstration that the distinctive punctate staining of the nuclear periphery detected at the level of immunofluorescence microscopy corresponded to an immunogold decoration exclusively of the pore complexes is of practical significance. It indicates that screening for monoclonal antibodies directed against other pore complex proteins can be done routinely by immunofluorescence microscopy. A punctate staining of the nuclear periphery of the type shown in Fig 4 would suggest that an antigen was located in the pore complex. However, definitive localization would still require immunoelectron microscopy, as punctate immunofluorescence may also indicate reactivity with other clustered structures in the nuclear periphery.

Our nuclear subfractionation studies have shown that p62 is part of the crude pore complex-lamina fraction. The fact that p62 can be extracted with salt but not with nonionic detergent indicates that it is a peripheral protein associated with the pore complex through ionic interactions. Curiously, only about half of the p62 molecules were extracted at 0.5 *M* salt. However, at 5 m*M* $MgCl_2$, the other half was not extracted,

even at 1 *M* salt. If p62 were in fact a constitutive protein of the rings or spokes, the extraction of only half of the p62 molecules could be explained by postulating that only the annulus facing the cytoplasm, or a component thereof, is sensitive to salt extraction. In contrast, the nuclear annulus might remain attached to the lamina and/or chromatin in a linkage resistant to extraction with high concentrations of monovalent ions. The consequences of salt extraction on the ultrastructural integrity of the pore complex have not yet been sufficiently and systematically documented to argue for or against such a possibility.

Chapter 4

Biosynthesis of p62 and its behavior during mitosis

To date, no biochemical studies have addressed the mechanism by which pore complexes are assembled. Two potentially different assembly processes must take place in all cells. The first is the apparently reversible disassembly of all NPC's during mitosis. As discussed in section ***1.4***, the fate of the NPC during mitosis is not known, but there are no ultrastructurally intact pore complexes in metaphase cells. There also appears to be a concerted assembly of new NPC's just before entry into S phase of the cell cycle (Maul *et al.*, 1972; Fry, 1976). The latter probably contributes to the two-fold increase in NPC's per nucleus that occurs during interphase, as the surface area of the nucleus is roughly doubled. Some turnover of pore complexes may also occur independent of the stage of the cell cycle.

One could imagine numerous ways that assembly of new envelope could occur. For example, the nuclear membrane could "grow" through recruitment of the endoplasmic reticular membranes continuous with the outer nuclear envelope. This mechanism would not require a fusion event. Alternatively, nuclear envelope components could be "preassembled" in the cytoplasm and then be inserted into the nuclear envelope through fusion. Indeed, the cytoplasm of oocytes (which contain large stores of material for subsequent cell division) as well as some rapidly dividing cells, contain annulate lammelae, which could well represent precursors to nuclear envelope. Either of the above mechanisms accommodate numerous possible ways in which pore complexes themselves could be formed. For example, the entire pore, or some part of it, may be preassembled in the cytoplasm and somehow inserted into the membrane. It is

perhaps more likely, however, that pore complex assembly occurs only within the context of the membrane. One possibility is that assembly is triggered through aggregation of integral membrane receptors both within each bilayer and across the two membranes, causing fusion of the inner and outer envelopes. This could then trigger ionic interaction of cytoplasmic pore components, some or all of which may be preassembled into macromolecular subunits. Whatever the mechanism, it is likely that the peripheral components of the pore are assembled from cytoplasmic precursors, some of which might be expected to exist as macromolecular complexes.

4.1 The behavior of p62 during mitosis

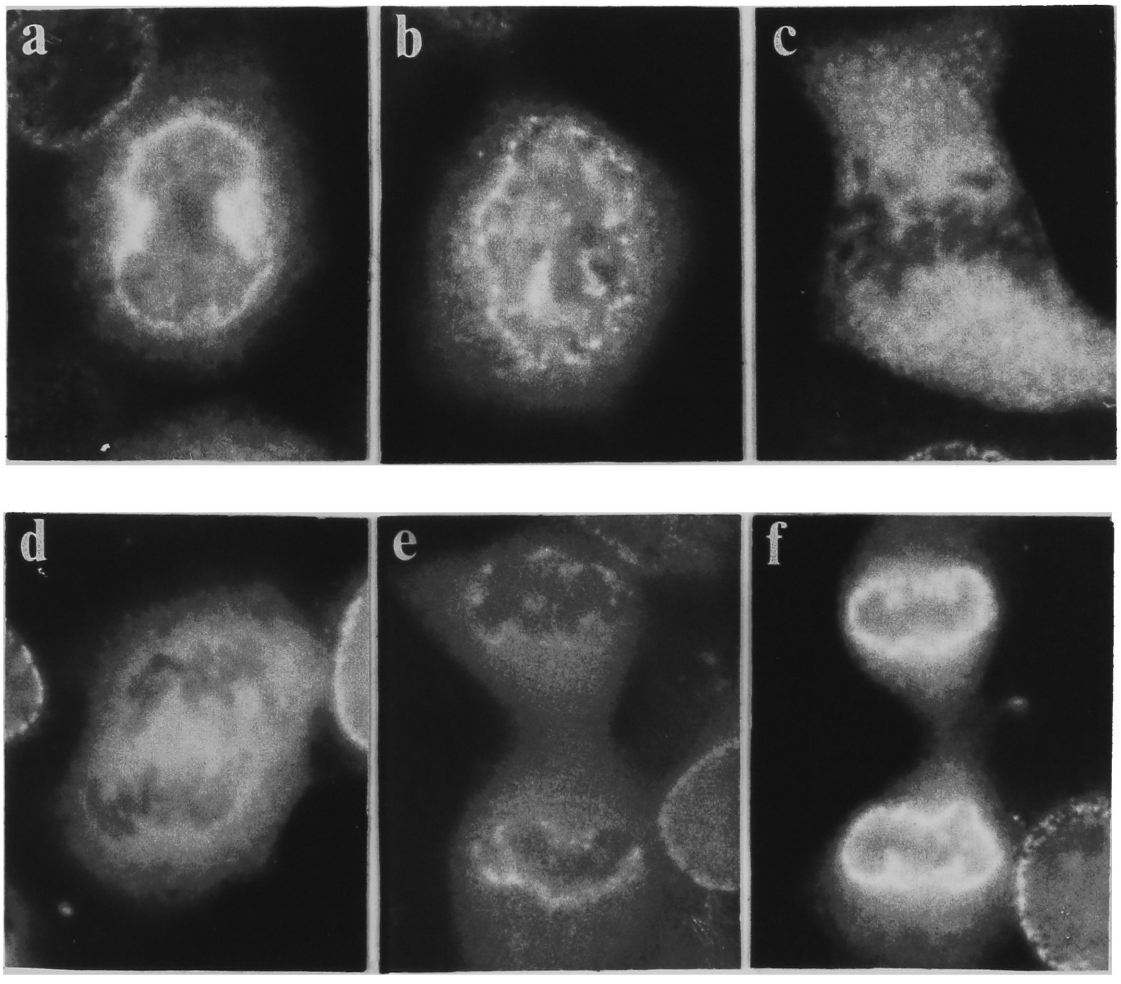
To look at what happens to p62 during mitosis, I examined the distribution of p62 by indirect immunofluorescence using BRL cell monolayers (Fig 9). Mitotic cells exhibited dramatic changes in the cellular localization of p62, which are analogous to the pattern of changes that were reported for the lamins (Gerace *et al.*, 1978, Krohne *et al.*, 1978) and for gp190 (Gerace *et al.*, 1982). During prophase (Fig 9a and b), the perinuclear staining became increasingly patchy, concomitant with an increased staining of the cytoplasm, presumably the result of gradual disassembly of the nuclear envelope into membrane vesicles. At metaphase (Fig 9c), staining became diffuse throughout the cell with no detectable staining of the chromatin. No further change in pattern occurred during anaphase (Fig 9d). During these stages, staining was almost completely abolished if cells were extracted *in situ* with Triton X-100 and DNase (data not shown). At telophase (Figure 9e and f), the process was reversed, with patchy staining observed around decondensing daughter chromosomes first, which gradually became more continuous as the chromatin decondensed.

This behavior is roughly similar to that of gp190 (an integral membrane protein located at the pore complex) and of the lamins, indicating that these pore

Figure 9

Immunofluorescence localization of p62 during mitosis

Buffalo rat liver tissue culture cell monolayers were fixed with 2% formaldehyde and permeabilized with methanol. They were then incubated with mAb 414 and subsequently with an FITC-conjugated goat anti-mouse IgG. Panels a-f represent: (a) early prophase; (b) late prophase; (c) metaphase; (d) anaphase; (e) early telophase; (f) late telophase.


a
b
c
d
e
f

complex constituents disassemble concomitant with vesiculation of the envelope and depolymerization of the lamina.

4.2 A soluble cytoplasmic precursor of p62

To look at the biosynthesis of p62, we labelled BRL cells for 16 hr with ^{35}S-methionine, and then fractionated them into postmitochondrial supernatant and pellet fractions. These samples were SDS solubilized and immunoprecipitated with mAb 414 (Fig 10, lanes 1 and 2). As expected, a polypeptide of ~62 kD was detected in the pellet fraction. However, a significant amount of a 62 kD polypeptide was also found in the postmitochondrial supernatant. This was not due to contamination of this fraction by nuclear material, or to the presence of disassembled p62 from mitotic cells, since immunoprecipitation with anti-lamin antibodies showed that the lamins were quantitatively recovered in the pellet fraction (Fig 10, lanes 3 and 4). Moreover, the supernatant form migrated slightly faster than the p62 in the pellet fraction.

To determine whether the supernatant form is a biosynthetic precursor of p62, we carried out a pulse-chase analysis. Exponentially growing BRL cells were pulse-labelled for 30 min with ^{35}S-methionine and chased for periods of up to 20 hr (Fig 11). There did indeed appear to be a precursor-product relationship between the soluble and nuclear components. Conversion of the soluble into the particulate form was, however, a very slow process: at chase periods of 6 hr only about half of the soluble form was converted to the particulate form (Fig 11, lanes 7 and 8). With increasing chase time the particulate form migrated progressively more slowly, indicating that it undergoes modification after incorporation into pore complexes (Fig 11). At chase periods of 10 hr, the precursor form had almost completely disappeared from the cytoplasm; however, at this time a small amount of the larger form began to appear in the postmitochondrial supernatant fraction (Fig 11, lanes 11, 13 and 15). This would be consistent with the idea

Figure 10

Immunoprecipitation of lamins and p62 from steady-state labelled BRL cells

BRL cells labelled for 16 hr with ^{35}S-methionine were subjected to hypotonic lysis, and centrifuged to yield postmitochondrial supernatant (S) and pellet (P) fractions, (see section *2.7*). After boiling in buffer containing 0.4% SDS, samples were immunoprecipitated with either mAb 414 (lanes 1 and 2), or anti-lamin antisera (lanes 3 and 4), and the immunoprecipitates were electrophoresed on a 10% polyacrylamide gel. The labelled proteins were visualized by fluorography. Arrows to the right of lane 4 indicate the three lamin proteins. Molecular weight markers, indicated by arrowheads, are as follows: Myosin, 200 kD; β-Galactosidase, 116.25 kD; Phosphorylase B, 92.5 kD; Bovine serum albumin, 68 kD; and Ovalbumin, 43 kD.

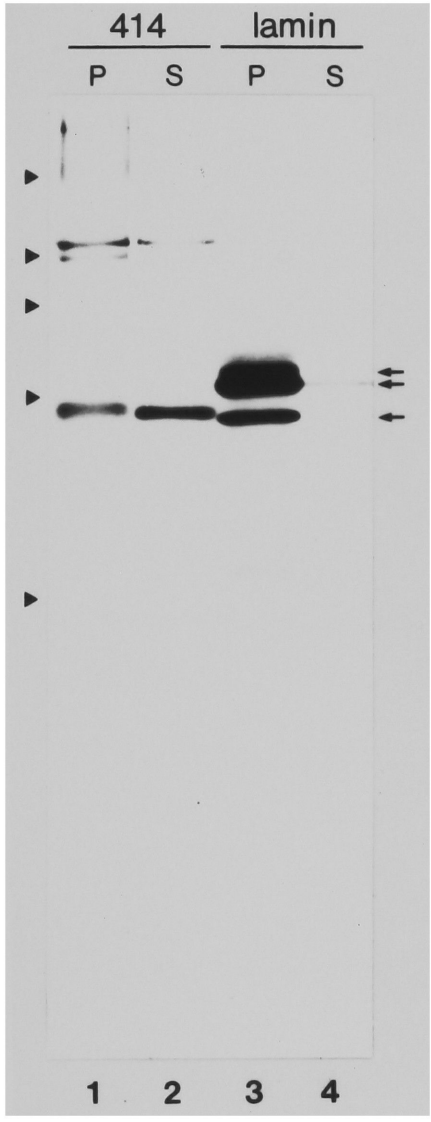
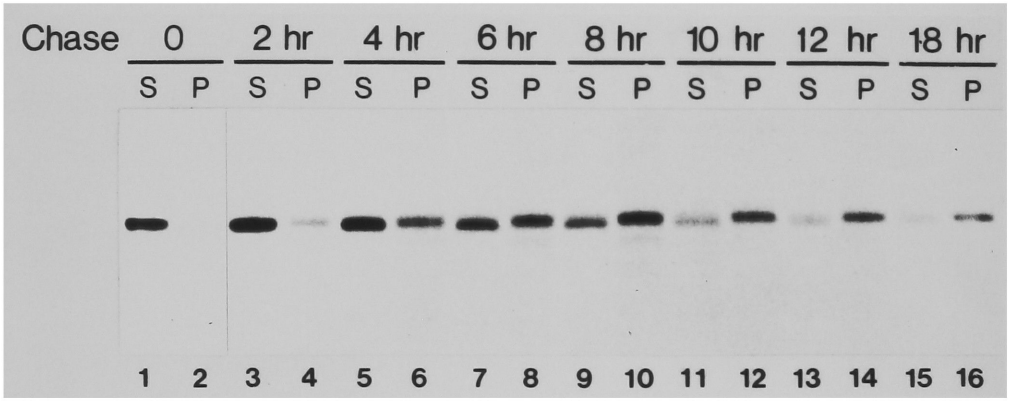

414
lamin
P
S
P
S
1
2
3
4

Figure 11

Pulse-chase analysis of p62 processing and incorporation into the nuclear fraction

BRL cells were labelled for 30 min with ^{35}S-methionine and chased for the indicated periods of time. Cells were then fractionated (see section ***2.7***) and postmitochondrial supernatants (S) and pellets (P) were boiled in SDS and immunoprecipitated with mAb 414. The immunoprecipitates were subjected to SDS-PAGE, and the labelled products visualized by fluorography.


Chase
0
2 hr
4 hr
6 hr
8 hr
10 hr
12 hr
18 hr
S P S P S P S P S P S P S P S P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

that incorporation of the precursor into the pore complex had been completed, and that mitotic disassembly of pore complexes containing labelled p62 had begun to occur, leading to release of "mature" p62 into the cytoplasm.

We next asked whether the supernatant form was indeed monomeric, or whether it might be part of a macromolecular pore precursor; either cytoplasmic or associated with cell membranes such as annulate lammelae or ER, both of which would fractionate with the crude postmitochondrial supernatant. To this end, an aliquot of this fraction from steady state-labelled BRL cells was analyzed by sucrose gradient centrifugation and fractions from the gradient, as well as the pellet fraction, were SDS solubilized and immunoprecipitated with mAb 414. All of the precursor was found to sediment at about 4S (Fig 12), a value that would be expected of a monomeric globular protein of that size.

4.3 p62 and its precursor have identical isoelectric points

We next addressed the question of the change in electrophoretic mobility that accompanied incorporation of p62 into the pore complex. Since this change was very slight, and since lamin assembly and disassembly appears to be mediated through changes in phosphorylation, it was logical to ask whether phosphorylation might be responsible for the observed shift. Thus, the soluble cytoplasmic form and the sedimentable p62 (which we assume represents material incorporated into the nuclear pore complex) from steady state-labelled BRL cells were analyzed by 2-dimensional electrophoresis (Fig 13). Both supernatant (Fig 13a) and pellet (Fig 13b) forms were present predominantly as single isoforms and, when coelectrophoresed, these two species exactly comigrated (Fig 13c). In Fig 13d, the immunoprecipitate from the pellet fraction was electrophoresed together with material immunoprecipitated by anti-lamin antibodies. P62 has an isoelectric point of ~5.9, just slightly less acidic than

Figure 12

Sucrose gradient centrifugation of the post-mitochondrial supernatant

The post-mitochondrial supernatant from BRL cells labelled for 16 hr with ^{35}S-methionine was fractionated by centrifugation on a 5-20% sucrose gradient for 6 hr at 280,000 xg_{av}. Each gradient fraction, including the pellet, was SDS denatured and immunoprecipitated with mAb 414, and the immunoprecipitates were subjected to SDS-PAGE on 10% acrylamide gels. The labelled proteins were visualized by fluorography. The direction of sedimentation is from right to left. Marker proteins (β-galactosidase, 16S; myosin, 6.4S; bovine serum albumin, 4.3S; and ovalbumin, 3.6S) were analyzed in parallel and their peak positions are indicated.

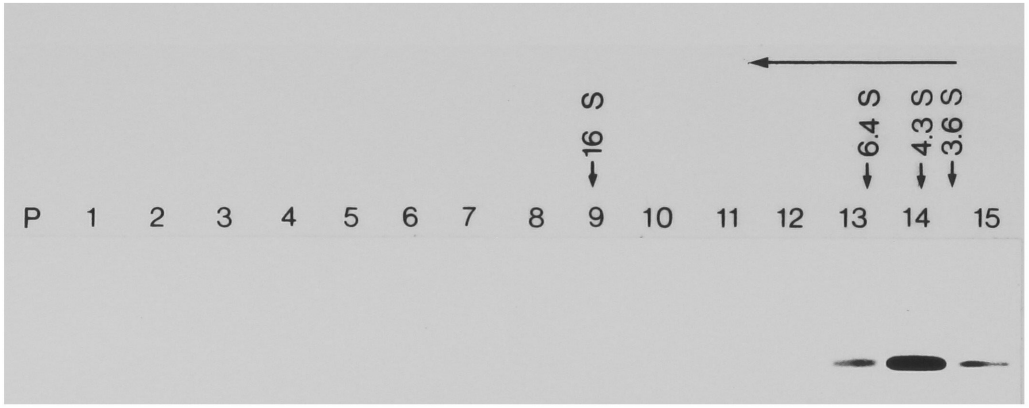
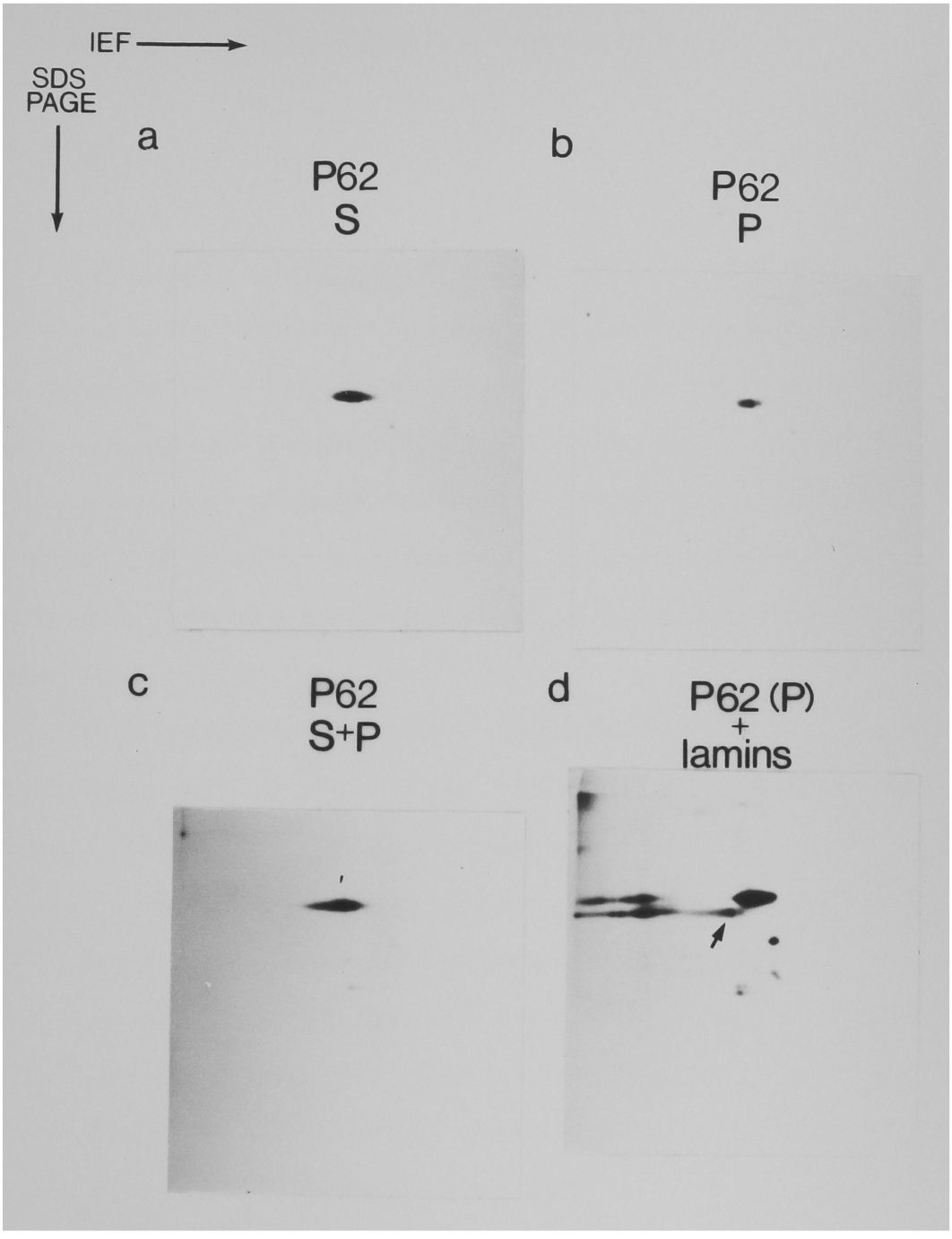

16 S
6.4 S
4.3 S
3.6 S
P 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 13

Two-dimensional gel electrophoresis of p62 immunoprecipitated from BRL cells

BRL cells labelled for 16 hr with ^{35}S-methionine were fractionated to yield a post-mitochondrial supernatant and pellet fractions (see section ***2.7***). Samples were solubilized in SDS/Triton X-100 wash buffer without boiling, sonicated briefly, and immunoprecipitated with mAb 414. The immunoadsorbed material was eluted from the Sepharose beads with 8 *M* urea containing 2% Triton X-100,β-mercaptoethanol, and pH 3.5-10 ampholines. The eluate was subjected to isoelectric focusing in the first dimension (O'Farrell *et al.*, 1977), followed by SDS-PAGE in the second dimension. Labelled proteins were visualized by fluorography. Panels a-c represent immunoprecipitates from postmitochondrial supernatant (panel a), pellet (panel b), or supernatant combined with pellet before electrophoresis (panel c). In panel d, material from the pellet fraction immunoprecipitated by mAb 414 was combined with material immunoprecipitated by anti-lamins A,B and C antisera prior to electrophoresis, to allow a direct comparison of their isoelectric points. The arrow in panel c points to p62


IEF
SDS
PAGE
a
P62
S
b
P62
P
c
P62
S+P
d
P62 (P)
+
lamins

that of lamin B. It appears, then, that the mobility difference between the soluble and particulate form of p62 is due to posttranslational modification(s) of the particulate form that affect its mass but not its charge.

4.4 Discussion

In this chapter, I have discussed the biosynthesis and assembly of p62, as well as its behavior during mitosis. I have followed the behavior of p62 during mitosis by immunofluorescence and have shown that, like the lamins and the gp190 glycoprotein, p62 becomes dispersed throughout the cytoplasm during mitosis. While it appears not to be associated with the chromosomes, it is not clear whether p62 is actually monodisperse or whether it might remain associated with other pore constituents. To answer this question, it will be necessary to perform sucrose gradient analysis of p62 from metaphase arrested cells.

P62 is synthesized as a soluble, cytoplasmic precursor, approximately 1,000 daltons lighter than the nuclear form. Incorporation of this precursor into the postmitochondrial pellet (presumably representing assembly into nuclear pore complexes) occurs over a surprisingly long time period: after a six hour chase only about half of the precursor molecule had been incorporated. In contrast, the half time for insertion of newly synthesized lamin A is five minutes, and that of lamins B and C is sixty minutes (Gerace *et al.*, 1984). Such kinetics of assembly may simply indicate that incorporation into the pore is very slow, perhaps because a large soluble pool is stored in the cell. Alternatively, if one were to assume that synthesis of p62 occurs throughout the cell cycle (which is about twelve hours in these cells), then the half time of six hours for incorporation of p62 into the pellet fraction may reflect assembly into the pore complex only during a specific phase of the cell cycle. This would be consistent with the electron microscopic studies of Maul *et al.* (1972) mentioned above, which found that pore

complexes roughly doubled in number at a specific point just prior to entry into S phase. Studies on synchronized cell populations will be necessary to further resolve this question.

One might have expected that p62 would have been assembled into a macromolecular pore "precursor" at some point during its residence in the cytosol prior to incorporation. However, since we found that the p62 in the cytosol was quantitatively recovered as a 4-5S particle after sucrose gradient sedimentation, we conclude that it probably remains soluble until incorporation into the nuclear pore complex, although we cannot rule out the possibility that a fleeting intermediate is present just before incorporation into the pore.

Chapter 5

P62 is glycosylated via a novel cellular pathway

In the last chapter I showed that p62 is synthesized as a soluble cytoplasmic precursor. This precursor is modified upon incorporation into the nuclear fraction (which we assume indicates assembly into the pore complex) such that its apparent molecular weight shifts up by about 500 daltons. This shift is not due to a charge altering modification, since both supernatant and pellet forms have identical isoelectric points.

One of the more common posttranslational modifications is glycosylation. A review of the literature showed that a nuclear protein of approximately the same molecular weight as p62 has been reported (Glass *et al.*, 1981) to bind to wheat germ agglutinin (WGA) but not to Concanavalin A (Con A), suggesting that this protein contains GlcNAc residues but no mannose or glucose. Modification by the addition of GlcNAc could be responsible for the increase in mass without charge alteration observed upon association of p62 with nuclei, and could therefore constitute the principle modification upon incorporation of p62 into pore complexes.

5.1 P62 binds to wheat germ agglutinin

To test this possibility, we affinity purified p62 from an SDS-solubilized pore complex-lamina fraction of rat liver nuclei using mAb 414 coupled to Sepharose (Fig 14). Triton X-100 treated nuclear envelopes (Fig 14, lane 1), were solubilized with buffer containing SDS and the sample was loaded onto a column consisting of mAb 414

Figure 14

Enrichment of p62 by affinity chromatography with mAb 414

Triton X-100 treated nuclear envelopes were prepared as described in section ***2.2***, and solubilized in buffer containing 2% SDS. After boiling, the sample (lane 1, 10 eq) was diluted into buffer containing Triton X-100 and loaded onto a column consisting of mAb 414 covalently coupled to CNBr-Sepharose (see section ***2.12***). The flowthrough was collected (lane 2, 10 eq) and the column was washed with 50 column volumes of load buffer (lane 3, 10 eq). The column was then eluted with 50% ethylene glycol, pH 11 (lane 4, 100 eq). Panel a: Coomassie Blue stained gel. Panel b: Protein blot of fractions, probed with mAb 414 (see section ***2.4***). Solid arrows in panel a indicate molecular weight markers as follows: myosin, 200 kD; β-galactosidase, 116.25 kD; phosphorylase B, 92.5 kD; bovine serum albumin, 68 kD; and ovalbumin, 43 kD. Open arrows indicate antibody molecules that bled from the affinity resin.

a
LOAD
FLOWTHROUGH
WASH (1M NaCl)
ELUATE
1
2
3
4
b
LOAD
FLOWTHROUGH
WASH (1M NaCl)
ELUATE
p175
p62
1
2
3
4

covalently coupled to CNBr-Sepharose. The flowthrough was collected (lane 2) and the column was washed with 50 column volumes of buffer (lane 3). The column was eluted with 50% ethylene glycol, pH 11 (lane 4). P62 and p175 were quantitatively recovered in the eluate (Fig 14b, lane 4). Thus, although not completely pure (as can be seen in Fig 14a, lane 4, the eluate probably contains some lamin proteins that bound nonspecifically), the eluate was highly enriched in p62. This material was subjected to SDS-PAGE, and the gel was blotted onto nitrocellulose. The blot was then probed with biotinylated WGA in the presence or absence of 200 m*M* GlcNAc (a competitive inhibitor) (Fig 15). In the absence of GlcNAc (Fig 15, lane 1), WGA bound to a polypeptide that exactly comigrates with p62 (Fig 15, lane 3), and which is also highly enriched in the affinity eluate, as WGA binding to this polypeptide on blots of crude rat liver nuclear envelopes containing equivalent amounts of material was barely detectable (data not shown). This binding was specific, because it was completely inhibited by the addition of GlcNAc. Probing with horseradish peroxidase conjugated Con A revealed no specific binding (data not shown), consistent with data of Glass *et al.* (1981), who find that the 62 kD WGA-binding protein does not bind to Con A.

To provide additional evidence that p62 is indeed glycosylated, as well as to gain more information as to the type of sugar and its disposition, we performed a number of further experiments.

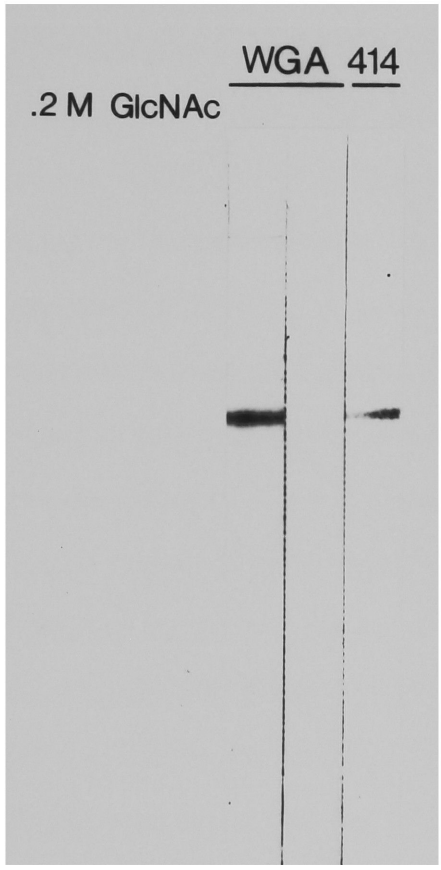
5.2 Both forms of p62 contain terminal GlcNAc residues

We first looked at the susceptibility of p62 to digestion with glycosidases. To this end, BRL tissue culture cells were incubated with ^{35}S-methionine for 16 hr before lysis by homogenization in hypotonic buffer. The homogenate was subjected to one differential centrifugation to prepare pellet and postmitochondrial supernatant fractions.

Figure 15

Protein blots of affinity purified p62 probed with WGA

P62 was purified by affinity chromatography with mAb 414-Sepharose, resolved by SDS-PAGE on preparative 10% gels (50 eq/cm gel width), and blotted onto nitrocellulose. Blots were probed with biotinylated WGA in the absence (lane 1) or presence (lane 2) of 0.2 *M* GlcNAc, or with mAb 414 (lane 3). Blots were then incubated either with an avidin-biotin-peroxidase conjugate (lanes 1 and 2), or a goat anti-mouse IGg conjugated to phosphatase (lane 3), after which strips were assayed for enzyme activity (see section ***2.11***).


WGA 414
.2 M GlcNAc

Both fractions were then immunoprecipitated with mAb 414 and the immunoprecipitates were digested with either of two different glycosidases (Fig 16). The undigested forms of p62 in the supernatant (Fig 16, lane 1) and the pellet (Fig 16, lane 2) fractions showed the expected 500 dalton difference in mobility. Therefore, if susceptible to a given glycosidase, we would expect that digestion would result in an increase in the mobility of the pellet fraction corresponding to a loss of ~500 daltons. We found instead that treatment of these fractions with N-Acetylglucosaminidase (GlcNAcase), an exoglycosidase which cleaves at terminal GlcNAc residues, reduced the apparent molecular weight of the pellet fraction by ~3 kD (Fig 16, lane 4). Moreover, the p62 in the supernatant was also reduced in mass, in this case by ~2.5 kD (Fig 16, lane 3), migrating with an M_r identical to that in the pellet fraction. The mobility difference between the treated and untreated forms of p62 was more clearly evident when the two were mixed prior to SDS-PAGE analysis (Fig 16, lanes 7-8). That the shift in mobility was due to the removal of GlcNAc by the enzyme and not, for example, to proteolytic cleavage by a contaminating protease was shown by inclusion of 0.5 *M* GlcNAc in the digestion mixture to inhibit the GlcNAcase activity (Fig 16, lanes 5 and 6). We therefore conclude that both the supernatant and the pellet fraction contain p62 that is modified by GlcNAc residues, the pellet form containing more GlcNAc than the supernatant form.

We also digested immunoprecipitates of p62 from both fractions with endo H [which cleaves between GlcNAc residues of high-mannose containing core oligosaccharides (Tarantino and Maley, 1974)]. As would be expected from its lack of reactivity with Con A, p62 was not digested by endo H (Fig 16, lanes 9 and 10) and therefore does not appear to contain N-linked high mannose oligosaccharides.

To provide further evidence that both forms of p62 contain GlcNAc residues, we labelled BRL cells with ^{3}H-glucosamine for 12 hr, and fractionated them into postmitochondrial supernatant and pellet fractions before immunoprecipitation with mAb

Figure 16

Digestion of p62 with glycosidases

BRL tissue culture cells were labelled with ^{35}S-methionine for 12 hr, and fractionated into postmitochondrial supernatant (lanes 1,3,5,7,9) and pellet (lanes 2,4,6,8,10) fractions, which were then immunoprecipitated with mAb 414 (see section ***2.7***). The washed immunoprecipitates were then either left untreated (lanes 1 and 2, arrow indicates the position of undigested p62), digested with GlcNAcase in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of 250 m*M* GlcNAc, or digested with endo H (lanes 9 and 10). In lanes 7 and 8, samples treated with GlcNAcase were electrophoresed in the same lane as untreated samples to show the difference in molecular weight more clearly. Labelled polypeptides were visualized by fluorography. Digestion conditions are described in section ***2.13***.

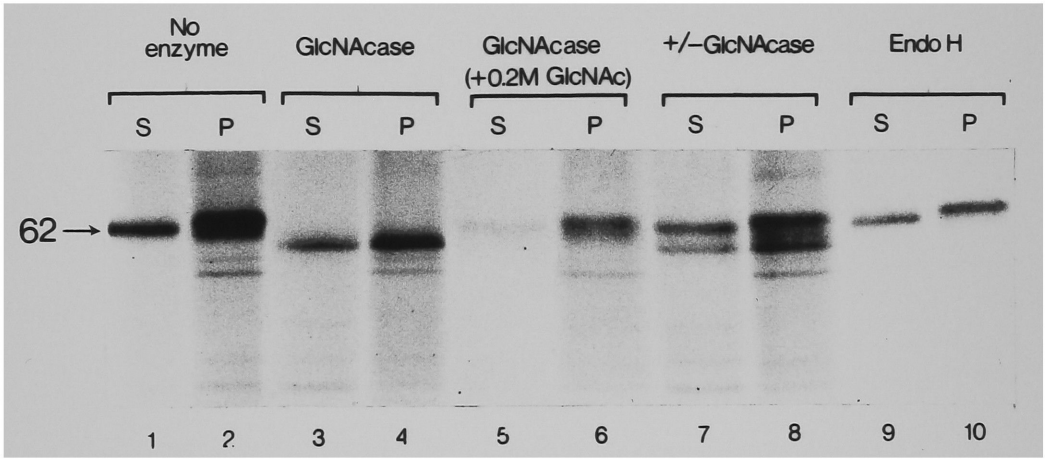
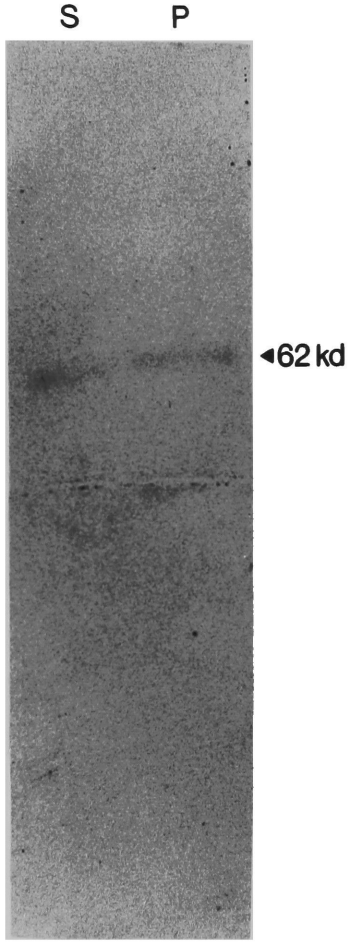

No enzyme
GlcNAcase
GlcNAcase (+0.2M GlcNAc)
+/−GlcNAcase
Endo H
S
P
S
P
S
P
S
P
S
P
62 →
1
2
3
4
5
6
7
8
9
10

Figure 17

In vivo incorporation of ^{3}H-glucosamine into p62.

BRL tissue culture cells were labelled for 12 hr with ^{3}H-glucosamine, after which they were fractionated into postmitochondrial supernatant (lane 1) and pellet (lane 2) fractions (see section ***2.7***). Samples were then solubilized with buffer containing 0.4% SDS, and immunoprecipitated with mAb 414 (see section ***2.7***). Immunoprecipitates were subjected to SDS-PAGE, and the labelled proteins visualized by fluorgraphy.


S
P
62 kd

414 (Fig 17). As expected, labelled p62 was immunoprecipitated from both supernatant (Fig 17, lane 1) and pellet (Fig 17, lane 2) fractions, indicating that both of these species contain GlcNAc residues.

5.3 The GlcNAc residues are not part of N-linked oligosaccharides

The insensitivity of p62 to endo H digestion suggested that it does not contain N-linked oligosaccharides; however it was still possible that it does contain such residues, but that they are highly trimmed. As the addition of N-linked oligosaccharides in the RER is mediated by dolichol phosphate, we could test whether this pathway is necessary for the glycosylation of p62 by blocking it with tunicamycin, an analog of GlcNAc that inhibits the formation of the lipid linked oligosaccharide (Takatsuki *et al.*, 1971, 1975; Tkacz and Lampen, 1975).

BRL cells were treated with tunicamycin prior to and during labelling with ^{35}S-methionine, and p62 was again immunoprecipitated from the postmitochondrial supernatant and pellet fractions (Fig 18). There was no difference in electrophoretic mobility between p62 from untreated (Fig 18, lanes 1 and 2) and from tunicamycin treated (Fig 18, lanes 3 and 4) cells. Moreover, p62 from tunicamycin treated cells was as sensitive to GlcNAcase treatment (Fig 18, lanes 5 and 6) as was that from untreated cells (Fig 18, lanes 7 and 8). Control samples precipitated with Con A-Sepharose showed that inhibition of synthesis of N-linked high-mannose glycans by tunicamycin was essentially complete (not shown).

Taken together, these data suggested that steady-state labelled p62 contains terminal GlcNAc residues that are not part of N-linked oligosaccharides.

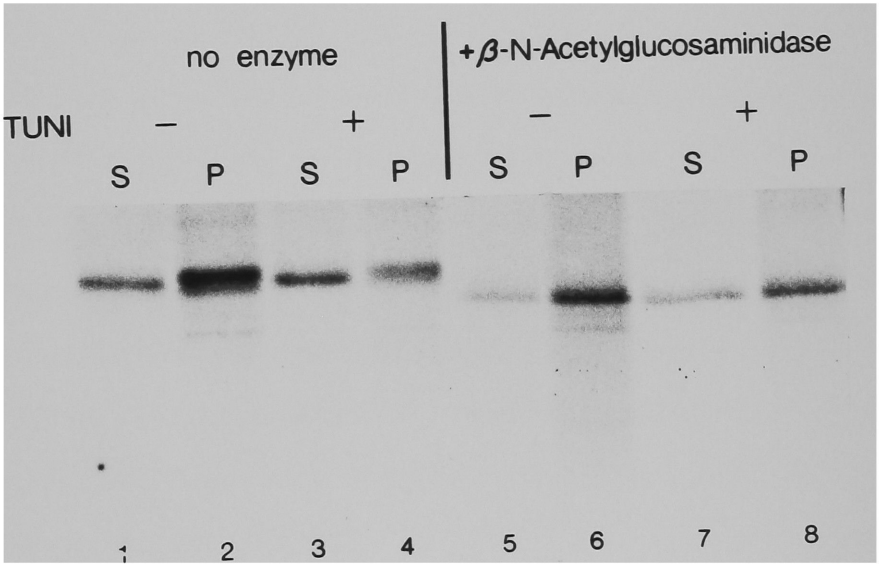
5.4 The timing of sugar addition to p62

In previously performed pulse-chase experiments we had "pulse" labelled cells for 90

Figure 18

Immunoprecipitation of p62 from tunicamycin treated cells.

BRL tissue culture cells were grown to subconfluency on one 60 mm^2 Petri dish and incubated with 1 ug/ml tunicamycin for 4 hr prior to labelling for 12 hr with 40 uCi/ml ^{35}S-methionine in the presence of tunicamycin (lanes 3, 4, 7, 8). A control dish was labelled in the absence of tunicamycin (lanes 1, 2, 5, 6). After homogenization in hypotonic buffer, cells were fractionated into postmitochondrial supernatant (S) and pellet (P) fractions. After boiling in SDS, samples were immunoprecipitated with mAb 414 (see section ***2.7***). Half of each immunoprecipitate was digested with GlcNAcase before SDS-PAGE (lanes 5-8, see section ***2.13***). Labelled polypeptides were visualized by fluorography.

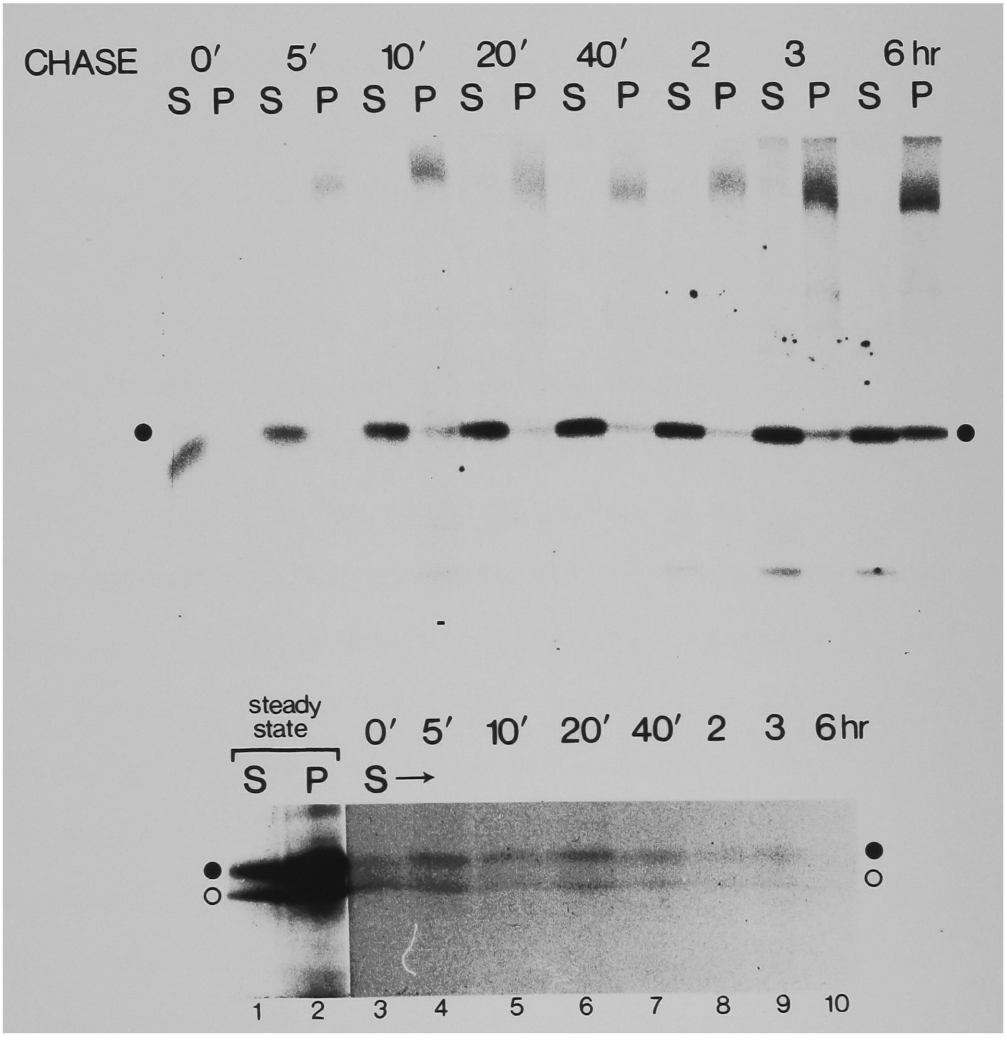

no enzyme
+β-N-Acetylglucosaminidase
TUNI
–
+
–
+
S
P
S
P
S
P
S
P
1
2
3
4
5
6
7
8

min followed by various chase periods (see Fig 11). These experiments showed that the increase in mass upon conversion of the cytosolic form of p62 to its nuclear form was accompanied by a shift of only about 500 daltons. Thus, since the total amount of sugar added to p62 amounted to a shift of 2500 daltons, the bulk of the sugar must have been added within 30 min of synthesis, since it was already present at 0 chase. To more precisely establish the time of addition of the bulk of GlcNAc to p62, we repeated the pulse-chase experiment but reduced the pulse with ^{35}S-methionine to 5 min (Fig 19). We also centrifuged the initial homogenate so as to sediment microsomes. P62 present in the pellet and postmicrosomal supernatant fractions was immunoprecipitated with mAb 414 (Fig 19a). In Fig 19b, half of the immunoprecipitate was treated with GlcNAcase and the treated and untreated samples were co-electrophoresed, in order to display the mobility differences and to estimate the amount of GlcNAc that had been added to p62 at each time point. Even at the earliest time point (0 chase), p62 appeared already to have received the bulk of its GlcNAc residues (Fig 19b, lane 3). There was no detectable unglycosylated p62 even at this point (Fig 19a, lanes 1 and 2). However, in these lanes the bands corresponding to p62 were lighter and more diffuse than at later points, indicating that glycosylation was probably not yet complete. Thus we conclude that the bulk of the GlcNAc residues are added within 5 min after translation, and perhaps even cotranslationally. The fact that all of the newly synthesized p62 at early time points was found in the postmicrosomal supernatant (Fig 19a), suggested that p62 is synthesized as a soluble cytosolic protein which is at no time associated with microsomal or Golgi membranes, and receives the bulk of its sugar complement while it is in this form. A small amount, however, appears to be added much later, after incorporation of p62 into the pore complex (Fig 11, and Fig 19a, lanes 15 and 16).

Figure 19

Short term pulse-chase labelling of BRL cells

BRL tissue culture cells were labelled for 5 min with ^{35}S-methionine and chased for the indicated periods of time with medium containing cold methionine (see section ***2.8***). The cells were fractionated into postmicrosomal supernatant (S) and pellet (P) fractions, which were then immunoprecipitated with mAb 414. In panel b, half of the immunoprecipitate from each supernatant fraction was treated with GlcNAcase (open circle, see section ***2.13***) and recombined with the corresponding untreated sample (closed circle) prior to SDS-PAGE (Panel b, lanes 3-11). For comparison, immunoprecipitation was also performed on supernatant (panel b, lane 1) and pellet (panel b, lane 2) fractions of cells labelled for 12 hr. Labelled proteins were visualized by fluorography.


CHASE
0′
5′
10′
20′
40′
2
3
6 hr
S P S P S P S P S P S P S P S P
steady state
S P
0′ 5′ 10′ 20′ 40′ 2 3 6hr
S →
1 2 3 4 5 6 7 8 9 10

5.5 P62 contains O-linked monosaccharidic GlcNAc residues

Galactosyl transferase can transfer galactose from UDP-galactose to terminal GlcNAc residues (Scwyzer and Hill, 1977; Beyer *et al.*, 1981). Using UDP-^{3}H-galactose and characterizing the labelled saccharide after β elimination, Hart and coworkers (Torres and Hart, 1984, Holt and Hart, 1986) have identified a novel family of glycoproteins that contain O-linked, monosaccharidic GlcNAc residues. Representatives of this family of glycoproteins were found in virtually all cell fractions, with the exception of mitochondria. Interestingly, most of the label in the nuclear fraction was associated with a single protein of 63 kD. To determine whether this protein was in fact identical to p62, we incubated rat liver nuclei with UDP-^{3}H-galactose and galactosyl transferase, solubilized the nuclei with SDS, and then immunoprecipitated with mAb 414 (Fig 20). Labelled p62 was in fact immunoprecipitated by mAb 414. Thus, consistent with the results presented in Figs 16-19, p62 appears to be O-glycosylated by the addition of monosaccharidic GlcNAc residues. MAb 414 also immunoprecipitated two other polypeptides labelled by *in vitro* ^{3}H-galactosylation, of molecular weights 175 and 270 kD. The relationship between these porteins and p62 will be discussed in Chapter 6.

The number of GlcNAc residues per p62 molecule remains to be determined. Most of the sugar, amounting to a shift in M_r of ~2,500 daltons, is added within a period of 5 min, either during or shortly after translation (Fig 4). More GlcNAc residues, amounting to a shift in M_r of ~500 daltons, are added at a later time, apparently only after incorporation into the pore complex (Fig 4). Assuming an increase in mass of ~220 daltons per GlcNAc residue, we estimate that ~12 residues of GlcNAc are present on each molecule of p62.

Figure 20

Immunoprecipitation of in vitro ^{3}H-galactosylated rat liver nuclear proteins with mAb 414

Isolated rat liver nuclei (20 equivalents) were incubated with UDP-^{3}H-galactose in the presence of galactosyl transferase (see section ***2.12***). After incubating 30 min at 37°C, nuclei were washed and boiled in buffer containing 0.4% SDS, before precipitating with mAb 414. The immunoprecipitate was subjected to SDS-PAGE, and the labelled proteins visualized by fluorography.


270
175
P62

5.6 Discussion

Two major pathways have been described thus far for the addition of carbohydrate to protein (for review, see Kornfeld and Kornfeld, 1980; Hubbard and Ivatt, 1981). The first is characterized by the dolichyl phosphate-mediated addition of asparagine-linked core oligosaccharides to protein in the lumen of the RER. In this case, a precursor ($Glc_3Man_9GlcNAc_2$) is assembled on the dolichyl phosphate in a complex process involving flipping of the lipid-linked molecule across the RER membrane (Perez and Hirschberg, 1986), before addition *en bloc* to an asparagine residue of the polypeptide through GlcNAc. It is then trimmed and in some cases further modified by the addition of other sugar residues during transit through the RER and Golgi apparatus, to yield "complex" oligosaccharides. The other major pathway takes place exclusively in the Golgi apparatus and is responsible for the addition of sugars via O-linked GalNAc to serine and threonine. These glycans may contain galactose, fucose and sialic acid residues.

Numerous studies have shown that glycosylation takes place on the lumenal side of the RER and Golgi apparatus (Katz *et al.*, 1977; Boulan *et al.*, 1974; Lingappa *et al.*, 1978; Hanover *et al.*, 1980; Snider and Robbins, 1982). It has been presumed, therefore, that only proteins which have been synthesized on membrane bound ribosomes and translocated across the RER membrane, i.e. plasma membrane, secretory or lysosomal proteins, are glycosylated via these pathways.

There have been numerous reports concerning the presence of glycans containing GlcNAc, GalNAc, sialic acid, mannose and fucose, in various nuclear and subnuclear fractions (see Stoddard, 1979 for review). Most of these were concerned solely with membrane glycoproteins of the nuclear envelope. Since this structure is contiguous with the endoplasmic reticulum, it is not surprising that it contains numerous glycoproteins. However, it has also been claimed that some nonmembrane intranuclear proteins, such as

the chromosomal proteins HMG 14 and 17 (Reeves *et al.*, 1981), histones (Levy-Wilson, 1983), and various unidentified chromatin-binding proteins (Rizzo *et al.*, 1977; Yeoman *et al.*, 1978; Goldberg *et al.*, 1978) are glycosylated. In all of these cases, however, the possibility of contamination by other subcellular fractions, most notably ER, has cast doubt on the validity of these findings.

In another approach, numerous groups have attempted to sublocalize glycoprotein moieties within the nucleus using various lectins as probes, yielding somewhat conflicting results. Some investigators (Monneron and Segretain, 1974; Virtanen and Wartiovaara, 1976) have found that Con A binds exclusively to the cisternal faces of both inner and outer nuclear membranes, as would be expected if only those compartments that are contiguous with the ER lumen contain glycans with mannose residues. Others, however, claim to have found intranuclear binding sites for Con A (Roth, 1983; Seve *et al.*, 1984; Kan and da Silva, 1986) WGA (Seve *et al.*, 1984) and Ulex Europaeus I (which binds specifically to fucose residues) (Kan and da Silva, 1986), although these differed in terms of the regions within the nucleus to which the lectins bound. To our knowledge, our study represents the first instance in which an antibody has been used to show that specific nuclear proteins (in a compartment topologically equivalent to cytoplasm) contain sugar residues.

Our data concerning the biosynthesis of p62 are inconsistent with glycosylation by either of the pathways thus described above. First, the insensitivity of sugar addition to treatment with tunicamycin indicates that the sugar moiety on p62 is not an N-linked oligosaccharide. This is consistent with the results of recent studies by Holt and Hart (1986) showing that the sugar on the group of proteins of which p62 is a member is composed of monosaccharidic GlcNAc residues in O-linkages to serine or threonine. Both of these findings indicate that glycosylation of p62 is not associated with the ER, but do not rule out addition of GlcNAc in the Golgi apparatus. We believe this to be unlikely for several reasons. First, the only O-linkage shown thus far to occur in the

Golgi apparatus is through GalNAc (see Kornfeld and Kornfeld, 1980 for review); O-linkage through GlcNAc has never been detected. Second, although different proteins migrate through the ER to the Golgi at different rates, it is generally at least 10 min between synthesis of the protein and addition of O-linked sugars in the Golgi. In contrast, we showed that p62 synthesized during a 5 min pulse already contained most of its sugar complement, indicating that a significant amount of glycosylation had occurred during or shortly after synthesis. Furthermore, since p62 was found in the postmitochondrial supernatant even at very early time points, when glycosylation was probably not yet completed, it seems highly unlikely that sugar addition could be occurring in the Golgi apparatus. Perhaps the strongest evidence that glycosylation of p62 occurs through a novel cellular pathway concerns the topology of the protein. If glycosylation took place on the lumenal side of the Golgi apparatus, p62 would be required to pass through the membrane after being glycosylated, since it is destined to become a cytoplasmically disposed, pore complex component. Even if this were to occur, it could not account for the further addition of GlcNAc that occurs much later, upon incorporation of p62 into the nuclear fraction. The data are more consistent with the hypothesis that p62 acquires GlcNAc through a transferase that is either a soluble cytoplasmic enzyme or, if membrane bound, has its catalytic site exposed to the cytosol. This cellular pathway then, is topologically distinct from the glycosylation pathways involving ER and Golgi membranes.

Chapter 6

A family of GlcNAc containing proteins in the nucleus

6.1 Localization of the other GlcNAc containing proteins of the nucleus.

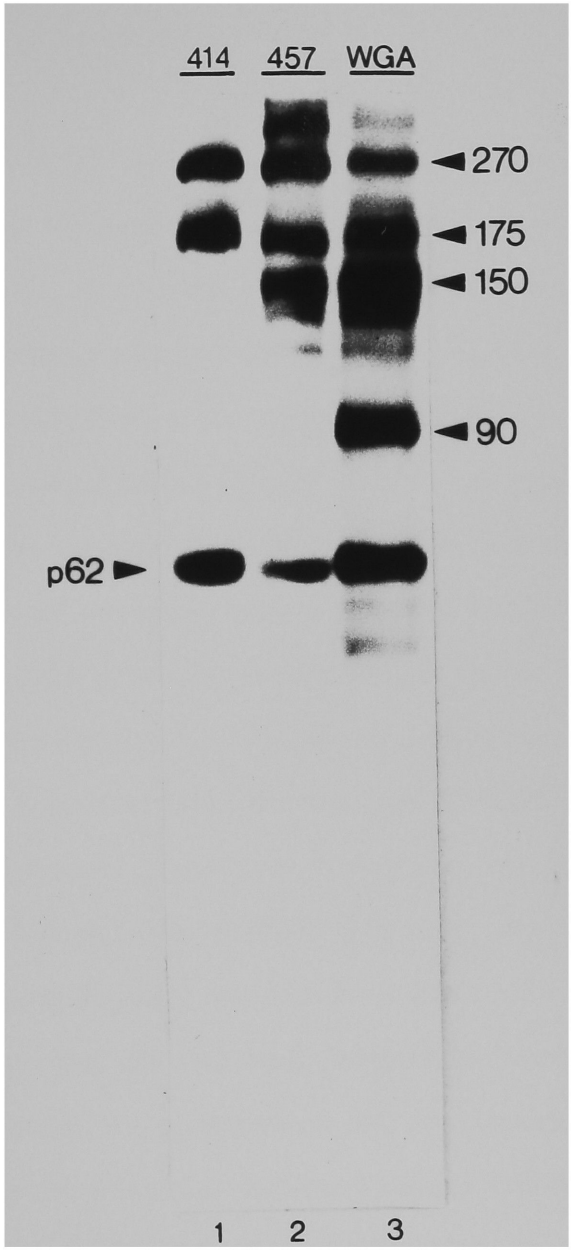
In the last chapter, I showed that mAb 414 immunoprecipitated labelled p62 from *in vitro* ^{3}H-galactosylated rat liver nuclei. Biochemical characterization of the sugars on the galactosylated products of such a reaction have indicated that they contain monosaccharidic GlcNAc residues in an O-linkage to serine and threonine (Holt and Hart, 1986). We also found that two other labelled proteins were immunoprecipitated by mAb 414 (see Fig 20).

The three polypeptides recognized by mAb 414 were not the only proteins susceptible to *in vitro* galactosylation. Fig 21 displays the proteins that were precipitated by two other probes, mAb 457 (Fig 21, lane 2), and WGA (Fig 21, lane 3). Since WGA binds to GlcNAc residues, it would be expected to recognize all of the galactosylated proteins. MAb 457 is a monoclonal antibody that was generated in the same fusion that gave rise to mAb 414. In addition to the three polypeptides precipitated by mAb 414 (Fig 21, lane 1), this antibody precipitated most of the labelled polypeptides recognized by WGA (Fig 21, compare lanes 2 and 3), albeit not always with the same apparent affinity. In the case of the 90 kD polypeptide, galactosylation apparently resulted in greatly diminished affinity for mAb 457 (Fig 21, lane 2), as the nongalactosylated species clearly reacted with mAb 457 when this antibody was used to probe protein blots of nuclei (see Fig 23). It is possible that galactosylation abolishes the antigenic site for mAb 457 on all of the reactive proteins, but that some of them are not completely galactosylated. We consider this unlikely because a time course experiment indicated

Figure 21

Immunoprecipitation of in vitro ^{3}H-galactosylated rat liver nuclear proteins WGA and mAb 457

Isolated rat liver nuclei (60 equivalents) were incubated with UDP-^{3}H-galactose in the presence of galactosyl transferase (see section ***2.12***). After incubating 30 min at 37°C, nuclei were washed and boiled in buffer containing 0.4% SDS, before precipitating with either mAb 414 (lane 1), mAb 457 (lane 2), or WGA (lane 3). After SDS-PAGE, the labelled proteins were visualized by fluorography.


414
457
WGA
270
175
150
90
p62
1
2
3

that no further sugars were added after 10 min of incubation with galactosyl transferase (data not shown), suggesting that the reaction had reached completion.

6.2 Immunofluorescence microscopy with mAb 457

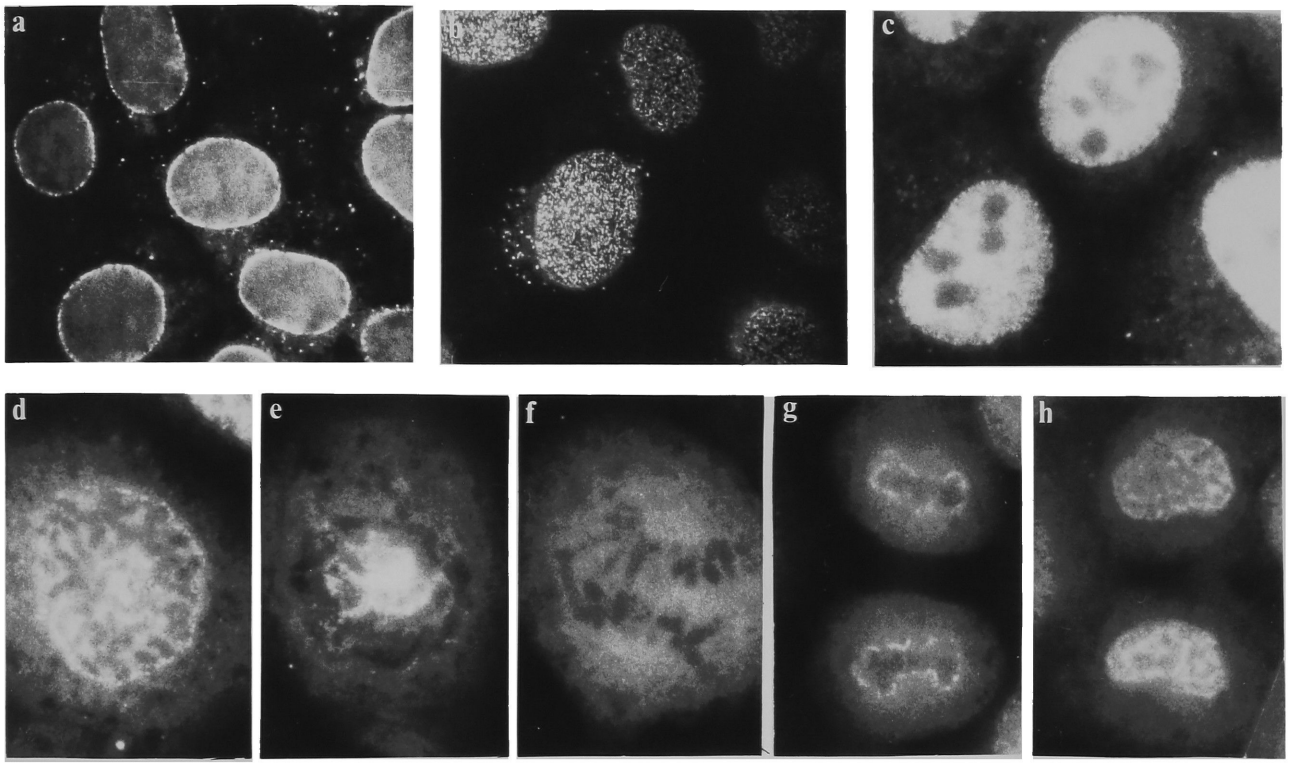
To investigate whether the additional galactosylated proteins reactive with mAb 457 were also located in the nuclear pore complex, we performed immunofluorescence microscopy on BRL cells using mAb 457 (Fig 22). Whereas mAb 414 yielded the previously observed characteristic rim staining pattern (Fig 22a), which could be seen to be finely punctate upon focusing at the top of the nucleus (Fig 22b), mAb 457 gave a granular intranuclear staining with a striking exclusion of staining from the nucleolar regions (Fig 22c). The expected punctate rim pattern was largely obscured by the intranuclear staining in the interphase nucleus (Fig 22c), but became clearly discernible in prophase cells (Fig 22d), as the intranuclear antigen became gradually dispersed. In anaphase (Fig 22f) and telophase (Fig 22g), the staining pattern obtained with mAb 457 was indistinguishable from that shown previously with mAb 414: diffuse cytoplasmic staining excluding condensed chromosomes in anaphase (Fig 22f) and punctate staining around decondensing chromatin in telophase (Fig 22g). As the chromosome masses expanded at late telophase (Fig 22h), irregular intranuclear staining reappeared.

Thus, it appears that the additional polypeptides reactive with mAb 457 are located within the nucleus during interphase and are excluded from the condensing chromatin during mitosis, when they become localized diffusely throughout the cell. Reassociation with the nuclear interior occurs concomitantly with expansion of the daughter chromosomes at telophase, and appears to occur after reformation of the pore complexes, since rim staining is apparent before intranuclear staining at early telophase (Fig 22g).

Figure 22

Indirect immunofluorescence labelling of BRL cells with mAb 457

BRL cells grown on cover slips were fixed with 2% formaldehyde and permeabilized with methanol (see section ***2.5***). The cells were incubated with either mAb 414 (a and b), or mAb 457 (c-h), and then with an FITC-conjugated goat anti-mouse IgG. Panels a-c show interphase cells while panels d-h show mitotic cells as follows: d and e, prophase; f, anaphase; g and h, telophase.


a
b
c
d
e
f
g
h

6.3 Protein blots of subnuclear fractions probed with mAb 457 and WGA

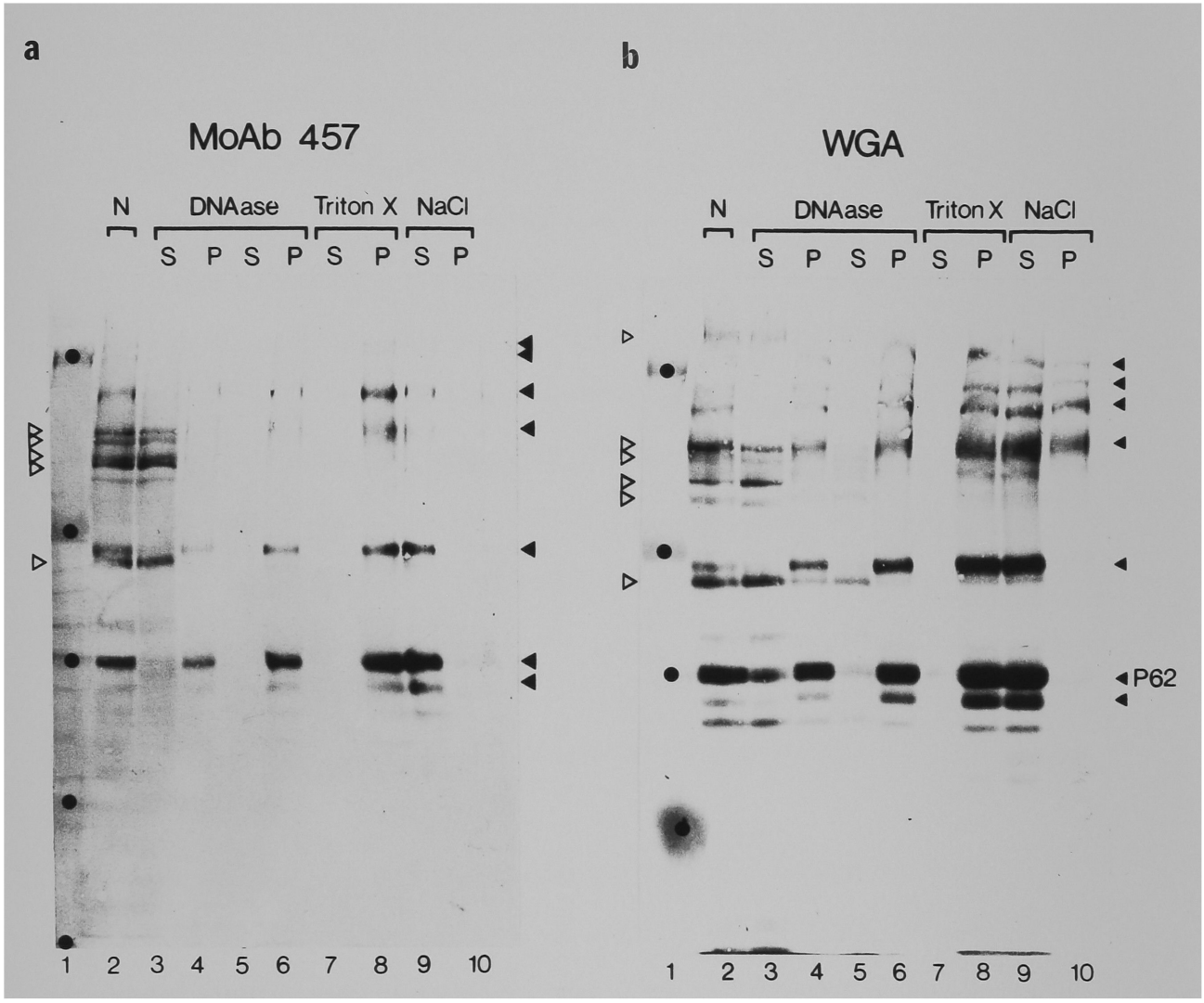
Proteins localized within the nucleus as opposed to the pore complex might be expected to be extracted during preparation of the pore complex-lamina fraction, since digestion with DNase I yields nuclear envelope ghosts devoid of intranuclear material (Dwyer and Blobel, 1976). Thus, to localize proteins reactive with mAb 457 and WGA to various subfractions of the nucleus, rat liver nuclei were extracted with DNase twice under different conditions, and then with Triton X-100 and 0.5 *M* NaCl (see section ***2.2***). The supernatant and pellet fractions resulting from each step were subjected to SDS-PAGE, after which proteins were transferred to nitrocellulose sheets. These were then probed with either mAb 457 (Fig 23a) or WGA (Fig 23b). Both the mAb 457 and the WGA profiles show that a discrete set of proteins was released during DNase digestion (open arrowheads), whereas another set was largely extracted with high salt, along with p62 (closed arrowheads). It is most likely that the set released upon DNase digestion is responsible for the intranuclear staining pattern detected by mAb 457. It is also likely that the other set, which includes the pore complex proteins recognized by mAb 414 (closed circles, data not shown) are all in the nuclear pore, since they fractionate with the nuclear envelope. Of course, proof for this will only be obtained when monospecific antibodies can be produced against each polypeptide.

It appears that mAb 457 has a specificity very similar to WGA, as it binds essentially the same group of polypeptides on protein blots (Fig 23, compare a and b). Note that the antibody reacted with the 90 kD polypeptide on protein blots even though it did not immunoprecipitate this polypeptide from ^{3}H-galactosylated nuclei. As mentioned, this is most likely due to the loss of the antigenic site for mAb 457 after addition of the labelled sugar residues.

Figure 23

Protein blots of subnuclear fractions probed with mAb 457 or WGA

Isolated rat liver nuclei were subfractionated as described in section ***2.2,*** and aliquots of 20,000 xg_{av} supernatant (S) and pellet (P) fractions. Samples were electrophoresed on 7% polyacrylamide gels, which were then blotted onto nitrocellulose and probed with either mAb 457 (panel a, see section ***2.4***) or biotinylated WGA (panel b, see section ***2.11***). Lane 2, whole nuclei (1 eq); lanes 3 and 4, first DNase digestion (1 eq); lanes 5 and 6, second DNase digestion (2 eq); lanes 7 and 8, Triton X-100 extraction (4 eq); lanes 9 and 10 (4 eq). Lane 1 contains prestained molecular weight markers indicated by filled circles: 200 kD (myosin), 97.4 kD (phosphorylase B), 68 kD (bovine serum albumin), 43 kD (ovalbumin) and 25.7 kD (α-chymotrypsinogen). Open triangles indicate those proteins extracted by nuclease digestion, whereas the closed triangles denote proteins fractionating with the pore complex-lamina.


a
MoAb 457
N
DNAase
Triton X
NaCl
S P S P S P S P
1 2 3 4 5 6 7 8 9 10
b
WGA
N
DNAase
Triton X
NaCl
S P S P S P S P
P62
1 2 3 4 5 6 7 8 9 10

6.4 Different determinants are recognized by each of the three probes

Because mAb 457 and WGA recognize the same set of nuclear proteins, it is likely that the GlcNAc residues form an important part, if not all, of the epitope recognized by mAb 457. Conversely, the narrower specificity of mAb 414 indicated that it might recognize protein epitopes common to only a subset of the GlcNAc-containing proteins, although it could also be specific for a particular configuration of sugars present only on some proteins. To investigate this question, rat liver nuclei were digested with GlcNAcase (see section 2.13), and then subjected to SDS-PAGE, after which the gel was transferred to nitrocellulose. The blot was probed with either mAb 414 (Fig 24, lanes 1 and 2), mAb 457 (Fig 24, lanes 3 an 4) or WGA (Fig 24, lanes 5 and 6). MAb 414 reacted with all of the digestion products of p62 (bracket, Fig 24, lane 2), indicating that this antibody does indeed recognize a determinant other than GlcNAc. On the other hand, mAb 457 and WGA reacted solely with p62 that had only a small amount of sugar removed.

6.5 One dimensional peptide mapping of mAb 414-binding proteins

Because mAb 414 still reacted after removal of the sugar residues, the shared epitope probably reflects related protein sequence in this case. To examine the relationship between these polypeptides, we performed Cleveland mapping of material immunoprecipitated by mAb 414. An immunoprecipitate from ^{3}H-galactose labelled rat liver nuclei was subjected to SDS-PAGE, after which the lane was excised and laid horizontally over a second SDS polyacrylamide gel. The gel slice was overlaid with *Staph aureus* V8 protease before reelectrophoresis essentially as described by Cleveland *et al.* (1977, see section ***2.15***). The result of this digestion is shown in Fig 25. The three polypeptides gave very distinct peptide maps, and in fact shared no peptides containing

Figure 24

Reactivity of WGA and mAbs 414 and 457 with N-Acetylglucosaminidase treated rat liver nuclei

Rat liver nuclei were incubated in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of GlcNAcase (see section ***2.13***), before SDS-PAGE. The gel was electroblotted onto nitrocellulose, which was then probed with mAb 414 (lanes 1 and 2) or 457 (lanes 3 and 4, see section ***2.4***), or with WGA (lanes 5 and 6, see section ***2.11***). Undigested p62 is indicated by the closed arrow; digested p62 by the bracket. Bands indicated by the open arrowheads indicate WGA-binding proteins present in the GlcNAcase preparation.

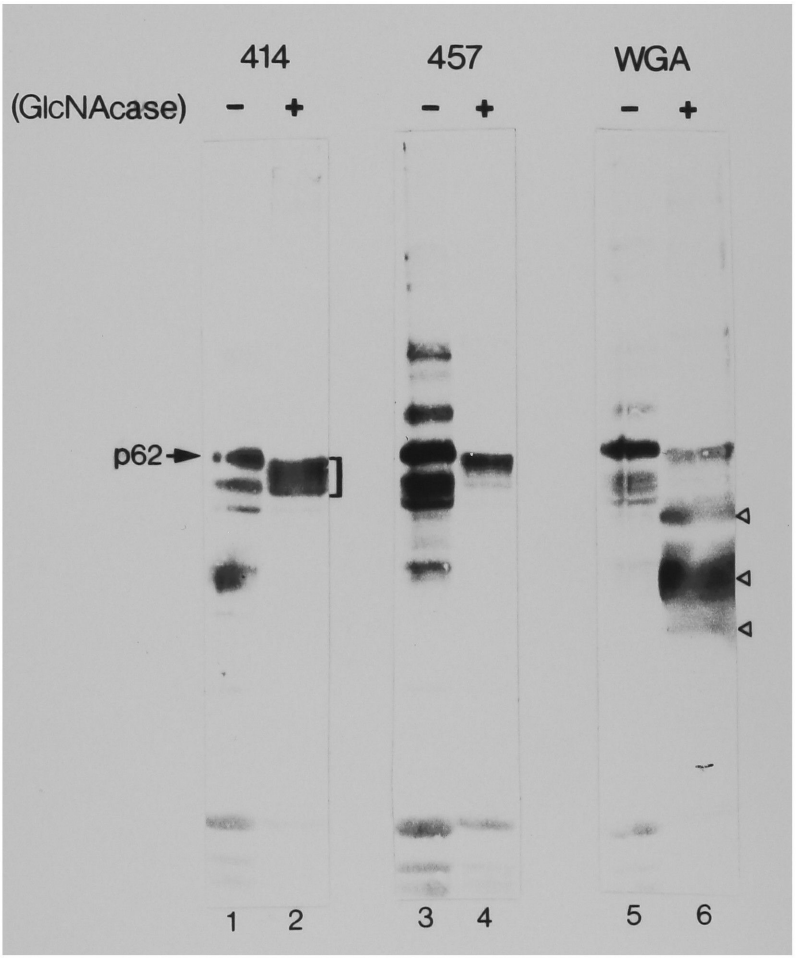
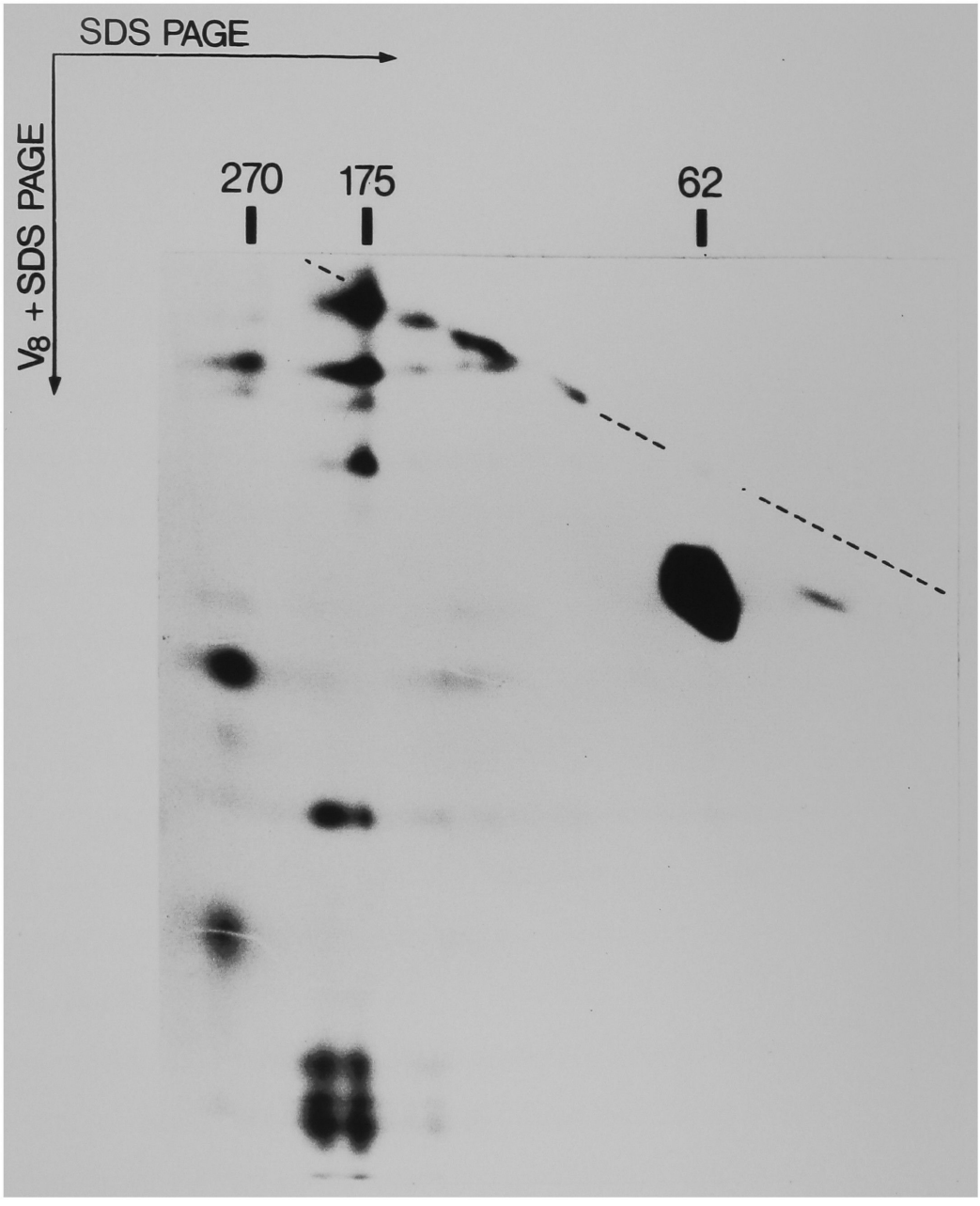

414
457
WGA
(GlcNAcase)
- + - + - +
p62
1 2 3 4 5 6

Figure 25

Protease mapping of proteins immunoprecipitated by mAb 414

Isolated rat liver nuclei were galactosylated *in vitro* by incubation with UDP-^{3}H-galactose in the presence of galactosyl transferase (see section ***2.12***), after which they were solubilized in buffer containing 0.4% SDS. The sample was then immunoprecipitated with mAb 414 (see section ***2.7***). The immunoprecipitate was electrophoresed on a 7% polyacrylamide gel, after which the lane was excised and laid horizontally over a 10-15% SDS-polyacrylamide gradient gel. Sample buffer containing *Staph aureus* V8 protease was layered over the gel slice, and the sample was reelectrophoresed. The polypeptides generating the fragments are indicated at the top by their molecular weight in the first dimension. The dotted line represents the diagonal upon which undigested material migrates in the second dimension.


SDS PAGE
V8 + SDS PAGE
270
175
62

labelled sugars. In addition, p62 was extremely resistant to proteolysis with V_8, yielding a single cleavage product. It is possible that it contains very few acidic residues, although its isoelectric point of ~5.9 (see Fig 13) makes that unlikely. Alternatively, it could have a very tightly folded conformation, perhaps due to its sugar content, that is resistant to denaturation with SDS.

6.6 WGA affinity chromatography

We were interested in isolating large amounts of p62 for further studies. However, because the affinity of mAb 414 for p62 is extremely high, it turned out not to be the optimum reagent to use for purification of p62, as evidenced by the extreme conditions required to elute the protein off the mAb 414 affinity column (see section ***5.1***). We thus turned to WGA-Sepharose as an affinity ligand, since we could presumably achieve elution merely by adding GlcNAc.

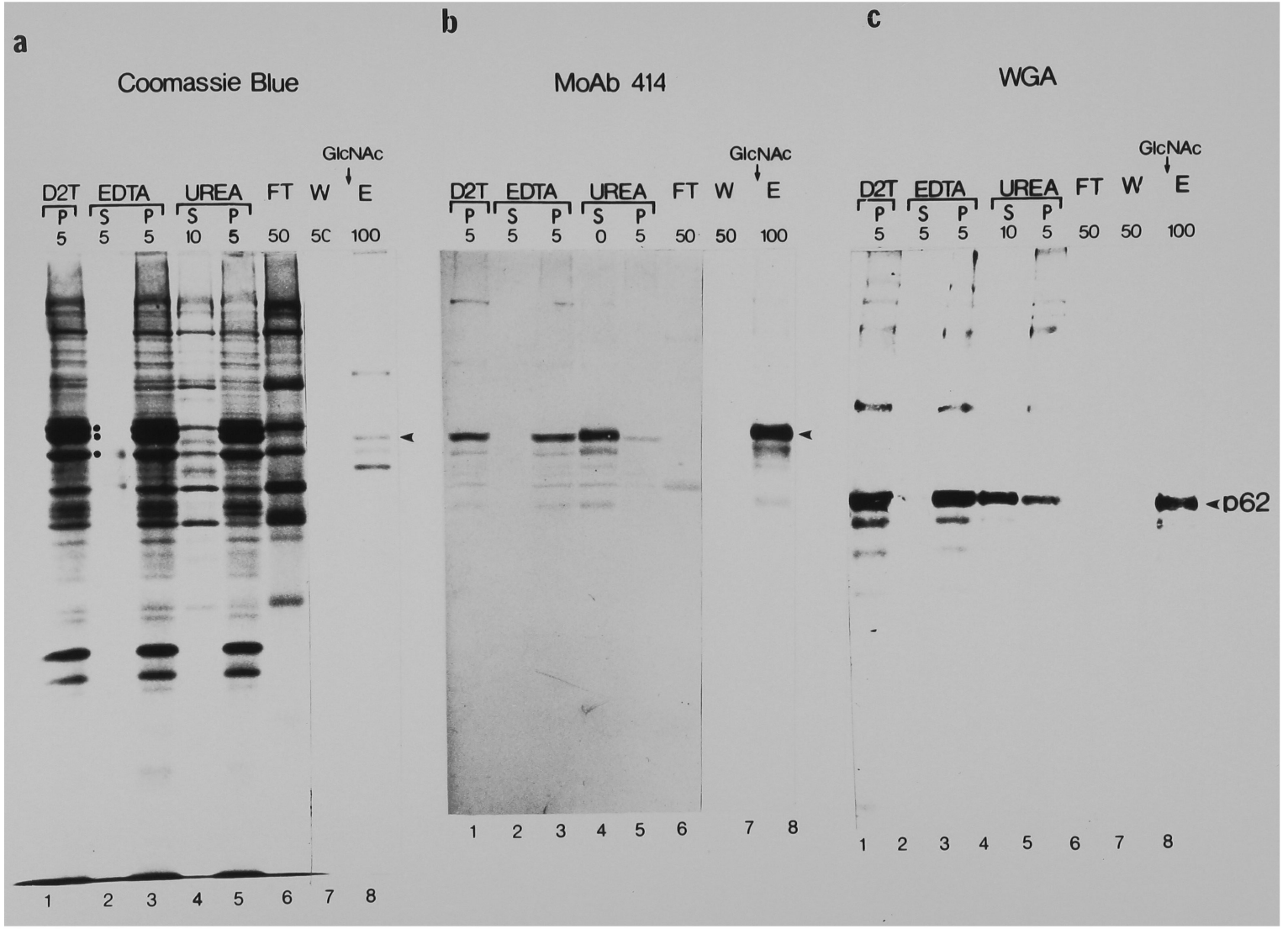
We took advantage of the fact that p62 was easily extracted by urea. At 2 *M* urea, it was almost completely released from the crude pore complex-lamina fraction (Fig 26b, lane 5), whereas the other WGA binding proteins were still largely unextracted (Fig 26c, lane 6). Thus, p62 was essentially the only WGA-binding protein loaded on the column (Fig 26c, lane 4). Urea extraction also represented a significant purification step in and of itself, since most of the lamins and the histones remained in the pellet fraction (Fig 26a, lane 5).

The urea extract was loaded onto a WGA-Sepharose column. The flowthrough was collected, (Fig 26, lane 6) and the column was washed with buffer containing 1 *M* NaCl (Fig 26, lane 7). It was then eluted with 100 mg/ml GlcNAc (Fig 26, lane 8). All of the p62 eluted in the first two column volumes. Interestingly, several other polypeptides coeluted with p62, the major ones of which are ~110, 60 and 55 kD (Fig 26a, lane 8). These polypeptides had no apparent affinity for WGA, since they could not

Figure 26

Affinity chromatography using WGA-Sepharose

Triton X-100 treated nuclear envelopes (lane 1) were prepared from rat liver nuclei as described in section ***2.2***. They were incubated with buffer containing 5 m*M* EDTA and centrifuged to yield supernatant (lane 2) and pellet (lane 3) fractions. The pellet was resuspended in buffer containing 2 *M* urea and the sample was centrifuged as described to yield supernatant (lane 4) and pellet (lane 5) fractions. The supernatant was loaded onto a column consisting of WGA-Sepharose (see section ***2.16***). The flowthrough was collected (lane 6) and the column was washed with 50 column volumes of load buffer (lane 7). Elution was accomplished with 100 mg/ml GlcNAc (lane 8). After electrophoresis, the gels were either stained with Coomassie Blue (panel **a**) or electroblotted to nitrocellulose. Blots were probed with either mAb 414 (panel **b**, see section ***2.4***) or WGA (panel **c**, see section ***2.11***). Closed circles denote lamins A,B and C. Arrowheads indicate p62. Numbers at the top of each lane indicate the number of eq loaded.


a
Coomassie Blue
b
MoAb 414
c
WGA
GlcNAc
D2T
EDTA
UREA
FT
W
E
P
S
P
S
P
5
5
5
10
5
50
50
100
0
1
2
3
4
5
6
7
8
p62

be detected on nitrocellulose blots probed with this lectin (Fig 26c, lane 8). Since the lectin binding assay is much more sensitive than Coomassie blue, one would have expected see very strong reactivity if these proteins did have affinity for WGA, since enough was present to be visualized by Coomassie blue. These polypeptides were specifically enriched in the eluate, since the flowthrough contained no major comigrating bands. Thus, their retention on the column does not appear to be due to nonspecific binding. One of two other explanations is more likely to account for this phenomenon. First, it is possible that they do have affinity for WGA in their native state, but lose it when they are denatured during SDS-PAGE. If this is true, they would be expected to bind to the WGA-Sepharose column, but not to be recognized by WGA after blotting. The other possibility is that the polypeptides coeluted by virtue of an association with p62 and not because they themselves contain sugar. Given the relatively mild conditions that were used for extraction, it would not be surprising if p62 were found to be associated with other polypeptides. Furthermore, the long time allowed for loading and the low urea concentration at which the column was loaded may have facilitated the association of proteins that might be bound to p62 *in situ*. If this is indeed the case, one should be able to subject the eluate to sucrose gradient sedimentation and show that all of the eluted polypeptides comigrate. If the WGA column can be used to "fish out" proteins that interact with p62, including other NPC proteins as well as proteins that might interact transiently, it will provide a very powerful approach towards characterizing the proteins of the NPC, both physically and functionally.

6.7 Discussion

Galactosylation of nuclei (Fig 23) revealed a number of other nuclear proteins with terminal GlcNAc residues. We assume that most if not all of these proteins belong to the family of novel glycoproteins containing O-linked monosaccharidic GlcNAc. It is

possible that some of them contain conventional oligosaccharides that happen to contain terminal GlcNAc residues. However, this is unlikely since all of them are extractable from nuclei under conditions that should not remove integral membrane proteins.

In addition to p62, mAb 414 immunoprecipitated two other proteins labelled by *in vitro* ^{3}H-galactosylation. One of these (p175) had appeared previously, both in immunoprecipitations from ^{35}S-methionine labelled tissue culture cells (see Fig 10), and on protein blots probed with mAb 414 (see Fig 7), but the reactivity was always low when compared to that with p62. It is probable that large size prevented efficient blotting. This is also the most likely explanation as to why p270 was not previously observed. It is also possible that labelling of both of these polypeptides with ^{3}H-galactose was very efficient and thus that they were more easily detectable in this assay than in assays using ^{35}S-methionine labelled or unlabelled material.

Since mAb 414 decorated only the nuclear pore complex in immunoelectron microscopy, we tentatively propose that all three proteins are localized to this structure. However, the assignment of p270 to the pore complex is subject to the caveat that it is only reliably detected after *in vitro* galactosylation. Thus, it could be the case that galactosylation of this protein is actually required for recognition by mAb 414. If so, mAb 414 would not be expected to react with p270 *in situ.*

Other GlcNAc containing nuclear proteins were identified using WGA and mAb 457, both of which precipitated at least six galactosylated proteins (Fig 21), among them p62, p175, and p270. Immunofluorescence with mAb 457 showed diffuse granular staining of the nucleus, with exclusion of the nucleolar region. The expected punctate nuclear "rim" staining (representing the pore complex proteins) was obscured in the interphase nucleus by the diffuse intranuclear staining, but was clearly evident in the mitotic prophase nucleus, where much of the internal staining was reduced as the chromosome condensation was initiated (Fig 22). Thus, mAb 457 reacts with polypeptides located in the nuclear pore complexes as well as others in the nuclear

interior.

This conclusion was supported by data on nuclear subfractionation (Fig 23). DNase treatment of rat liver nuclei quantitatively extracted at least four major mAb 457-reactive polypeptides, all of which also bound to WGA. These presumably represent the proteins which generate internal nuclear staining in immunofluorescence using mAb 457. The remainder of the mAb 457-reactive proteins fractionated with the crude pore complex-lamina fraction, as did the pore complex proteins p62 and p175, and are thus likely to represent pore complex proteins. As with the internal polypeptides, all of these proteins also bound to WGA. Thus it appears that mAb 457 has a specificity similar to WGA, a hypothesis supported by the observation that mAb 457 appears to require the presence of GlcNAc residues for recognition. However, this antibody probably also recognizes some polypeptide sequence, since it is specific for the nuclear proteins, even though there are proteins containing the same type of O-linked GlcNAcs in every cell compartment except mitochondria (Holt and Hart, 1986)

We would like to emphasize that some of the conclusions concerning the various proteins remain conjectures. Purification of each of the mAb and WGA-reactive polypeptides will be necessary to permit extensive characterization and thus to establish whether they are in fact *bona fide* members of this novel family of glycoproteins containing O-linked GlcNAc residues, and whether they are synthesized in the cytosol. Monospecific antibodies to each of the purified proteins will be necessary also for definitive sublocalization of each of these proteins within the nucleus.

Chapter 7

Conclusions and perspectives

In spite of fact that the nuclear pore complex most likely plays an important role in cellular events as fundamental as nucleocytoplasmic transport, almost nothing is known about its biochemistry, let alone the regulation and mechanics of its biosynthesis and assembly. In this thesis, I have described the production of monoclonal antibodies specific for the nuclear pore complex, and the use of these probes to identify some of the constitutive proteins of the pore. At the inception of this project, the production of such probes was predicted to be a somewhat difficult task, since the identification of antibodies directed against the pore complex would ultimately necessitate the use of immunoelectron microscopy. For this reason, our demonstration that antibodies against the pore complex exhibit a strikingly punctate pattern by immunofluorescence microscopy represents a significant step forward, since it means that one can be fairly confident whether a particular antibody is directed against the nuclear pore complex after performing a relatively simple procedure. Thus, it should now be possible to generate antibodies to all of the components of the pore complex with some expedience, provided that they in fact are able to elicit an immune response. This last point is a caveat that bears some consideration. Retrospectively, it is clear that the predominant immune response was against the set of GlcNAc-containing proteins that are discussed in this thesis. All of the five mice we injected with detergent extracted nuclei gave a serum response that showed reactivity mainly with p62, suggesting that the sugar residues are highly immunogenic. Thus, finding antibodies against pore constituents that do not contain such residues may be somewhat difficult if whole pore complexes are injected.

One of the antibodies that we generated (mAb 414) has been used to look at the biosynthesis of a pore complex protein (p62), and these studies have yielded some surprising results. Not unexpectedly, we found that p62 is present as a soluble, cytoplasmic species prior to assembly into the pore complex. However, the residence time in the cytoplasm is very long; the $t_{1/2}$ for incorporation into the nuclear fraction is approximately 6 hr. This might indicate that incorporation of p62 into the pore complex occurs at a specific point in the cell cycle. This hypothesis would agree with the findings of Maul *et al.* (1972) that pore complexes are formed at a specific point just before entry into S phase. To follow up on this, we would like to ask whether labelled, synchronized cell populations incorporate p62 into the pore complex at a specific time. Synchronized cell studies will also be useful to ask a number of other questions about the assembly of p62 into the pore. For example, by subjecting mitotic extracts to sucrose gradient sedimentation, we can ask whether mitotic p62 is monomeric, or whether it might in fact be associated with a macromolecular pore subunit structure.

Another surprising finding was that p62 is glycosylated. This was unexpected since we had shown that it is present as a soluble, cytoplasmic protein prior to incorporation into the NPC. Furthermore, once in the pore complex, p62 is extractable with salt or urea, indicating that it is not a membrane protein. To our knowledge, this represents the first study that uses a specific probe to demonstrate that there are glycoproteins in cytoplasmically disposed regions of the cell, and that such proteins are not restricted to the lumen of intracellular membranes or the cellular exterior. The fact that p62 does not contain high mannose residues and that glycosylation is resistant to treatment with tunicamycin suggest that the sugar is not part of an N-linked oligosaccharide. In agreement with these findings, Hart and coworkers (Torres and Hart, 1984; Holt and Hart, 1986) have shown that the sugars on p62 consist of monosaccharidic GlcNAc residues O-linked to serine and threonine, a linkage that had not previously been identified. Indeed, all of the evidence so far indicates strongly that

p62 is glycosylated through a novel cellular pathway.

P62 is not the only protein that contains such residues. In fact, at least fifteen other nuclear proteins appear to contain "O-linked GlcNAc" (Holt and Hart, 1986, and chapter 6). At least two of these, p175 and p270, also bind to the pore complex-specific antibody mAb 414, and thus are almost certain to be localized to the pore complex. Five others fractionate biochemically with the pore complex-lamina and thus may also be pore complex proteins. If this is indeed the case, and if we assume that there is one copy of each protein in each of the annular subunits of the pore, then these GlcNAc containing proteins could account for as much as 16 million daltons, or roughly 20% of the mass of the pore complex. Obviously, this estimate is extremely rough, but it illustrates that the total contribution of proteins containing this modification is probably quite significant.

Another set of proteins containing O-linked GlcNAc is quantitatively released upon DNase digestion. A monoclonal antibody that recognizes these proteins, as well as the others, generates strong intranuclear staining when used in immunofluorescence. Thus, these species are most likely present in the interior of the nucleus. It will be interesting to ascertain with what internal structures these proteins might be associated.

The function of the sugar residues remains obscure. It may be that, like other glycoproteins, all of these polypeptides have diverse functions that are not necessarily dependent on the presence of the sugar. On the other hand, the sugar may be indicative of a common function of some kind. The suggestion that the GlcNAc provides some sort of karyophilic targeting signal for these proteins has been discussed (Holt and Hart, 1986). It was concluded that such a function was unlikely since proteins containing O-linked GlcNAc are found in almost every compartment of the cell. However, it is possible that the configuration of sugars is not the same on all of these polypeptides, and the sugars could indeed provide some sort of localization signal if there were lectin-like binding proteins in different compartments that recognized different configurations. In this regard, it is interesting that one of our antibodies, mAb 457, seems to recognize all

of the nuclear proteins containing O-linked GlcNAc, but none of the those in any other cellular compartments, suggesting that the nuclear proteins do indeed contain a common determinant. We know that this epitope must include the sugar residues, since mAb 457 no longer binds after removal of the sugar. There is precedent for intracellular targeting through sugar residues, since the targeting of lysosomal enzymes has been shown to be mediated at least in part by mannose-6-phosphate residues (Sly and Fisher, 1982; Creek and Sly, 1984). It has also been postulated that the sugar residues might serve to protect p62 from digestion by proteases during its protracted residence in the cytosol (Schindler *et al.*, 1987). Our observation that p62 is extremely resistant to protease digestion would tend to support this notion. However, the susceptibility of the other GlcNAc-containing proteins to digestion argues against its being a general characteristic of this group of proteins.

Numerous other functions can be imagined. The sugar could also be necessary for association of the proteins with each other, or with other proteins which serve as lectins, to form macromolecular complexes such as pore subunits or intranuclear particles. Such an aggregation phenomenon might even play some role in fusing membrane vesicles to form the intact nuclear envelope. Another intriguing possibility is that the GlcNAc residues are on proteins that are part of a conveyor-like apparatus that extends from the inside of the nucleus, through the nuclear pore complex, and perhaps even into the cytoplasm, and that they provide a common recognition site on different proteins along the route for macromolecules that are shuttled to and from the nucleus. Recently, results have been obtained from two separate laboratories suggesting that these proteins are indeed involved in nucleocytoplasmic transport. An antiserum raised against a 67 kDa protein of clam oocytes was found to inhibit both nuclear envelope associated NTPase activity and ATP-dependent RNA transport out of isolated nuclei (Baglia and Maul, 1983). In the same report WGA was found to have a similar affect. Because the antiserum was presumed to react with lamin B, the authors concluded that lamin B was

probably glycosylated, and seemed to play some role in RNA transport. In light of the data presented here, and considering the fact that there is no other evidence that lamin B is glycosylated, we believe it to be more likely that the antiserum used for these studies contained antibodies not only against lamin B, but also against the equivalent of p62 in the clam oocyte nuclear envelope. Since the presence of p62 is highly conserved, one might expect it to be found in the clam. More recently, the addition of WGA was also found to inhibit the import of karyophilic proteins into isolated nuclei (Finlay *et al.*, 1987).

These studies suggest that one or more of the GlcNAc-containing proteins plays an active role in nucleocytoplasmic traffic of both protein and RNA. This interpretation should be regarded as tentative, however, because of the possibility that WGA causes inhibition of transport simply because it physically blocks the channel by binding to pore complex proteins. Finlay *et al.* (1987) addressed this question in a preliminary manner by stating that WGA did not inhibit the passive diffusion of dextran molecules in their transport assay, however no data was presented to substantiate this. The two possibilities can be differentiated in a number of ways. First, one could use probes of different sizes, ranging for example from F_{ab} fragments of mAb 414 to large multimeric aggregates of mAb 414 bound to a polyclonal anti-mouse IgG, and test the relative efficiency with which these reagents inhibit transport. If inhibition is indeed due to the binding of a specific protein involved in transport, one should see that F_{ab} are just as inhibitory as larger molecules. Another way to answer the question would be to add purified GlcNAc containing proteins to an *in vitro* transport assay, and then to ask whether any of them can inhibit import by competing with their counterparts in the pore complex for binding of the protein to be transported.

Molecular cloning of the GlcNAc containing pore proteins may also provide some insight into the function of these molecules, as well as their relationship, if any, to one another. Transcripts from a cDNA clone could also be useful for *in vitro* translation,

in order to further study early biosynthetic events. Specifically, translation products would provide a substrate for the putative GlcNAc transferase, so that various cell fractions could be assayed for their ability to add GlcNAc residues. This should allow us to prove whether glycosylation is indeed a cytoplasmic event, and eventually to purify the transferase.

We also intend to continue using WGA affinity chromatography to identify other proteins that are associated with the GlcNAc containing polypeptides. We have already shown that a specific set of proteins, none of which have apparent affinity for WGA, coelute with p62 after chromatography on WGA-Sepharose. Some of these proteins may be other components of the pore complex, and some of them may be proteins that have a functional interaction with the pore complex. To differentiate between these two possibilities, we plan to raise antiserum against these proteins and look at their localization within the cell. The same approach can also be used to ask with what the intranuclear GlcNAc containing proteins are associated.

The prospect of an entire family of proteins that appear to located in the nuclear pore complex and that may in fact constitute a large amount of its mass, is an exciting one. Purification and further characterization of these proteins promises to yield significant progress in our understanding of the function of this organelle.

REFERENCES

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

Aaronson, R.P., and G. Blobel. 1975. Isolation of nuclear pore complexes in association with a lamina. *Proc. Nat. Acad. Sci. USA* **72**, 1007-1011.

Adlakha, R.C., D.A Wright, C.G. Sahasrabuddhe, F.M. Davis, N. Prashad, H. Bigo, and P.N. Rao. 1985. Partial purification and characterization of mitotic factors from HeLa cells. *Exp. Cell Res.* **160,** 471-482.

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

Afzelius, B.A. 1955. The ultrastructure of the nuclear membrane of the sea urchin oocyte as studied with the electron microscope. *Exp. Cell Res.* **8**, 147-158.

Agutter, P.S., H.J. McArdle, and B. McCaldin. 1976. Evidence for involvement of nuclear envelope nucleoside triphosphatase translocation of ribonucleoprotein. *Nature* **263**, 165-167.

Agutter, P.S., J.R. Harris, and I. Stevenson. 1977. Ribonucleic acid stimulation of mammalian liver nuclear-envelope nucleoside triphosphatase. *Biochem J.* **162**, 671-679.

Agutter, P.S., B. McCaldin, and H.S. McArdle. 1979. Importance of mammalian nuclear-envelope nucleoside triphosphatase in nucleo-cytoplasmic transport of ribonucleoproteins. *Biochem J.* **182**, 811-819.

Avrameas, S., and M. Bouteille. 1968. Ultrastructural localization of antibody by antigen label with peroxidase. *Exp. Cell Res.* **53**, 166-176.

Baca, M. and L. Zamboni. 1967. The fine structure of human follicular oocytes. *J. Ultrastruct. Res.* **19**, 354-381.

Baglia, F.A., and G. Maul. 1983. Nuclear ribonucleoprotein release and nucleoside triphosphatase activity are inhibited by antibodies directed against one nuclear matrix glycoprotein. *Proc. Nat. Acad. Sci. USA* **80**, 2285-2289.

Bantle, J.A., and W.E. Hahn. 1976. Complexity and characterization of polyadenylated RNA in the mouse brain. *Cell* **8**, 139-150.

Berezney, R., L.K. Funk, and F.L. Crane. 1970. The isolation of nuclear membrane from a large-scale preparation of bovine liver nuclei. *Biochem. Biophys. acta* **203**, 531-546.

Berget, S., C. Moore, and P. Sharp. 1977. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc. Nat. Acad. Sci. USA* **74**, 3171-3175.

Beyer, T.A., J.E. Sadler, J.I. Rearick, J.C. Paulson, and R.L. Hill. 1981. Glycosyltransferases and their use in assessing oligosaccharide structure and structure-function relationships. *Adv. Enzymol.* **52**, 23-175.

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

Blobel, G., and V.R. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science.* **154**, 1662-1665.

Bonner, W.M. 1975a. Protein migration into nuclei. I.Frog oocyte nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. *J. Cell Biol.* **64**, 421-430.

Bonner, W.M. 1975*b*. Protein migration into nuclei II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. *J. Cell Biol.* **64** 431-437.

Bornens, M. 1973. Action of heparin on nuclei: Solubilization of chromatin enabling the isolation of nuclear membranes. *Nature* **244**, 28-30.

Bornens, M. and J. Courvalin. 1978. Isolation of nuclear envelopes with poly anions. *J. Cell Biol.* **76**, 191-206.

Boulan, E.R., G. Kreibich, and D.D. Sabatini. 1974. Spatial organization of glycoproteins in membranes of rat liver rough microsomes I. Localization of lectin-binding sites in microsomal membranes. *J. Cell Biol.* **78**, 874-893.

Branton, D., and H. Moor. 1964. Fine structure in freeze-etched *Allium cepa* L. root tips. *J. Ultrastruct. Res.* **11**, 401-411.

Callan, H.G. and S.G.Tomlin. 1950. Experimental studies on amphibian oocyte nuclei. I. Investigation of the structure of the nuclear membrane by means of the electron microscope. *Proc. Roy. Acad. Soc. Lon. B* **137**, 367-378.

Chow, L.T., R.E. Gelina, T.R. Broker, and R.J. Roberts. 1977. An amazing sequence rearrangement at the 5;' ends of adenovirus 2 messenger RNA. *Cell* **12**, 1-8.

Clawson, G.A., M. Koplitz, B. Castler-Schechter, and E.A. Smuckler. 1978. Energy utilization and RNA transport: their interdependence. *Biochemistry* **17**, 3747-3752.

Clawson, G.A., J. James, C.H. Woo, D.S. Friend D. Moody, and E.A. Smuckler. 1980. Pertinence of nuclear envelope nucleoside triphosphatase activity to ribonucleic acid transport. *Biochemistry* **19**, 2748-2756.

Cleveland, D.W., S.G. Fischer, M.W. Kirschner, and U.R. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**, 1102-1106.

Creek, K.E. and W.S. Sly. 1984. *In* Lysosomes in Biology and Pathology (J.T. Dingle, R.T. Dean and W. Sly *eds.*) Elsevier, New York. pp 63-82.

Dabauville, M.C., and W.W. Franke. 1982. Karyophilic proteins: polypeptides synthesized *in vitro* accumulate in the nucleus upon microinjection into the cytoplasm of amphibian oocytes. *Proc. Nat. Acad. Sci. USA* **79**, 5302-5306.

Davey, J., N.J. Dimmock, and A. Colman. 1985. Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in *Xenopus* oocytes. *Cell* **40**, 667-675.

Dawson, J.M., J. Hossack, and G.M. Wyburn. 1955. Observations on the Nissl's substance, cytoplasmic filaments and the nuclear membrane of spinal ganglion cells. *Proc. Roy. Soc. B* **144**, 132-142.

dePetris, S., G. Karlsbad, and B. Pernis. 1963. Localization of antibodies in plasma cells by electron microscopy. *J. Exp. Med.* **117**, 849-862.

DeRobertis, E. 1983. Nucleocytoplasmic segregation of proteins and RNAs. *Cell* **32**, 1021-1025.

De Robertis, E.M., R.F. Longthorne, and J.B. Gurdon. 1978. Intracellular migration of nuclear proteins in *Xenopus* oocytes. *Nature* **272**, 254-256.

DeRobertis, E., S. Leinhard, and R. Parisot. 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. *Nature* **295**, 572-577.

Dingwall, C., S.V. Sharnick, and R.A. Laskey. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* **30**, 449-458.

Drury, K.C. 1978. Method for the preparation of active maturation promoting factor (MPF) from *in vitro* matured oocytes of *Xenopus laevis*. *Differentiation* **10**, 181-186.

Dwyer, N., and G. Blobel. 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* **70**, 581-591.

Feldherr, C.M. 1965. The effect of the electron-opaque pore material on exchanges through the nuclear annuli. *J. Cell Biol.* **25**, 43-53.

Feldherr, C.M. 1975. The uptake of endogenous proteins by oocyte nuclei. *Exp. Cell Res.* **93**, 411-419.

Feldherr, C.M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* **99**, 2216-2222.

Feldherr, C.M., R.J. Cohen, and J.A. Ogburn. 1983. Evidence for mediated protein uptake by amphibian oocyte nuclei. *J. Cell Biol.* **96**, 1486-1490.

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

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

Franke, W.W. 1966. Isolated nuclear membranes. *J. Cell Biol.* **31,** 619.

Franke, W.W. 1974. Structure, biochemistry and functions of the nuclear envelope. *Int. Rev. Cytol* suppl. **4**, 71-236.

Franke, W.W and U. Scheer. 1974*a*. Pathways of nucleocytoplasmic translocation of ribonucleoproteins. *Symp. Soc. Exp. Biol.* **28**, 249-282.

Franke, W.W. and U. Scheer. 1974*b*. Structures and functions of the nuclear envelope. *In* The cell nucleus. vol. 1 (H. Busch ed.) Academic Press, New York. pp. 219-347.

Franke, W.W., B. Deumling, B. Ermen, E.D. Jarasch, and H. Kleinig. 1970. Nuclear membranes from mammalian liver I. Isolation procedure and general characterization. *J. Cell Biol.* **46**, 379-395.

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

Fry, D.J. 1976. The nuclear envelope in mammalian cells. *In* Mammalian Cell Membranes, vol. 2 (G.A. Jamieson and D.M. Robinson eds) Butterwoth Publishers inc, Woburn. pp. 197-287.

Gall, J.G. 1954. Observations on the nuclear membrane with the electron microscope. *Exp. Cell Res.* **7**, 197-200.

Gall, J.G. 1956. Small granules in the amphibian oocyte nucleus and their relationship to RNA. *J. Biophys. Biochem. cytol.* **2s**, 393-397.

Gerace, L., and G. Blobel. 1980. The nuclear envelope is reversibly depolymerized during mitosis. *Cell* **19**, 277-287.

Gerace, L. and G. Blobel. 1982. Nuclear lamina and the structural organization of the nuclear envelope. *Cold Spring Harbor Symp. Quant. Biol.* **46**, 967-978.

Gerace, L., A. Blum, and G. Blobel. 1978. Immunochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. *J. Cell Biol.* **79**, 546-566.

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

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

Gerhart, J., M. Wu , and M.W. Kirschner. 1984. Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **98** 1247-1255.

Getz, M., G. Bernie, B. Young, E. MacPhail, and J. Paul. 1975. A kinetic estimation of base sequence complexity of nuclear poly (A)-containing RNA in mouse Friend cells. *Cell* **4**, 121-129.

Glass, W.F., R.C. Briggs, and L.S. Hnilica. 1981. Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. *Anal. Biochem.* **115**, 219-224.

Goldberg, A.H., L.C. Teoman, and H. Busch. 1978. Chromatin-associated glycoproteins of normal rat liver and Novikoff hepatoma ascites cells. *Cancer Res.* **38**, 1052-1056.

Goldfarb, D.S., J. Gariepy, G. Schoolnik, and R.D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. *Nature* **322**, 641-644.

Gurdon, J.B. 1970. Nuclear transplantation and the control of gene activity in animal development. *Proc. Roy. Soc. Lond. B* **176**, 303-314.

Hancock, R. 1982. Topological organization of interphase DNA: the nuclear matrix and other skeletal structures. *Biol. Cell.* **46**, 105-122.

Hanover, J.A., and W.J. Lennarz. 1980. N-linked glycoprotein assembly. *J. Cell Biol.* **255**, 3600-3604.

Hartmann, F. 1953. An electron optical study of sections of nervous system. *J. Comp. Neurol.* **99**, 201-249.

Herman, R.C., J.G. Williams, and S. Penman. 1976. Message and non-message sequences adjacent to poly(A) in steady state heterogeneous nuclear RNA of HeLa cells. *Cell* **7**, 429-437.

Hertig, A.T. 1968. The primary human oocyte: Some observations on the fine structure of Balbiani's vitelline body and the iorigin of the annulate lamellae. *Am. J. Anat.* **122**, 107-138.

Holt, G.D. and G. W. Hart. 1986. The subcellular distribution of terminal GlcNAc moieties. *J. Biol. Chem.* **261**, 8049-8057.

Horowitz, S.B, and L.C. Moore. 1974. The nuclear permeability, intracellular distribution, and diffusion of inulin in the amphibian oocyte. *J. Cell Biol.* **60**, 405-415.

Hough, B.R., M.J. Smith, R.J. BRitten, and E.H. Davidson. 1975. Sequence complexity of heterogeneous nuclear RNA in sea urchin embryos. *Cell* **5**, 291-299.

Hubbard, S.C., and R.J. Ivatt. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* **50**, 555-583.

Jackson, R.C. 1976. Polypeptides of the nuclear envelope. *Biochemistry* **15**, 5641-5651.

Johnson, G.D. and G.M. de C. Nogueira Araujo. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Meth.* **43**, 349-350.

Kalderon, D. and A.E. Smith. 1984. *In vitro* mutagenesis of a putative DNA binding domain of SV40 large-T. *Virology* **139**, 109-137.

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

Kalderon, D., W.D. Richardson, A.F. Markham, and A.E. Smith. 1984b. Sequence requirements for nuclear location of Simian virus 40 large-T antigen. *Nature* **311**, 33-38.

Kan, F.W.K., and P.P. da Silva. 1986. Preferential association of glycoproteins to the euchromatic regions of cross-fractured nuclei is revealed by fracture label. *J. Cell Biol.* **102**, 576-586.

Kashnig, D.M., and C.B. Kasper. 1969. Isolation, morphology, and composition of the nuclear membrane from rat liver. *J. Biol. Chem.* **244**, 3786-3792.

Kasper, C.B. 1984. Nuclear envelope proteins: selected biochemical aspects. *In* Chromosomal nonhistone proteins. vol. 4 (S. Hnilica *ed.*) CRC press, Boca Raton, FA, pp 1-20.

Katz, F.N., J.E. Rothman, V.R. Lingappa, and G. Blobel. 1977. Membrane assembly *in vitro*: Synthesis, glycosylation and asymmetric insertion of a transmembrane protein. *Proc. Nat. Acad. Sci. USA* **74**, 3278-3282.

Kay, R.R., D. Fraser, and I.R. Johnston. 1972. A method for the rapid isolation of nuclear membranes from rat liver. *Eur. J. Biochem.* **30**, 145-154.

Keenan, T.W., R. Berezney, L.K. Funk, and F.L. Crane. 1970. Lipid composition of nuclear membranes isolated from bovine liver. *Biochem. Biophys. acta* **203**, 547-554.

Kessel, R.G. 1968. Annulate Lamellae. *J. Ultrastruc. Res.* **10**s, 1-82

Kishimoto, T., R. Kuriyama, H. Kondo, H. Kanatani. 1982. Generality of the action of various maturation-promoting factors. *Exp. Cell Res.* **137**, 121-126.

Kleinig, H. 1970. Nuclear membranes from mammalian liver. II. lipid composition. *J. Cell Biol.* **46**, 396-402.

Klessig, D.F. 1977. Two adenovirus mRNA's have a common 5' terminal leader sequence encoded at least 10 kb upstream from their main coding regions. *Cell* **12**, 9-21.

Kornfeld, R. and S. Kornfeld. 1980. Structure of glycoproteins and their oligosaccharide units. *In* The biochemistry of glycoproteins and proteoglycans (W. Lennarz ed.) Plenum Press, New York. pp 85-160.

Krohne, G. and R. Benavente. 1986. The nuclear lamins: A multigene family of proteins in evolution and differentiation. *Exp. Cell Res.* **162**, 1-10.

Krohne, G., W.W. Franke, S. Ely, A. D'Arcy, and E. Jost. , 1978. Localization of a nuclear envelope-associated protein by indirect immunofluorescence microscopy using antibodies against a major polypeptide from rat liver fractions enriched in nuclear envelope-associated material. *Cytobiologie* **18**, 22-38.

Lanford, R.E. and J.S. Butel. 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37**, 801-813.

Leduc, E.H., S. Avrameas, and M. Bouteille. 1968. Ultrastructural localization of antibody in differentiating plasma cells. *J. Exp. Med.* **127**, 109-119.

Leduc, E.H., G.B. Scott, and S. Avrameas. 1969. Ultrastructural localization of intracellular immune globulins in plasma cells and lymphoblasts by enzyme-labelled antibodies. *J. Histochem. Cytochem.* **17**, 211-224.

Levy, B.W., C.B. Johnson, and B.J. McCarthy. 1976. Diversity of sequences in total and polyadenylated nuclear RNA from Drosophila cells. *Nucl. Acids Res.* **3**, 1777-1789.

Levy-Wilson, B. 1983. Glycosylation, ADP-ribosylation, and methylation of Tetrahymena histones. *Biochemistry* **22**, 484-489.

Lingappa, V.R., J.R. Lingappa, R. Prasad, K.E. Ebner, and G. Blobel. 1978. Coupled cell-free synthesis, segregation, and core glycosylation of a secretory protein. *Proc. Nat. Acad. Sci. USA* **75**, 2338-2342.

Maller, J., M. Wu, and J.C. Gerhart. 1977. Changes in protein phosphorylation accompanying maturation of *Xenopus laevis* oocytes. *Dev. Biol.* **58**, 295-312.

Masui, Y. and C.L. Markert, 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129-147.

Mattaj, I.W., S. Lienhard, R. Zeller, and E.M. DeRobertis. 1983. Nuclear exclusion of transcription factor IIIA and the 42S particle transfer RNA-binding protein in *Xenopus* oocytes: a possible mechanism for gene control? *J. Cell Biol.* **97**, 1261-1265.

Maul, G.G. 1977. The nuclear and cytoplasmic pore complex. Structure, dynamics, distribution, and evolution. *Int. Rev. Cytol.* Suppl. **6**, 76-186.

Maul, G.G. and L. Deaven. 1977. Quantitative determination of nuclear pore complexes in cycling cells with differing DNA content. *J. Cell Biol.* **73**, 748-760.

Maul, G.G., J.W. Price, and M.W. Lieberman. 1971. Formation and distribution of nuclear pore complexes in interphase. *J. Cell Biol.* **51**, 405-418.

Maul, G.G., H.M. Maul, J.E. Scogna, M.W. Lieberman, G.S. Stein, B.Y. Hsu, and T.W. Borun. 1972. Time sequence of nuclear pore formation in phytohemagglutinin-stimulated lymphocytes and in HeLa cells during the cell cycle. *J. Cell Biol.* **55**, 433-447.

Mc Gadey, J. 1970. A Tetrazolium method for non-specific alkaline phosphatase. *Histochemie* **23**, 180-184.

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

Miake-Lye, R. and M.W. Kirschner. 1985. Induction of early mitotic events in a cell-free system. *Cell* **41**, 165-175.

Miake-Lye, R., J. Newport, and M. Kirschner. 1983. Maturation-promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of *Xenopus laevis*. *J. Cell Biol.* **97**, 81-91.

Mills, A.D., R.A. Laskey, P. Black, and E.M. De Robertis. 1980. An acidic protein which assembles nucleosomes *in vitro* is the most abundant protein in Xenopus oocyte nuclei. *J. Mol. Biol.* **139**, 561-568.

Monneron, A. and D. Segretain. 1974. Extensive binding of Concanavalin A to the nuclear membrane. *FEBS letters* **42**, 209-213.

Monneron, A, G. Blobel, and G.E. Palade. 1972. Fractionation of the nucleus by divalent cations. *J. Cell Biol.* **55**, 104-125.

Newport, J.W. and M.W. Kirschner. 1984. Regulation of the cell cycle during early *Xenopus* development. *Cell* **37**, 731-742.

O'Farrell, P., H. Goodman, and P. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133-1142.

Ottaviano, Y. and L. Gerace. 1984. Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* **148**, 624-632.

Paine, P.L. 1975. Nucleocytoplasmic movement of fluorescent tracers microinjected into salivary gland cells. *J. Cell Biol.* **66**, 652-657.

Paine, P.L., and C.M. Feldherr. 1972. Nucleocytoplasmic exchange of macromolecules. *Exp. Cell Res.* **74**, 81-98.

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

Paine, P.L., T.W. Pearson, L.J.M. Tluczek, and S.B. Horowitz. 1981. Nuclear sodium and potassium. *Nature* **291**, 258-261.

Palay, S., and E. Palade. 1955. The fine structure of neurons. *J. Biophys. biochem. cytol.* **1**, 69-89.

Perez, M., and C.B. Hirschberg. 1986. Topography of glycosylation reactions in the rough endoplasmic reticulum membrane. *J. Cell Biol.* **261**, 6822-6830.

Philipp, E.I., W.W. Franke, T.W. Keenan, J. Stadler, and E. Jarasch. 1976. Characterization of nuclear membranes and endoplasmic reticulum isolated from plant tissue. *J. Cell Biol.* **68**, 11-29.

Puddington, L., M.O. Lively, and D.S. Lyles. 1985. Role of the nuclear envelope in synthesis, processing, and transport of membrane glycoproteins. *J. Biol. Chem.* **260**, 5641-5647.

Reeves, R., D. Chang, and S. Chung. 1981. Carbohydrate modifications of the HMG proteins. *Proc. Nat. Acad. Sci. USA* **78**, 6704-6708.

Reynhout, J.K. and L.D. Smith. 1974. Studies on the appearance and nature of a maturation-inducing factor in the cytoplasm of amphibian oocytes exposed to progesterone. *Dev. Biol.* **38**, 394-400.

Richardson, W.D., B.L. Roberts, and A.E. Smith. 1986. Nuclear location signals in polyoma virus large-T. *Cell* **44**, 77-85.

Rizzo, W.B., and M. Bustin. 1977. Lectins as probes of chromatin structure. *J. Cell Biol.* **252**, 7062-7067.

Roth, J. 1983. Application of lectin-gold complexes for electron microscopic localization of glycoconjugates on thin sections. *J. Histochem. cytochem.* **31**, 987-999.

Russo, C., L. Callegaro, L., E. Lanza, and S. Ferrone. 1983. Purification of IgG monoclonal antibody by caprylic acid precipitation. *J. Imm. Meth.* **65**, 269-271.

Salditt-Georgieff, M., and J.E. Darnell Jr. 1982. Further evidence that the majority of primary nuclear RNA transcripts in mammalian cells do not contribute to mRNA. *Mol. Cell Biol.* **2**, 701-707.

Scheer, U. 1973. Nuclear pore flow rate of ribosomal RNA and chain growth rate of its precursor during oogenesis of *Xenopus laevis*. *Dev. Biol.* **30**, 13-28.

Schindler, M., M. Hogan, R. Miller, and D. DeGaetano. 1987. A nuclear specific glycoprotein representative of a unique pattern of glycosylation. *J. Biol. Chem.* **262**, 1254-1260.

Schwyzer, M. and R.L. Hill. 1977. Porcine A blood group-specific N-acetylgalactosaminyltransferase. II. Enzymatic properties *J. Biol. Chem.* **252**, 2346-2355.

Seve, A.P., J. Hubert, D. Bouvier, C. Masson, G. Geraud, and M. Bouteille. 1984. *In situ* distribution in different cell types of nuclear glycoconjugates detected by two lectins. *J. Submicr. Cytol.* **16**, 631-641.

Siebert, G. 1978. Compartmentalization and metabolic control. *Biochem. Soc. Trans.* **6**, 5-9.

Silver, P.A., L.P. Keegan, and M. Ptashne. 1984. Amino terminus of the yeast *GAL4* gene product is sufficient for nuclear localization. *Proc. Nat. Acad. Sci. USA* **81**, 5951-5955.

Sjostrand, E.S., and J. Rhodin. 1953. The ultrastructure of the proximal convoluted tubules of the mouse kidney as revealed by high resolution electron microscopy. *Exp. Cell Res.* **4**, 426.

Sly, W.S., and H.D. Fisher. 1982. The phosphomannosyl recognition system for intracellular transport of lysosomal enzymes. *J. Cell. Biochem.* **18**, 67-85.

Smith, A.E., D. Kalderon, B.L. Roberts, W.H. Colledge, M. Edge, P. Gillett, A. Markham, E. Paucha, and W.D. Richardson. 1985. The nuclear localization signal. *Proc. R. Soc. Lond. B* **226**, 43-58.

Snider, M.D. and D.W. Robbins. 1982. Transmembrane organization of protein glycosylation. *J. Biol. Chem.* **257**, 6796-6801.

Soeiro, R., M.H. Vaughan, J.R. Warner, and J.E. Darnell, Jr. 1968. The turnover of nuclear DNA-like RNA in HeLa cells. *J. Cell Biol.* **39**, 112-118.

Staufenbiel, M., and W. Deppert. 1984. Preparation of nuclear matrices from cultured cells: Subfractionation of nuclei *in situ*. *J. Cell Biol.* **98**, 1886-1894.

Stevens, B.J., and H. Swift. 1966. RNA transport from nucleus to cytoplasm in *Chironomus* salivary glands. *J. Cell Biol.* **31**, 55-77.

Stick, R. and D. Hausen. 1980. Immunological analysis of nuclear lamina proteins. *Chromosoma* **80**, 219-236.

Stoddard, R.W. 1979. Nuclear glycoconjugates and their relation to malignancy. *Biol. Rev.* **54**, 199-235.

Suprynowicz, F.A. and L. Gerace. 1986. A fractionated cell-free system for analysis of prophase nuclear disassembly. *J. Cell Biol.* **103**, 2073-2081.

Swift, H. 1956. The fine structure of annulate lamellae. *J. Biophys. Biochem. Cytol.* **2**s, 415-418.

Takatsuki, A., K. Arima, and G. Tamura. 1971. Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *J. Antibiotics* **24**, 215-233.

Takatsuki, A., K. Kohno, and G. Tamura. 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick-embryo microsomes by tunicamycin. *Agri. Biol. Chem.* **39**, 2089-2091.

Tarantino, A.L. and F. Maley. 1974. Purification and properties of an endo-β-N-acetylglucosaminidase from *Streptomyces griseus*. *J. Biol. Chem.* **249**, 811-817.

Tkacz, J.S. and J.O. Lampen. 1975. Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. *Biochem. Biophys. Res. Comm.* **65**, 248-257.

Tobian, J.A., L. Drinkard, and M. Zasloff. 1985. tRNA nuclear transport: defining the critical regions of human $tRNA_i^{met}$ by point mutagenesis. *Cell* **43**, 415-422.

Torres, C. and G.W. Hart. 1984. Topography and polypeptide distribution of terminal GlcNAc residues on the surface of intact lymphocytes. *J Biol. Chem.* **259**, 3308-3317.

Unwin, P.T., and R.A. Milligan. 1982. A large particle associated with the perimeter of the nuclear pore complex. *J. Cell Biol.* **93**, 63-75.

Virtanen, I. and J. Wartiovaara. 1976. Lectin receptor sites on rat liver nuclear membranes. *J. Cell Sci.* **22**, 335-344.

Wasserman, W.J., and Y. Masui. 1975. Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. *Exp. Cell Res.* **91**, 381-388.

Wasserman, W.J., and L.D. Smith. 1978. The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. *J. Cell Biol.* **78**, R15-R22.

Watson, M.L. 1954. Pores in the mammalian nuclear membrane. *Biochim. Biophys. acta* **15**, 475-479.

Watson, M.L. 1955. The nuclear envelope: its structure and relation to cytoplasmic membranes. *J. Biophys. Biochem. Cytol.* **1**, 257-271.

Watson, M.L. 1959. Further observations on the nuclear envelope of the animal cell. *J. Biophys. Biochem. Cytol.* **6**, 147-156.

Wold, B.J., W.H. Klein, B.R. Hough-Evans, R.J. Britten, and E.H. Davidson. 1978. Sea urchin embryo mRNA sequences expressed in the nuclear RNA of adult tissues. *Cell* **14**, 941-950.

Wu, M., and J.C. Gerhart. 1980. Partial purification and characterization of the maturation-promoting factor from eggs of *Xenopus laevis*. *Dev. Biol.* **79**, 465-477.

Yeoman, L.C., J.J. Jordan, R.K. Busch, C.W. Taylor, H.E. Savage, and H. Busch. 1976. A fetal protein in chromatin of Novikoff hepatoma and Walker 256 carcinosarcoma tumors that is absent from normal and regenerating rat liver. *Proc. Nat. Acad. Sci. USA* **73**, 3258-3262.

Zasloff, M. 1983. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Nat. Acad. Sci. USA* **80**, 6436-6440.

Zbarsky, I.B., K.A. Perevoshchikova, L.N. Delektorskaya, I.N. Delektorskaya, and V.V. Delektovsky. 1969. Isolation and biochemical characteristics of the nuclear envelope. *Nature* **221**, 257.

Zeller, R., T. Nyffenegger, and E. DeRobertis. 1983. Nucleocytoplasmic distribution of snRNP's and stockpiled snRNA binding proteins during oogenesis and early development in *Xenopus laevis*. *Cell* **32**, 425-434.

Zentgraf, H., B. Deumling, E. Jarasch, and W.W. Franke. 1971. Nuclear membranes and plasma membranes from hen erythrocytes. *J. Biol. Chem.* **246**, 2986-2995.

End