Molecular Characterization of Components of the Nuclear Pore Complex and the Nuclear Import System in *Saccharomyces cerevisiae*

by

Jonathan David Joshua Loeb

A.B., Molecular Biology
University of California, Berkeley, 1987

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
in Biology

at the

Massachusetts Institute of Technology

May 1995

Signature of Author_____
Department of Biology
, May, 12, 1995

Certified by_____
Gerald R. Fink
Professor of Biology
Thesis Supervisor

Accepted by_____
Frank Solomon
Biology Graduate Committee

Molecular Characterization of Components of the Nuclear Pore Complex and the Nuclear Import System in *Saccharomyces cerevisiae*

by
Jonathan David Joshua Loeb

Submitted to the Department of Biology
on May 12, 1995 in partial fulfillment of the requirements for
the Degree of Doctor of Philosophy in Biology

Abstract

The nuclear pore complex (NPC) is the site of the bidirectional traffic of macromolecules into and out of the nucleus. Components of the NPC are responsible for regulating the active transport of macromolecules as well as organizing the structure of the nuclear envelope. This work documents the identification and characterization of two components of the NPC in the budding yeast, *Saccharomyces cerevisiae* that function directly in the nuclear import reaction, but also affect nuclear structure. Furthermore, I show that defects in the nuclear import of proteins lead to unexpected effects on the cell cycle, which may be evidence for a new level of regulation of mitosis.

The *NUP2* gene encodes a 78kD protein that belongs to a family of NPC components known as XFXFG nucleoporins. *NUP2* was cloned by its cross-reactivity with an antibody raised against mammalian nucleoporins. Immunofluorescence microscopy reveals that Nup2 is located in the NPC in yeast. *NUP2* is not essential, but *NUP2* is required when either of the two other XFXFG nucleoporins, *NSP1* or *NUP1* are mutated. In mammalian cells it has been shown that XFXFG nucleoporins participate in the nuclear import of proteins. *NUP2* also exhibits homology to another recently identified group of soluble proteins that participate in nuclear import, known as RAN binding proteins (RanBPs). These are proteins that have been identified by their ability to bind to the small GTPase required for nuclear import, RAN/TC4.

SRP1 encodes the nuclear import signal sequence receptor in yeast. Mutations in this gene are unable to transport proteins into the nucleus. *SRP1* mutants interact genetically with mutations in both families of nucleoporins: the XFXFG nucleoporins *NUP1* and *NUP2* and the GLFG nucleporin *NUP116*. Also Srp1 is in physical association with the nucleoporins Nup1 and Nup2. Furthermore, a mutation in *SRP1* causes conditional arrest during the cell cycle at the G2/M stage. We propose that the reason this mutant arrests is that the transport of a particular substrate for nuclear import is affected in the mutant. Candidates for the affected substrate are likely to be involved in the degradation pathway that destroys the mitotic kinase because the *srp1* mutant does not properly degrade the B-type cyclin Clb2.

Thesis supervisor: Dr. Gerald R. Fink
Title: Professor of Biology

For my best professors: Mom and Dad;
and for my littlest professor, Elana Yosefa.

Acknowledgments

My five years in the Fink lab have been a very valuable time. Professionally and personally I am far removed from the enthusiastic young fellow who arrived on these bleak shores, looking to learn a little science. I learned more than a little science, thanks to the MIT faculty, to Gerry Fink, and mostly to the students and post-docs with whom I shared my many days and nights in the lab. The members of the lab were very generous to me with their time, their enthusiasm and their encouragement, and I hope that I have returned these in kind.

I would like to especially thank those colleagues whose help made my scientific life first possible, then enjoyable. I thank Bonnie Bartel, Per Ljungdahl, and Dave Miller for their molecular biological genius, which I exploited many times. I thank Adam Antebi, Judy Bender, Julie Brill, Chip Celenza, Paula Grisafi, Julia Kohler, Daniel Kornitzer, Nikki Levin, Hans-Ueli Mösch, Kris Niyogi, David Pellman, Cora Styles and about 50 others for their sage advice and genuine friendship. I thank Drs. Bartel, Bender and Mösch for making it cool to be tense. Furthermore, I am endebted to Patrick McCaw for his company on those 500 dinners out and to Brenda Schulman for climbing those endless hills with me. I especially thank Gabriel Schlenstedt for his help both scientific and otherwise, and for being the guy with whom I could finally talk about nuclear transport.

Most importantly, I thank Tish. This saintly woman withstood years of stress and complaint with solid love, patience and understanding. I am so grateful. I will spend the next thirty years trying to make it up to you!

Table of contents

List of Figures

List of Tables

Abbreviations

DAPI:	4',6'-diamidino-2-phenylindole
5-FOA:	5-Fluoroorotic Acid
GFP:	green fluorescent protein
NBP:	nuclear localization sequence binding protein
NE:	nuclear envelope
NEM:	N-ethylmaleimide
NLS:	nuclear localization sequence
NPC:	nuclear pore complex
WGA:	wheat germ agglutinin

Chapter 1

The nuclear pore complex and nuclear transport

1.1 The nuclear envelope

The major feature that separates the eukaryotic kingdoms from prokaryotes is the nucleus, a complex membranous organelle that encapsulates the cell's chromosomal DNA. The nucleus provides physical separation of the genetic material from the site of most cellular processes. Its contents can therefore be maintained as a unique biochemical environment distinct from the rest of the cell contents (Franke et al., 1981). This separation provides eukaryotic cells with the opportunity to exploit several new levels of regulation that are unavailable to prokaryotes. Unlike bacteria, where polyribosomes accumulate on messenger RNA prior to the completion of synthesis, eukaryotic translation is removed in time and space from transcription. Regulation of mRNA processing, mRNA transport from the nucleus and mRNA stability are thereby available as novel means to modulate gene expression. A good example of regulation on this level is that of tissue specific alternative splicing of mRNAs in animals (Sharp, 1994). In this process tissue specific factors act on nascent mRNA prior to its departure from the nucleus, modifying it in order to produce different proteins from the same gene. This sort of regulation is not possible without the removal of translation to a distant site within the cell. Another case of regulated gene expression dependent on the existence of the nucleus is the DNA binding transcription factors whose entry into the nucleus requires extracellular signals, which include a broad range of proteins, such as the glucocorticoid hormone receptor and NF-kB (Nigg et al., 1991; Picard and Yamamoto, 1987).

The defining feature of the nucleus is the nuclear envelope (NE) membrane. The evolution of the NE as an impermeable barrier between the nuclear interior and the cytoplasm has driven the development of a mechanism to convey molecules between these two compartments that has been termed "nucleocytoplasmic transport". Unlike other cellular systems that transport macromolecules across membranes, this system is capable of selective, bi-directional traffic of a broad variety of molecules, including RNAs, proteins and RNPs. Transport is rapid, energy dependent and regulated. In this chapter I will describe the structure and functions of the NE, focusing on the nuclear transport process, which is mediated by a unique organelle, the nuclear pore complex (NPC).

The nuclear envelope membrane

The nuclear envelope (NE) is composed of two concentric lipid bilayers. The outer sheaf of the membrane is contiguous with the endoplasmic reticulum (Dingwall and Laskey, 1992). Therefore the cisternal space between the inner and outer sheaves of the NE is part of the ER lumen. As a consequence of this arrangement, the nuclear envelope serves as part of the secretory apparatus as well as insulating the nucleoplasm from the cytoplasm. The inner nuclear envelope is often found to be in close association with a highly organized cytoskeletal network termed the nuclear lamina. The nuclear lamina is composed of intermediate filament proteins known as nuclear lamins (Gerace et al., 1978). In some organisms the nuclear envelope breaks down at mitosis and later reforms. In these cells lamins disassemble at the onset of mitosis prior to NE breakdown and after mitosis are thought to nucleate the reassembly of nuclear envelope vesicles onto chromatin (Nigg, 1992). The cell cycle regulation of lamin polymerization is known to be controlled directly by phosphorylation by the cyclin dependent kinase (Enoch et al., 1991). The nuclear lamina also serves as an attachment point for chromatin to the nuclear envelope in interphase cells and may thereby participate in determining the three-dimensional organization of the interphase nucleus (Nigg, 1992).

Differences between organisms

Eukaryotes undergo complex morphological changes as they pass through the cell cycle. After DNA replication, the chromosomes must be organized, then segregated into two new daughter nuclei. The mitotic spindle apparatus has evolved to achieve the required physical separation between homologous chromosomes prior to cytokinesis. Interestingly, there is a great deal of diversity in the behavior of the NE during mitosis among different species. The NE and the cytoskeletal structures that are the force generating mechanisms for mitosis take on many forms, particularly in the fungal and protist kingdoms (Heath, 1980). In many eukaryotes, including mammals, the nuclear envelope disperses at the beginning of mitosis and reforms around the chromatin after anaphase. Obviously, in these organisms the distinction between the nucleoplasmic and cytoplasmic compartments is lost every cell cycle and must be reestablished in the next. However, nuclear

envelope breakdown is not a prerequisite for mitosis. Fungi perform "closed" mitosis within the nuclear envelope (Byers, 1981; Heath, 1980) and other organisms, such as *Drosophila*, only partially disassemble the nuclear envelope during some mitoses (Harel et al., 1989; Stafstrom and Staehelin, 1984). Closed mitosis dictates several differences between the nuclear envelopes of fungi and that of eukaryotes that break down their NE: the microtubule organizing centers (MTOC) of fungi (spindle pole bodies) must be embedded within the NE in order to nucleate both nuclear and cytoplasmic microtubules whereas the MTOCs of other eukaryotes (centrosomes) reside in the cytoplasm near the NE and do not nucleate the spindle microtubules until after the NE dissolves. In many species where the NE breaks down during mitosis, the NPCs also disassemble, and reform as the nuclear membrane encases the chromatin.

1.2 Nuclear transport

A large variety of molecules travel across the nuclear envelope (Figure 1.1). Ions, metabolites and peptides freely pass across the NE (Paine et al., 1975). Messenger RNA, snRNA, tRNA, ribosomes and some nuclear proteins are actively exported to the cytoplasm (Zasloff, 1983; Bataille et al., 1990; Elliott et al., 1994). Simultaneously, proteins, snRNPs and virus particles (De Robertis et al., 1982; Yamada and Kasamatsu, 1993) are imported into the nucleus. Much of the early characterization of the nuclear transport process was achieved by assays involving microinjection of labeled nuclear transport substrates into frog oocytes or mammalian tissue culture cells. This type of assay is useful because one can determine the rate, the extent and the specificity of movement of molecules between compartments *in vivo*, using natural or synthetic substrates. The transport of macromolecules to their proper destinations is highly specific. Large concentration gradients of specific substrates can be maintained across the nuclear envelope (Bonner, 1975; De Robertis et al., 1978). For example, some proteins are 100 fold more concentrated in the nucleus than the cytosol (Dingwall et al., 1982). The quantity and rate of flow of macromolecules across the NE is astronomical. In addition to other transport substrates, histone proteins sufficient to double the chromatin content of the nucleus must be imported every cell cycle. This translates to approximately 100-500 histone molecules/minute/pore (Silver,

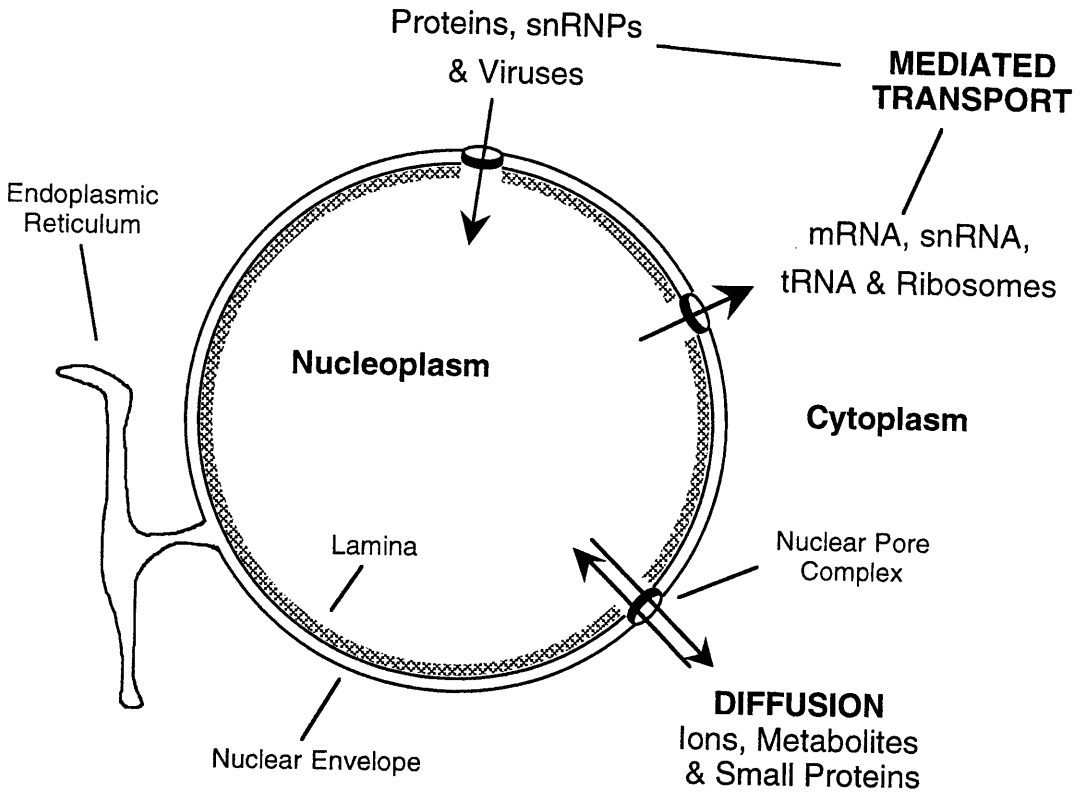
1991). There are two possible mechanisms for this rapid and specific exchange: a) high affinity receptors for each substrate within the target compartments and passive diffusion until equilibrium is reached, or b) mediated, energy dependent, substrate specific transport. The second model is favored based on kinetic data determined for injected proteins. For example, uptake of the *Rana pipiens* nuclear protein RN1 was determined to be at least 20-fold faster than the maximum possible rate of diffusion of a protein of this size (Feldherr et al., 1983). This import is saturable: addition of a large excess of free signal peptide inhibits the import of the labeled conjugate, further suggesting that import is a receptor mediated process. Mechanistic evidence discussed below confirms that the import of proteins is signal mediated and depends on receptors in the cytoplasm and/or NPC .

Import into nuclei is a saturable (Goldfarb et al., 1986), nucleotide triphosphate dependent process that occurs in two separable steps: binding to the NPC, followed by translocation into the nuclear interior (Newmeyer et al., 1986; Richardson et al., 1988). Both *in vivo* and *in vitro*, deprivation of ATP or chilling leads to accumulation of import substrate on the cytoplasmic face of the NPC. This accumulation has been interpreted as evidence for binding to the NPC as an intermediate step in import. Blocks at the binding step are rapidly reversible: addition of ATP or increase in temperature stimulate transport of the substrate into the nucleus.

The NPC is the site of transport

The best evidence that the nuclear pore is the site of transport of macromolecules across the NE comes from the analysis of synthetic transport substrates conjugated onto gold beads that have been injected into either the cytoplasm or the nucleoplasm. These gold particles can be observed traversing the NE via NPCs by transmission electron microscopy (Feldherr et al., 1984). Particles are never observed in the NE lumen, which would be an expected intermediate if import occurred by a vesicular budding process. The NPC can accommodate a wide range of particles including gold beads bearing targeting signals as large as 26nm (Feldherr and Akin, 1990). Constitutive, passive channels accept diffusion of unconjugated particles up to 9nm (Dworetzky et al., 1988). Each NPC is apparently competent for both the

Figure 1.1 Transport across the nuclear envelope


Proteins, snRNPs
& Viruses
MEDIATED
TRANSPORT
Endoplasmic
Reticulum
mRNA, snRNA,
tRNA & Ribosomes
Nucleoplasm
Cytoplasm
Lamina
Nuclear Pore
Complex
DIFFUSION
Ions, Metabolites
& Small Proteins
Nuclear Envelope

import and the export function (Dworetzky and Feldherr, 1988) because when protein and RNA coated gold beads are simultaneously injected into the same cell, both types of particles associate with the same NPCs. In some studies, import substrates conjugated to gold particles have been observed "queuing" on cytoplasmic fibrils emanating from the NPC (Richardson et al., 1988). These fibrils could be an extension of the NPC that is involved in the recognition step of import. Once the import substrate reaches the NPC, it may interact with a series of sites in the NPC prior to translocation (Akey and Goldfarb, 1989). By injecting nucleoplasmin-gold into oocytes, dissecting out the nuclear envelopes, and visualizing NPCs and bound particles by cryo-electron microscopy, it was shown that the substrate binds to three discrete rings on the surface of the NPC. It was suggested that these rings represent progressive movement through sites that correspond to a series of transport intermediates.

Signals for protein import

Labeled nuclear proteins injected into the cytoplasm of Xenopus oocytes accumulate rapidly into the germinal vesicle (nucleus) (Bonner, 1975; De Robertis et al., 1978). The fact that the uptake of proteins is specific implies the existence of cis-acting signal sequences in proteins that are recognized by the import mechanism. Such a signal sequence or "nuclear localization sequence" (NLS) was first identified by microinjecting radiolabeled derivatives of nucleoplasmin into the cytoplasm of Xenopus oocytes (Dingwall et al., 1982). It was determined that the carboxy terminal tail of nucleoplasmin is necessary for concentration of the labeled protein into the nucleus because a proteolytically derived amino-terminal fragment of nucleoplasmin was not imported. It was also suggested that the import mechanism was a form of selective migration rather than selective affinity for the nuclear interior because nucleoplasmin missing the tail injected into the nucleus was retained, demonstrating that nuclear affinity is not sufficient for transport.

The NLS sequences of many proteins have since been characterized. Although a consensus sequence *per se* has not been identified, NLS sequences tend to be highly basic stretches of 7-10 amino acids that have the capability of targeting a heterologous protein to the nucleus. An active signal sequence

with the property of directing a heterologous protein to the nucleus was first shown for the Matα2 transcriptional repressor (Hall et al., 1984). The best characterized NLS sequence to date is that of the SV40 large T antigen (Lanford and Butel, 1984; Kalderon et al., 1984). This signal is very effective for targeting heterologous proteins to the nucleus. A V_{max} of $6.4X10^9$ molecules/cell/min was measured for a SV40 virus large T antigen signal peptide/bovine serum albumin conjugate injected into Xenopus oocytes (Goldfarb et al., 1986). A single residue change in the T antigen sequence (Lys128->Thr) leads to cytoplasmic mislocalization of the protein. The NLS of the nucleoplasmin protein was more difficult to determine because it requires a bipartite set of sequences to function effectively (Burglin and De Robertis, 1987; Dingwall et al., 1988). Interestingly, some NLS's may consist in part of sugar residues, because some sugars are sufficient to direct import of albumin into the nucleus (Duverger et al., 1993). However, most nuclear protein NLS sequences probably interact with a single recognition system, because the import of many nuclear proteins can be inhibited by SV40 T antigen NLS peptide (Michaud and Goldfarb, 1993). It is possible that not every nuclear protein has a specific sequence that is recognized by the uptake machinery. It is clear that some multiprotein complexes traverse the pore as a unit (e.g. the ribosome), therefore some proteins may rely on the NLS sequences of other proteins with which they are associated.

Although these defined short NLS sequence segments are capable of directing heterologous proteins into the nucleus, they are not necessarily completely independent of their context. Fusing the same NLS sequence to different reporter proteins can lead to different rates of import (Nelson and Silver, 1989). Moreover, well-defined sequences such as SV40 large T antigen NLS may not be the only determinant within the protein that is normally recognized by the import apparatus, because careful measurement of the kinetics of import of derivatives reveals the minimal signal is significantly less efficient at nuclear entry than the whole T antigen molecule or a derivative that contains residues 111-125 in addition to the canonical signal sequences (Nelson and Silver, 1989). This stretch of sequence is not a NLS by itself, suggesting that this sequence affects recognition through the previously defined NLS region.

The recognition function of the nuclear transport mechanism is well conserved. In fact, the nuclear localization sequences of many proteins will

function in heterologous organisms. The NLS from the SV40 mammalian tumor virus can direct import of protein fusions into the yeast nucleus *in vivo* and *in vitro* (Nelson and Silver, 1989; Schlenstedt et al., 1993). The NLS of the yeast transcription factor Mcm1 is sufficient to direct it into the nucleus of frog oocytes (Wagner and Hall, 1993), and the gluticocorticoid receptor transcription factor functions in yeast (Privalsky et al., 1990; Schena and Yamamoto, 1988), which is strong evidence that its NLS is functional.

The import of some snRNPs into the nucleus probably depends on separate sets of recognition factors from those involved in protein import (Michaud and Goldfarb, 1992; Michaud and Goldfarb, 1991; Baserga et al., 1992; Fischer et al., 1991). The lectin wheat germ agglutinin (WGA), which is known to prevent the import of many proteins into the nucleus, inhibits nuclear uptake of U6, but not of U1, U3 or U5 snRNPs. Conversely, a 2,2,7mGpppG dinucleotide analog of the trimethyl cap structure added to snRNAs in the cytoplasm inhibits transport of the polII U snRNAs, but does not detectably affect the transport of U3, U6 snRNA or a karyophilic protein. Therefore U6 enters the nucleus by a pathway similar or identical to that used by karyophilic proteins whereas U1 and U5 are recognized by a distinct pathway and U3 by a third.

Shuttling proteins

Some proteins continuously cycle between the cytoplasm and the nucleus (Goldfarb, 1991; Laskey and Dingwall, 1993). This behavior has been termed "shuttling". A diverse group of proteins including the catalytic subunit of cAMP-dependent protein kinase type II (Nigg et al., 1985), nucleolar proteins nucleolin and No38 (Borer et al., 1989), the yeast RNA binding protein Npl3 (Flach et al., 1994), pre-mRNA binding proteins (Borer et al., 1989; Pinol-Roma and Dreyfuss, 1992) and HSP70 homologs (Mandell and Feldherr, 1990) have been shown to shuttle. In most cases the role of shuttling in terms of protein function is unclear. In the case of the heat shock proteins, it has been suggested that they act as chaperones that accompany nuclear proteins through the NPC in order to prevent their aggregation, whereas the RNA binding proteins are proposed to have an analogous role in RNA export. Proteins that shuttle may not need specific signals for export from the nucleus, because some NLS sequences from proteins that are

retained within the nucleus induce shuttling behavior when fused to reporter proteins (Schmidt-Zachmann et al., 1993). It was concluded that export of proteins from the nucleus may be a general process that is inhibited in some nuclear proteins as a function of their affinity for intra-nuclear structures. Therefore the nucleocytoplasmic equilibrium of an imported protein is probably a function of its intrinsic rate of import and its affinity for sites within the nucleus.

Nuclear export

Although the mechanisms of protein import into the nucleus are increasingly well understood, the properties of the reciprocal passage of RNA from the nucleus has been more difficult to elucidate. This has been a complicated problem because RNA trafficking is tightly intertwined with RNA processing. This interdependence clouds experimental results derived from the injection or addition of mature RNA transport substrate to the cell. Furthermore, it has not been possible to develop an *in vitro* system that mimics export for the simple topological fact that it is not possible to add exogenous substrate to the inside of nuclei. Furthermore, genetic approaches towards this problem have suffered from the fact that mutants selected for RNA export defects tend to have extremely pleiotropic phenotypes that make it difficult to extrapolate a mechanism. Despite these technical difficulties, it is clear that RNA export is also a selective, mediated process. Furthermore, recent evidence suggests there is considerable overlap between the components of the import and export pathways.

Messenger RNAs have been observed passing out from the nucleus into the cytoplasm via the NPC using electron microscope tomography on the very large transcripts encoded by *Chironomus tentans* Balbiani rings (Mehlin et al., 1992). In this case it was shown that mRNA travels through the pore in the 5' to 3' direction and that the RNA is packaged as an RNP particle. It is not known whether this polarity in transport is a general phenomenon. Like protein import, mRNA export is saturable and specific, as mRNAs injected in Xenopus oocyte nuclei compete with each other for export (Dargemont and Kuhn, 1992), and intron containing messages are largely restricted to the nucleus. Pre-mRNAs are organized into discrete domains within the nucleus that probably represent sites of processing (Carter et al., 1993; Xing et al., 1993).

Some mRNAs can be seen as "tracks" that originate at their site of transcription, pass through processing foci and ultimately lead to a single NPC (Lawrence et al., 1989; Rosbash and Singer, 1993).

Little is known about the cis-acting signals required for the export of processed mRNA (Izaurralde and Mattaj, 1992). Monomethlyated 5' caps on mRNA are necessary for nuclear export (Hamm and Mattaj, 1990). Export of polymerase II-transcribed RNAs were inhibited by the cap analog m7GpppG. However, it is unlikely that this signal is sufficient for export, as capped splicing intermediates are rarely detected in the cytoplasm. Recognition steps in the export of various classes of nuclear RNA appear to be mediated by different factors. Using microinjection into Xenopus oocytes, it was found that a tRNA, a U snRNA, and a mRNA competitively inhibit their own export at concentrations which do not affect the export of heterologous RNAs (Jarmolowski et al., 1994) . Therefore, there are at least three pathways for the targeting of RNA to the cytoplasm. A candidate nuclear protein has been isolated that binds to monomethylated mRNA caps *in vitro* (Izaurralde et al., 1992). This protein may be required for the recognition of mRNAs for export, however a direct functional role has yet to be demonstrated.

In order to elucidate the mechanism of nuclear transport, and to identify other as yet unknown functions of the NPC, three general approaches have been employed. The first approach has been to determine the structure of the nuclear pore complex with ever increasing resolution. Clues towards the mechanism of transport such as the interaction between transport apparatus components, binding sites for substrate or gross movements made by the NPC during the transport reaction may be found in detailed study of the three-dimensional structure of this large organelle. Another means to study this process has been to try to methodically isolate and clone all the components of the NPC based purely on their intracellular localization. This strategy does not presuppose any function for a given component, but the assumption is that cataloguing each subunit of the NPC is a necessary step towards ascribing function and mechanism. Furthermore, identifying these genes enables a variety of forms of "reverse genetics" that permit determination of function by both *in vivo* and *in vitro* experiments. Recently, *in vitro* assays that mimic all or part of the protein import reaction have been developed. Because these assays use crude extracts of cytoplasm to support import, it has been possible to fractionate these extracts, and purify

and clone factors that are essential for nuclear transport. This technique has been very successful for definition of the soluble part of the import system. The following sections will describe progress with each of these approaches in turn, followed by description of some recent experiments that are beginning to establish links between proteins identified purely by localization and those isolated by function that lead to an emerging model for the import mechanism.

1.3 The ultrastructure of the nuclear pore complex

The NPC is the major feature of all eukaryotic nuclear envelopes. It is the only observable structure on the surface of nuclei that could be responsible for nucleocytoplasmic trafficking. The first observation of the NPC by electron microscopy was by Callan and colleagues in 1949 (Callan et al., 1949). The morphology of the NPC has been refined several times by increasingly sophisticated electron microscopic techniques. The general ultrastructure of the nuclear pore complex does not vary significantly among different organisms (Allen and Douglas, 1989; Newport and Forbes, 1987). Therefore, conclusions drawn from the structure of the NPC from any species may be applied to others with reasonable confidence.

The NPC has been estimated by scanning transmission electron microscopy to have a molecular mass of 10^8 daltons (for comparison, a ribosome is about $4X10^6$ daltons) (Reichelt et al., 1990). Greater than 80 different proteins can be identified in highly purified yeast nuclear pores (Rout and Blobel, 1993). The NPC is an extremely large cellular transporter: the reported molecular mass is approximately 300 fold that of a gap junction (Rout and Wente, 1994). The density of NPCs on the NE varies by species and cell type in a range between 3 and 80 NPC/μm^2 (Franke, 1974). The nuclear pore density is thought to reflect the metabolic activity of the cell (Maul et al., 1980). In vertebrates, NPCs disassemble during the entry into mitosis. The components of the NPC become dispersed throughout the cytoplasm. Following mitosis, the nuclear envelope reforms around the chromatin and new pores assemble into the membrane (Maul, 1977). Therefore, the NPC is a dynamic complex, whose assembly and disassembly must be tightly regulated.

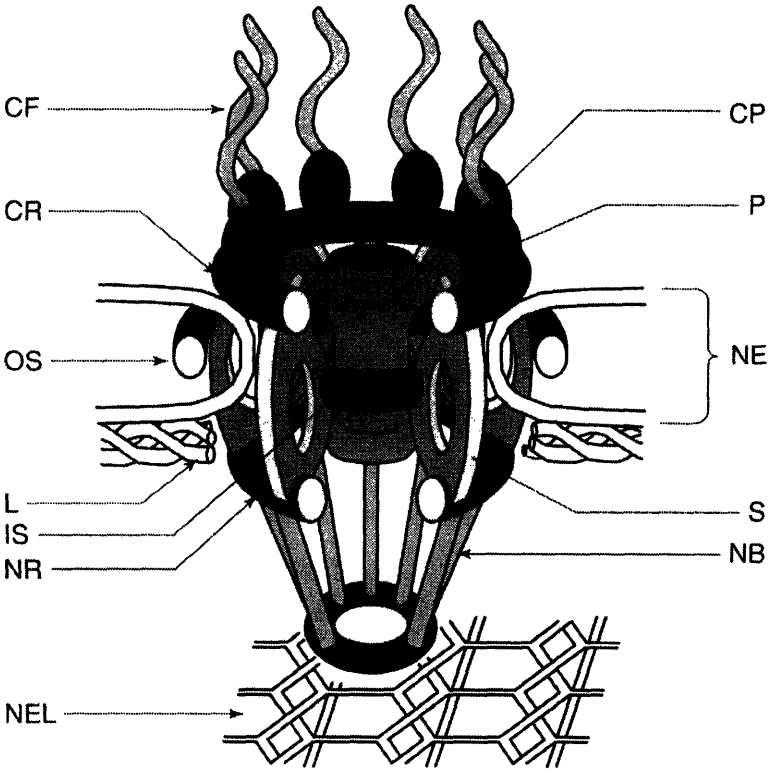
The current model for the structure of the NPC is shown in Figure 1.2. The development of this model is reviewed by Pante and Aebi (1993) and Rout and Wente (1994). The most striking feature of the NPC are two rings,

Figure 1.2 The stucture of the nuclear pore complex

A summary of the current model for the three dimensional structure of the NPC and associated structures is shown. The cytoplasmic side is uppermost.

CF: cytoplasmic filament
P: cytoplasmic particle
CR: cytoplasmic ring
OS: outer spoke ring
IS: inner spoke ring
NR: nuclear ring
S: spoke
CP: central plug
NB: nuclear basket (fishbasket)
NE: nuclear envelople
L: nuclear lamina
NEL: nuclear envelope lattice

Adapted from Rout and Wente, 1994.


CF
CP
CR
P
OS
NE
L
IS
NR
S
NB
NEL

each of eight globular subunits positioned on opposing surfaces of the nuclear envelope membranes. The outer diameter of the rings is 130nm and the inner diameter is 80nm (Unwin and Milligan, 1982; Akey, 1989; Akey, 1990; Akey and Radermacher, 1993; Goldberg and Allen, 1993; Hinshaw et al., 1992). The NPC cylinder is about 70 nm thick. A group of spokes or struts extends from each subunit into the center of the pore. The overall structure of the spoke complex has 8-2-2 symmetry (Hinshaw et al., 1992). Each spoke subunit may consist of as many as 20 polypeptides (Hinshaw et al., 1992). Between each spoke subunit is a 10 nm "peripheral channel" that may be the site of diffusion of small molecules through the NPC. The center of the pore is filled with a less well-defined structure sometimes called the "transporter" or central granule. Fibrils have been observed emanating from both the inner and outer rings (Richardson et al., 1988; Whytock and Stewart, 1988). The inner ring is the base for another structure with eight fold symmetry known as the fishbasket or cage (Aebi et al., 1990; Jarnik and Aebi, 1991; Ris, 1989; Ris, 1991; Stewart, 1992). This structure extends as much as 100 nm into the nucleoplasm. The fishbasket has been shown to interact with nucleoskeletal elements (Goldberg and Allen, 1992).

1.4 Components of the nuclear pore complex

The isolation of proteins on the basis of their position within the nuclear pore complex has been an extremely fruitful approach towards unraveling the nucleocytoplasmic transport process. The foundation for this line of research was the availability of monoclonal antibodies specific for nuclear pore antigens. These were produced by immunizing mice with the nuclear envelopes or nuclei of rat liver (Gerace et al., 1982; Davis and Blobel, 1986). Once appropriate antibodies were identified, it was possible to characterize the protein products that they recognize, and to begin to address their function. The next advance was the use of these antibodies raised against mammalian nucleoporins to isolate the genes for homologs to this protein family from the budding yeast *Saccharomyces cerevisiae*. Two families of nucleoporin proteins have been identified in yeast to date. Because the NPC of Saccharomyces has a similar ultrastructure to that of vertebrates (Allen and Douglas, 1989), it has been assumed that the structure of the NPC would be conserved on a protein by protein basis. Yeast have

proved a facile system to determine phenotypes of loss of function mutations of these genes, to develop genetic screens to identify new interacting and related genes, and to study physical interactions among cloned genes. Because intact NPCs have been purified from yeast (Rout and Blobel, 1993), and the DNA sequence of the Saccharomyces genome by the Yeast Genome Project (Dujon, 1993) is nearly complete, most yeast nuclear pore constituents should be identified, cloned and sequenced in the near future.

Complementing the work on yeast NPC constituents, several vertebrate nucleoporins have now been cloned. Comparison of the genes from different species has indicated significant differences, although the same gene families are represented. Although it is more difficult to perform functional studies in vertebrate cells, a number of interesting observations have been made particularly in the areas of biochemical interactions between pore components and sublocalization of theses proteins within the NPC. Recent evidence from both yeast and vertebrate systems demonstrates that these families of nucleoporin genes are directly involved in the nuclear transport process. A catalog of NPC constituents that have been purified and/or cloned to date is shown in Tables 1.1 and 1.2.

Nucleoporins

The immunological approach to the identification of nuclear pore components was initiated by raising monoclonal antibodies against partially purified fractions containing the nuclear envelope, the NPC and the nuclear lamina from rat liver nuclei (Davis and Blobel, 1986; Park et al., 1987; Snow et al., 1987). Because the NPC represents a major portion of this fraction many antigens present in this fraction are NPC components. These monoclonal lines were screened by indirect immunofluorescence for pore specific antibodies. The specificity of some of these antibodies for the NPC was confirmed by immunoelectron microscopy. Monoclonal antibodies that decorated the NPC recognized families of proteins, which were dubbed "nucleoporins". During mitosis in vertebrates, the localization of these antigens changes to a dispersed, cytoplasmic pattern as would be expected for NPC antigens in cells where the NE and NPCs are completely disassembled at each division. The most thoroughly studied nucleoporin protein is an approximately 62 kilodalton protein named p62. This protein is synthesized

Table 1.1 ***Saccharomyces*** **NPC proteins**

protein	sequence motifs	structure/function	reference
Nup49	GLFG	essential, required for docking step in import in vitro, import defect *in vivo*, accumulates poly(A)$^+$ RNA in nucleus, in Nsp1 complex	Wente et al., 1992 Rout and Blobel, 1993; Schlenstedt et al., 1993; Wimmer et al., 1992 Grandi et al., 1993 Doye et al., 1994; Grandi et al., 1995
Nup57	GLFG	in Nsp1 complex, essential	Wente et al., 1992 Rout and Blobel, 1993 Grandi et al., 1993; Grandi et al., 1995
Nup1	GLFG & XFXFG	essential, mutants affect protein import, RNA export and nuclear structure. physically interacts with Srp1	Davis and Fink, 1990; Loeb et al., 1993 Belanger et al., 1994; Bogerd et al., 1994 Grandi et al., 1995
Nup2	XFXFG	physically interacts with Srp1, Gsp1, genetically interacts with Nup1 and Nsp1	Belanger et al., 1994; Loeb et al., 1993
Nsp1	GLFG & XFXFG	in 240kD complex with Nup49, Nic96 and other proteins	Hurt, 1988; Hurt, 1990 Nehrbass et al., 1990 Wimmer et al., 1992 Schlenstedt et al., 1993 Grandi et al., 1993; Rout and Blobel, 1993
Nic96	no homologies	in Nsp1 complex, depletion leads to accumulation of nuclear proteins in the cytoplasm	Grandi et al., 1993; Grandi et al., 1995
Nup100	GLFG	interacts genetically with Nup116, binds RNA	Wente and Blobel, 1993; Wente et al., 1992
NUP133/ Rat3	no homologies	interacts genetically with NUP49, RNA export defect, deletion is temperature sensitive, NPC clustering defect	Doye et al., 1994; Li et al., 1995
Nup116	GLFG	deletion mutants affect local NE structure, binds RNA	Wente and Blobel, 1993; Wente et al., 1992
Nup145/ Rat 10	GLFG	deletion mutants affect local NE structure, binds RNA, essential	Wente and Blobel, 1994
Pom152	no homologies	membrane protein	Wozniak et al., 1994
Srp1	ARM repeats	nuclear import receptor: interacts genetically and physically with Nup1 and Nup2	(Yano, et al. 1992) Belanger et al., 1994; Yano et al., 1994
Cse1	no homologies	interacts genetically with Srp1	Xiao et al., 1993

Animal NPC proteins

protein	mass (kD)	sequence motifs	structure/function	reference
xp54			in complex with p58 and p62	Finlay et al., 1991
xp58			in complex with p54 and p62	Finlay et al., 1991
r,xp62		XFXFG	anti-p62 antibodies inhibit nuclear import, in 230kD complex with p54 and p58, carboxy terminal domain has heptad repeats, O-glycosylated	Davis and Blobel, 1986 Carmo-Fonseca et al., 1991; D'Onofrio et al., 1988; Starr et al., 1990 Cordes et al., 1991 D'Onofrio et al., 1991 Finlay et al., 1991 Vigers and Lohka, 1992 Kita et al., 1993 Pante et al., 1994
r,Xp97		GLFG	required for DNA synthesis in vitro, binds to importin/Srp1 homolog	Powers et al., 1995 Radu et al., 1995
rNUP107			unique sequence, putative leucine zipper	Radu et al., 1994
rPOM121		XFXFG	membrane protein	Snow et al., 1987 Hallberg et al., 1993
rNUP153		XFXFG	binds DNA	Cordes et al., 1993; McMorrow et al., 1994; Pante et al., 1994; Sukegawa and Blobel, 1993
rNUP155			unique sequence	Radu et al., 1993
rNUP180			localized in cytoplasmic rings/filaments	Wilken et al., 1993
rgp210	190		membrane protein, no homology to other pore membrane proteins, major membrane protein of the NPC antibodies against the lumenal domain block import	Gerace et al., 1982; Greber et al., 1990; Wozniak et al., 1989; Wozniak and Blobel, 1992
hNUP214	225-250		localized to cytoplasmic face of NPC may be an oncogene	Kraemer et al., 1994; Pante et al., 1994
rp260 h tpr-L	260			Pante et al., 1994
d-germ cell-less			localized to NPC in germ cells in drosophila embryo, required for germ cell development	Jongens et al., 1994; Jongens et al., 1992
c elegans nup with RanBP1 domain		XFXFG	homologous to RanBP1 and Nup2	

as a 61kD precursor which is slowly modified to the mature 62kD size prior to its incorporation into the NPC (Davis and Blobel, 1986). It was subsequently determined that the modification of p62 and other nucleoporins was the addition of a novel form of O-linked glycoslylation (Cordes and Krohne, 1993; Davis and Blobel, 1987; Holt et al., 1987; Miller and Hanover, 1994). Single N-acetyl glucosoamine residues are covalently added to nucleoporins in the cytoplasm. This modification has been very useful for the study of nucleoporins because the lectin wheat germ aggluntin (WGA) specifically binds to O-linked N-acetyl glucosamine with high affinity and because this modification is found in very few proteins. Therefore it has been possible to label or purify this family of proteins as a group using WGA as an affinity probe.

Monoclonal antibodies directed against mammalian nucleoporins cross-react with a number of yeast proteins (Aris and Blobel, 1989). It was concluded that these proteins are constituents of the NPC because these antibodies specifically labeled NPCs by both immunofluorescence and immunoelectron microscopy. Furthermore, some of these yeast proteins have similar fractionation properties to their mammalian counterparts. A 130kD protein (Nup1) was cloned from a lambda phage expression library using an antibody against mammalian nucleoporins (Davis and Fink, 1990). *NUP1* was sequenced and found to be divided into three domains, two charged terminal regions with no homologies to other proteins and a central repetitive domain. This central domain is comprised of more than twenty repeats of nine residues centered on the sequence phenylalanine-polar-phenylalanine. The repeats (which are now known as XFXFG motifs) are separated by highly charged sequences of varying lengths. The XFXFG domain was found to be homologous to another gene, *NSP1*, which was isolated by reactivity with polyclonal sera produced against an insoluble fraction purified from yeast nuclei (Hurt, 1988). Nsp1 is a 100 kilodalton protein that was originally thought to be a component of the nucleoskeleton. This protein is also recognized by some of the antibodies raised against mammalian proteins. The repeats are likely to constitute the antigen recognized by anti-nucleoporin antibodies, because the homology between Nsp1, Nup1 and other nucleoporins is confined to this domain and because derivatives of Nsp1 that lack the repeated domain are not recognized by these antibodies (Davis and Fink, 1990; Loeb et al., 1993).

Both the *NSP1* and *NUP1* genes were demonstrated to be essential for viability by replacement of one copy of their coding sequence by a selectable marker gene by homologous recombination in a diploid, followed by tetrad analysis (Hurt, 1988; Davis and Fink, 1990). I isolated a gene for a third yeast XFXFG nucleoporin, Nup2 (Loeb et al., 1993); described in detail in Chapter 2. Unlike *NSP1* and *NUP1*, deletion of the *NUP2* coding sequence has no detrimental effect on the growth of yeast during standard laboratory culture. The protein products of these three XFXFG nucleoporins were shown to be localized in the NPC by indirect immunofluorescence with specific antibodies (Nehrbass et al., 1990; Davis and Fink, 1990). Unambiguous localization of Nup1 and Nup2 required the introduction of foreign epitopes into these proteins (Kolodziej and Young, 1991), because monospecific antibodies were not available. The position of these proteins within the NPC was not possible to ascertain due to the poor preservation of NPC structure in yeast immunoloelectron microscopy preparations. Anti-nucleoporin antisera have been useful in yeast to document the dynamics of the NE during the cell cycle by immunofluorescence. Consistent with the results determined by electron microscopy (Byers, 1981), it was found that neither the nuclear pores nor the NE break down during mitosis. Therefore, these antibodies can be used as a marker for nuclear behavior throughout the cell cycle (Davis and Fink, 1990; Copeland and Snyder, 1993).

The carboxy terminal domain of Nsp1 has a heptad repeat structure and therefore has been predicted to form an alpha-helical coiled coil structure (Nehrbass et al., 1990). Expression of this domain is sufficient to complement the deletion of the *NSP1* gene, so the conserved XFXFG repeated domain is dispensable in this protein. Mutation of glutamate 706 to proline within the heptad repeat region, theoretically a helix breaking change, results in temperature sensitive growth. This was taken as evidence that the carboxy terminal domain indeed forms a helix *in vivo*. Furthermore, the carboxy terminal domain is sufficient to target a heterologous protein, dihydrofolate reductase, to the NE (Hurt, 1990). Surprisingly, this fusion protein can also complement the deletion of the *NSP1* gene.

A number of genes for mammalian members of the XFXFG family have been cloned. The first was p62 from rat (D'Onofrio et al., 1988; Starr et al., 1990). p62 was first immunopurified from rat liver nuclei, and tryptic fragments were microsequenced. The p62 cDNA encodes a protein 525 amino

acids in length that is very similar in overall structure to Nsp1. Although p62 does not have a homologous amino-terminal domain, it has a XFXFG domain followed by heptad repeats homologous to *NSP1*. Furthermore, antibodies specific to Nsp1 in yeast extracts recognized p62 in human cell extracts (Carmo-Fonseca et al., 1991). p62 has subsequently been cloned from human, mouse and Xenopus cells (Carmo-Fonseca et al., 1991; Cordes et al., 1991). The heptad repeat regions of these proteins are about 80% identical whereas the XFXFG domains are more variable.

Although the primary sequence of yeast nucleoporins has not been particularly useful in terms of providing functional information via homology, some intriguing information has been uncovered within the sequence of mammalian nucleoporins. pom121 (**p**ore **m**embrane 121) is an interesting member of the XFXFG class because in addition to a weak set of repeats it also has a single transmembrane domain. It was purified from rat liver on the basis of its affinity for WGA (Hallberg et al., 1993). Most of the pom121 protein, including the XFXFG repeats, is on the cytoplasmic side of the NE membrane, consistent with a role in NPC structure, perhaps linking the NPC into the NE membrane (Soderqvist and Hallberg, 1994). Another XFXFG nucleoporin that has been recently cloned is nup214 (Kraemer et al., 1994). Interestingly this protein is closely related to the human CAN gene that was identified as an oncogene involved in the development of myeloid leukemias. It is unclear whether the role of CAN in oncogenesis is related to its normal function within the NPC.

Rat nup153 was isolated on the basis of its reactivity with an anti-nucleoporin antibody (Sukegawa and Blobel, 1993; McMorrow et al., 1994). This gene encodes the 180kD nucleoporin first described by Snow *et al.* (1987). Embedded within its 37 iterations of the XFXFG motif is a domain with four repeats similar to the Cys_2-Cys_2 family of zinc finger DNA binding proteins. The Cys_2-Cys_2 domain of Nup153 expressed in *E. coli* was shown to bind non-specifically to DNA in a zinc-dependent fashion. Furthermore, it was found that the nup153 protein is limited to the nucleoplasmic face of the NPC by immuno-electron microscopy with a monospecific antibody. It was concluded that nup153 might be a linkage point between the NPC and chromatin based on its DNA binding activity and its location on the nuclear side of the NPC. However, it has not been shown that nup153 binds DNA *in*

vivo, therefore the possibility exists that the DNA binding observed *in vitro* was an artifact due to production of this domain out of its normal context.

A second, unrelated family of nucleoporin proteins has also been identified by anti-NPC antibodies. This family is characterized by a different repeated domain, typified by the repeated GLFG motif (Wente et al., 1992; Wimmer et al., 1992). Genes for the Nup49, Nup100 and Nup116 proteins were isolated by an immunological screen with a monoclonal antibody raised against rat nuclear envelopes with non-overlapping specificity to those that recognize XFXFG nucleoporins (Wente et al., 1992). *NUP49* and *NUP116* were also isolated as mutations that are incompatible with a temperature sensitive mutation in *NSP1* (Wimmer et al., 1992). *NUP49* was found to be essential for cell viability, *NUP100* non-essential, and deletions of *NUP116* were found to have a temperature sensitive phenotype (Wente and Blobel, 1993). Two more members of this family have since been identified Nup145 (Wente and Blobel, 1994; Fabre et al., 1994) and Nup57 (Grandi et al., 1995). These five proteins are likely to represent all of the GLFG nucleoporins in yeast because together they account for all the bands on western blot of total yeast protein probed with a monoclonal antibody raised against the GFLG domain (Grandi et al., 1995).

One GLFG nucleoporin from vertebrate cells has been reported (Powers et al., 1995). Like pom121, the nup97 protein was purified on the basis of its binding to WGA sepharose. Immunofluorescence with a specific antibody reveals the signature punctate rim staining common to all NPC antigens. Microsequence was obtained that includes a peptide with one GLFG motif and other peptides with weak homology to conserved regions in the yeast GLFG proteins. Conclusive assignment of this protein to the GLFG family awaits the cloning of its gene.

The immunological cross reactivity that characterizes these families of proteins is clearly due to the conservation of repeated domains among the family members. Two subfamilies exist that display limited overlap, both families are modified in vertebrates by the addition of O-linked N-acetyl glucosamine. It is unclear whether these proteins are also glycosylated in yeast. It is also unknown whether these modifications are required for assembly or function of nucleoporins. Although both families are conserved from *Saccharomyces* to vertebrates, there is only one case where significant homology outside the repetitive domains makes it possible to conclude that a

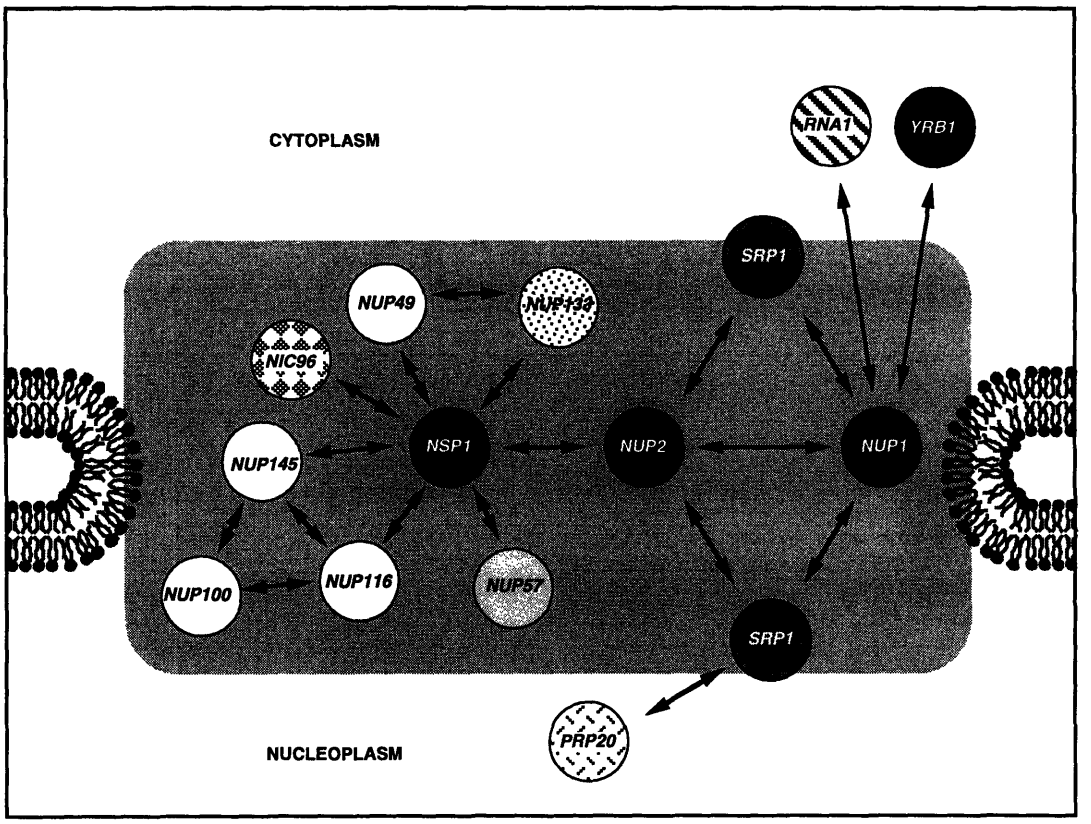
mammalian and a yeast protein are analogous. Furthermore, no vertebrate genes have been isolated that can complement any of the yeast mutants. Therefore, while the overall form of the NPC is conserved among all eukaryotes, there are significant differences on the molecular level that complicate generalizations about NPC function based on the results from either system.

Nucleoporin genetics

Once the first nucleoporin proteins were cloned from *Saccharomyces*, it became possible to use reverse genetic approaches to construct mutations in these genes *in vitro* and to subsequently analyze their effects. Two classes of mutations have been made in these genes: knock-outs, removal of the wild-type gene from the genome produced by homologous recombination, and point mutations that preserve the gene product but introduce a small defect. In addition to phenotypic analysis it also is feasible to study interactions between mutations in these genes and to screen for mutations in additional nuclear pore constituents by utilizing the powerful genetic techniques available for this organism (a summary of genetic relationships among nucleoporins and between nucleoporins and other genes is shown in Figure 1.3).

One obvious question to address by genetic means was the relationships between members of the nucleoporin gene families. Why does the cell require a family of similar proteins? In the case of the XFXFG nucleoporins, we found that while Nup2 is not essential, deletion of the *NUP2* gene is not tolerated in cells that also have mutations in the termini of either *NUP1* or *NSP1* (Loeb et al., 1993). Synthetic lethality such as this has been a useful method to identify genes that have one of two general relationships with the target gene. Mutations in genes that have redundant functions, or genes that participate in the same pathway may be lethal in combination. Therefore, based upon their similarity, we proposed that the three XFXFG nucleoporins have redundant, or overlapping function. However, these three proteins do not have significant homology outside of their central repetitive domains and they cannot completely substitute for one another, so they must have unique functions, in addition to those they share. Similarly, the GLFG nucleoporins Nup100 and Nup116 are homologous over their length. Because neither is essential for life, it was

Figure 1.3 Genetic interactions between nuclear pore components. A schematic diagram of the NPC depicting some components from which mutants have been isolated. Synthetic lethality between pairs of mutants is shown by an arrow. Shading of components indicates sequence similarity.


CYTOPLASM
RNA1
YRB1
SRP1
NUP49
NUP159
NIC96
NSP1
NUP2
NUP1
NUP145
NUP100
NUP116
NUP57
SRP1
PRP20
NUCLEOPLASM

informative to demonstrate that simultaneous deletion of both genes was inviable (Wente et al., 1992).

The first attempt to isolate additional nuclear pore components by a genetic approach was a synthetic lethal screen based on a temperature sensitive mutant of the *NSP1* gene (Wimmer et al., 1992). Mutations were isolated that depended on the presence of the wild type *NSP1* gene for viability. Initially the genes for two such mutants were cloned and they were found to be identical to the *NUP49* and *NUP116* genes described by Wente *et al.* (1992). This result also demonstrated that the two subclasses of nucleoporins, the XFXFG family represented by *NSP1* and the GLFG family, are somehow involved in the same processes. This screen finally resulted in 11 complementation groups including *nup49, nup57, nic96, nup116, nup145, nup84, nup85, nup188* and *los1* (Grandi et al., 1995; Shen et al., 1993). A similar screen based on a temperature sensitive mutation in *NUP49* yielded a temperature sensitive mutation in the *NUP133* gene (Doye et al., 1994). Nup133 is a novel nuclear pore protein that does not have signature nucleoporin repeated domains.

Another genetic screen that has yielded interesting components of the transport apparatus was described by Belanger, et al. (1994). This screen utilized the observation that the deletion of the *NUP1* gene is not lethal in all laboratory strains. Therefore it was possible to look for mutations in other genes that lead to inviability in combination with the *nup1* deletion. Sixteen complementation groups (*nle1-16*) synthetically lethal with *nup1Δ* were identified using a similar sectoring screen to that of Wimmer *et al.* (1992). At least three of these mutants are directly involved in nuclear import as determined by the comparison of their sequences to those of other known components of the transport apparatus. *NLE3* has significant similarity to rat nup153 and therefore is likely to be another XFXFG nucleoporin. Of particular interest are two genes for proteins of the soluble component of the import system. *NLE1* was found to be allelic to *SRP1*, which has since been shown to be the yeast NLS receptor (Chapters 3 and 4) and *NLE7* is allelic to *YRB1*, the yeast RanBP1 homolog, which has been shown to be a cytoplasmic factor that mediates the function of the small G protein Ran, involved in both import and export (L. Davis, personal communication). Furthermore, temperature sensitive mutations in the *nup1* gene were found to be synthetically lethal with *rna1-1*, the yeast homolog of the human RanGTPase-

activating protein RanGAP1 (Bogerd, et al., 1994; Bischoff, et al., 1995). These proteins and other soluble import factors are described in more depth in a following section.

Phenotypes associated with nucleoporin mutations

Conditional mutations in yeast are extremely useful as a method to determine function of their corresponding gene products. A number of techniques have been utilized to engineer conditional function of yeast genes. Two approaches that have been useful with nucleoporins are conditional expression, employing an inducible/repressible promoter and temperature sensitive mutations created by *in vitro* mutagenesis. Mutant lines can be maintained under the permissive conditions, and then shifted to the non-permissive condition, whereupon their defect will be manifested. By analysis of the resulting phenotypes with a variety of cell biological techniques, a great deal of information can be obtained with respect to the cause of death in conditional mutants. From these phenotypes the essential function of the protein can be extrapolated. One pitfall of this technique is the difficulty in discriminating between primary defects directly due to the loss of gene function, and secondary effects that are indirect consequences of the mutation. Therefore, it is important to interpret these phenotypes carefully, and to focus on those that appear rapidly after the shift.

Conditional expression of a gene from the *GAL1* promoter in yeast can be employed in two ways: (i) because this promoter is capable of very high levels of inducible expression, one can assess the consequences of overproduction of a protein, and (ii) one can determine the effect of depletion of an essential gene product by repressing its expression by the addition of glucose to the culture. Overexpression of the genes encoding Nup1, Nup2, Nup116 or Pom152 is toxic, leading to slow growth or cell death (Davis and Fink, 1990; Loeb et al., 1993; Wente and Blobel, 1993; Wozniak et al., 1994). Presumably this effect is due to excess concentration of one member of a multi-subunit complex titrating other components away from productive complexes. Repression of expression of the *NSP1* gene leads to gradual depletion of Nsp1 protein, which in turn induces a very slow decrease in the number of NPCs, as visualized by freeze-fracture electron microscopy (Mutvei et al., 1992). Over the course of a 20 hour time course, the density of pores on the NE surface decreases from $15/\mu m^2$ to $5/\mu m^2$. While informative, these

phenotypes derived from modulating expression have not directly illuminated the function of these genes, so more elaborate efforts have been made to ascertain their roles within the NPC.

Deletion of the *NUP116* gene leads to a temperature sensitive phenotype (Wente and Blobel, 1993). The viability of the mutant cells diminishes rapidly after shift to 37°C. When examined by transmission electron microscopy, it was found that these cells form membranous seals over the cytoplasmic face of the NPC. In severe cases, many NPCs become incorporated into one membrane enclosed space, which is extremely electron dense. It was postulated that the material filling this membrane "hernia" is an accumulation of export substrates that are trapped from passage into the cytoplasm. Perhaps the Nup116 protein is involved in anchoring the NPC to the adjacent membrane. In the absence of Nup116, the membrane near the pore may be more fluid, and thereby capable of forming protrusions into the cytosol that can fuse and seal the pore. Because the position of the Nup116 protein within the pore is unknown, it is difficult to gauge whether this is a plausible model for Nup116 function.

Mutants in *NUP145* and *NUP133* also have defects in the interaction between the NPC and the NE membrane. When these nucleoporin mutants were examined by immunofluorescence with anti-nucleoporin antisera, or by transmission electron microscopy on both thin sections and freeze-fractured preparations, it was noted that the NPCs were limited to a small part of the nuclear surface. Yeast normally have an even distribution of NPCs of about 10-15 pores/μm^2, whereas in clustering mutants the local concentration can be as high as 60 pores/μm^2 (Doye et al., 1994; Wente and Blobel, 1994). It was further shown that the amino-terminal part of Nup133 is sufficient to maintain proper distribution of NPCs (Doye et al., 1994). The nuclear pores themselves are morphologically normal as determined by transmission electron microscopy. Mutants in the *NUP145* gene display a related phenomenon where the NPCs are clustered along with extra NE in "grape-like clusters" (Wente and Blobel, 1994). NPC clustering *per se* does not lead to cell death, since *nup133* temperature sensitive mutants cluster at all temperatures (Doye et al., 1994).

A conditional mutation in the XFXFG nucleoporin Nup1 leads to a different terminal phenotype. After temperature shift, the *nup1* mutant begins to accumulate misshapen and multiple nuclei (Bogerd et al., 1994).

When examined by electron microscopy, it is apparent that the mechanism that maintains the normal spherical topology of the nucleus is impaired. Projections of NE membrane, with NPCs included extend great distances into the cytoplasm, and occasionally surround other organelles. Unlike the mutations in nucleoporins described above, these nuclei have random distributions of NPCs and the morphology of the membrane in the vicinity of each NPC is normal. The orientation of the mitotic spindle in these mutant cells is often misaligned, which probably accounts for the accumulation of binucleate cells. I have also found a similar phenotype in a conditional *nup2Δ srp1* double mutant that is described in Chapter 3.

The role of nucleoporins in transport

Upon the identification of NPC constituents, the foremost question was whether they participate in the transport reaction. It was demonstrated both *in vitro* and *in vivo* that interfering with nucleoporin function leads to cessation of protein import. The O-linked glycosylation of vertebrate nucleoporins enabled several groups to inactivate the entire family of proteins by treatment with the lectin WGA. Injection of WGA into the cytoplasm of mammalian tissue culture cells inhibited the accumulation of a co-injected import substrate (Dabauvalle et al., 1988; Yoneda et al., 1987). WGA binds to nuclear pores when added to isolated rat liver nuclei, and prevents the uptake of a fluorescent transport substrate in the presence of oocyte cytosol (Finlay et al., 1987). *In vitro*, binding of substrate to pores is still apparent in presence of WGA, which suggests that the substrate can still be recognized, but is incapable of translocation under these conditions (Newmeyer and Forbes, 1988). It must be pointed out that inhibition of nuclear import by the addition of WGA or anti-nucleoporin antibodies could be due to a steric block for the substrate, merely because the nucleoporins are in the vicinity of the transport machinery. Injection of neutralizing anti-nucleoporin antibodies also prevents nuclear import, but has no effect on diffusion of small proteins (Featherstone et al., 1988). Depleting oocyte extracts of WGA binding proteins prior to nuclear reconstitution *in vitro* also leads to transport incompetence (Finlay and Forbes, 1990). Better biochemical evidence that the nucleoporins directly participate in the transport reaction is the finding that there is an essential cytosolic import factor that can be

depleted from extracts by its affinity to WGA binding proteins (Sterne-Marr et al., 1992).

Determining the function of a particular NPC component is not straightforward. Assays for the individual steps of nuclear import or export are just now becoming available. Furthermore NPC assembly has not been dissected *in vitro*. Because the NPC is an enormous complex of some one hundred different proteins, it may be only possible to infer function of individual components from the phenotypes of mutants *in vivo*. This has been the strength of the yeast system; however, the same caveats that apply to analysis of morphological phenotypes also apply to nuclear transport. It is always difficult to conclude that the import defect is directly tied to the loss of a gene's function rather than a downstream consequence of a different primary defect. Thus far it has been possible to define genes into classes on the basis of the types of phenotypes their mutations reveal. Gratifyingly, these classes roughly correlate with the groupings of genes that have been defined by physical and genetic interactions.

In yeast, three main assays for nucleocytoplasmic exchange have been developed: *in vivo* measurements of mRNA and protein localization and an *in vitro* assay for protein import. The steady state localization of polyadenylated mRNAs can be measured by *in situ* hybridization with a digoxygenin conjugated oligo(dT) probe (Amberg et al., 1992). In wild-type cells the majority of poly(A)+ RNA is in the cytoplasm, however in the nucleoporin mutants described below, the poly(A)+ RNA accumulates within the nucleus. This result is interpreted as a block in RNA export, however it could also be due to faulty RNA processing or changes in the relative stability of different mRNA pools. To determine the effects on protein import *in vivo*, an assay has been developed based on the conditional expression of a heterologous NLS containing reporter protein, generally a β-galactosidase derivative (Moreland et al., 1987; Sadler et al., 1989). There is also an *in vitro* permeabilized cell assay (Schlenstedt et al., 1993) that can be used in conjunction with mutant strains that will be described in detail in a later section.

Conditional mutations in nucleoporin genes of both repeat classes lead to the accumulation of poly(A)+ RNA in the nucleus. In addition to its aberrant NE morphology, the *nup1* temperature sensitive mutant exhibits a strong block in cytoplasmic RNA localization after 3.5 hours at 36°C. Among

the GLFG nucleoporins, *nup145* (Fabre et al., 1994) *nup116* (Wente and Blobel, 1993) and *nup49* (Doye et al., 1994) mutants fail to transfer poly(A)+ RNA to the cytoplasm. Wente and Blobel (1993) postulated that the seal over the nuclear envelope that forms in the *nup116* mutant prevents the poly(A)+ RNA from escaping the nucleus. However, the Nup100, Nup116 and Nup145 proteins contain sequences homologous to RNP-1 RNA binding motifs and it has been shown that Nup116 and Nup145 bind to polyguanine RNA homopolymer. Simultaneous deletion of the RNA binding domains of Nup100, Nup116 and Nup145 leads to temperature sensitive growth (Fabre et al., 1994). Therefore, these GLFG nucleoporins might be more directly involved in the recognition or translocation of RNA across the NPC. If this is the case, then the NPC herniations observed in *nup116* mutants may be a secondary effect of loss of RNA transport. Mutations in the *NUP133* gene also lead to a block in poly(A)+ RNA export. The RNA mislocalization phenotype can be separated from the NPC clustering phenotype, because the RNA phenotype can be complemented with an amino-terminal fragment of Nup133 that does not restore normal NPC spacing. Unlike Nup100, Nup116, and Nup145, Nup133 and Nup49 do not have identifiable RNA binding domains. It must be noted that it has not been shown that the transport defect observed in these mutants is specific to mRNA, because no assay for the export of ribosomes or snRNAs has been developed.

Mislocalization of nuclear proteins is also a common phenotype among nucleoporin mutants. These constitute a partially overlapping set of mutants with those that accumulate poly(A)+ RNA in their nuclei. The *nup1, nup49* mutants mislocalize both RNA and protein (Bogerd et al., 1994; Doye et al., 1994). Nup49 is intriguing because import and export are separable by different conditional alleles. Therefore as more data about the interactions of Nup49 with other proteins is collected, a clearer model of its functions may become apparent. The *nsp1* conditional mutation also has effects on the localization of nuclear proteins, but the extent of the defect varies by nuclear protein substrate (Nehrbass et al., 1993). The differences in these localization phenotypes may reflect different roles in the various transport functions of the NPC or they may be a consequence of the varying effects of a particular mutant on a process. Therefore the phenotypic results must be considered along with the genetic and physical interactions between these proteins in order to establish a model for their functions.

Physical associations between nucleoporins

Another approach to extending the menagerie of NPC components has been to isolate novel proteins that form complexes with known nucleoporins. A variety of immunoprecipitation and affinity chromatography techniques have been used successfully to purify complexes of nucleoporin proteins. The most important technical issue has been the relative insolubility of NPC components. Therefore most experiments with nucleoporin complexes have been performed in the presence of denaturants or high concentrations of salt to extract NPC components away from the NE fraction. As a consequence, probably only rather stable complexes will be detected. Presumably, proteins that form specific sub-complexes with the NPC must participate in the same function(s). Several complexes of nucleoporin proteins have been identified from vertebrates and yeast.

In Xenopus oocytes, it is possible to purify complexes of nucleoporin proteins readily because they are abundantly present in a soluble form as precursors for the rapid synthesis of new nuclear envelope that occurs during the early cell divisions of embryonic development. The p62 protein is in a 600kD complex with 54 and 58 kilodalton proteins that also bind WGA (Finlay et al., 1991; Kita et al., 1993; Macaulay et al., 1995). The p200 protein also forms a different 1000kD, stable complex with p62 (Macaulay et al., 1995). nup97 exists in a 450 kilodalton complex with other as yet unidentified proteins (Macaulay et al., 1995). The p62-p58-p54 complex has been shown to be required for nuclear protein import in the sense that oocyte extracts from which it has been depleted cannot reconstitute import competent nuclei (Finlay et al., 1991).

In yeast at least three different nucleoporin complexes have been observed. Each includes one XFXFG nucleoporin with a different cohort of other proteins, some of which have been identified. The Nup1 and Nup2 proteins exist in similar complexes that include the nuclear import receptor Srp1 (Belanger et al., 1994). Interestingly fusions between either amino or carboxy terminal fragments of Nup1 and glutathione S-transferase associate with Srp1. A Nsp1 containing complex has been studied in more detail. This complex was isolated by expressing a fusion of the carboxy-terminal coiled-coil domain of Nsp1 and protein A (Grandi et al., 1993). Included in this complex were Nup49, Nup57 and a novel protein named Nic96 (for **N**ucleoporin-**I**nteracting **C**omponent of 96 kilodaltons). When sequenced, it

was found to be a unique protein without any nucleoporin repeats. Depletion of the Nic96 protein or expression of a temperature sensitive truncation mutant leads to defects in protein import but not poly(A)+ RNA localization (Grandi et al., 1995). It is unclear why Nup1 and Nup2 form similar complexes and Nsp1 forms a completely different complex, because Nup1 and Nup2 are no more related to each other than each is to Nsp1.

In yeast, these physical interactions can also be compared to interactions discovered genetically and the phenotypes of mutants. Together, these comparisons lead to a variety of conclusions. (i) Genetic interactions can be found between proteins that are in a single complex, and also between proteins that are never found in complex together. These interactions probably reflect different functional relationships on the molecular level. Genetic interactions between mutants in different complexes are evidence for functional redundancy, whereas interactions within a complex probably reflect participation in different roles in the same mechanism. (ii) The mutant phenotypes of genes whose proteins are in a single complex are similar, but not identical. For example, *nsp1* mutants have defects in nuclear import, whereas *nup49* mutants have defects in import and export. This diversity may reflect multiple functions for the Nsp1 complex or alternatively some of the defects observed with one or the other mutant may be indirect.

Nucleoporin structure and modification

The XFXFG repeats found in p62, Nup1, Nup2 and Nsp1 were predicted to form short, amphipathic beta sheets with alpha-helical spacers (Davis and Fink, 1990). In addition, the homologous carboxy terminal domain of p62 and Nsp1 is predicted to form a coiled-coil structure. Although definitive structural information awaits X-ray crystallographic study of these proteins, electron microscopy and circular dichroism spectroscopy studies of the p62 protein purified from *E. coli* are consistent with a rod-shaped molecule with an alpha-helical coiled-coil domain at its C terminus and a cross-beta structure at its N terminus (Buss et al., 1994).

Another area of research concerning nucleoporins has been their post translational modification. As described above, vertebrate nucleoporins are modified by O-linked glycosylation. It is unclear whether yeast nucleoporins have similar modifications. The functional significance of the added sugar moieties is unknown. Masking the sugars with WGA inhibits transport, but

this may be due to steric hindrance rather than a direct role for the sugar residues in import. Thus far it has not been possible to test directly whether glycosylation of p62 or other nucleoporins is required for their function.

Nucleoporins are also modified by phosphorylation. p200 and p97 are phosphorylated during mitosis in Xenopus egg extracts. p62 is not phosphorylated (Macaulay et al., 1995). It was also shown that these nucleoporins are direct targets of the mitotic $p34^{cdc2}$ kinase *in vitro*. By analogy to nuclear lamins, it is very likely that phosphorylation by $p34^{cdc2}$ kinase transmits the signal for NPC breakdown at the beginning of mitosis. The small complexes that these proteins form do not change with the cell cycle, therefore they might represent the units that nucleoporins disassemble into at mitosis. I have also found that the yeast nucleoporins Nup2 and Nsp1 are cyclically phosphorylated with maximum phosphorylation levels at mitosis. Because yeast NPCs do not breakdown during mitosis, it is difficult to imagine their need for some type of cell cycle regulation. Perhaps these modifications serve to regulate the substrate specificity of the nuclear pore, or alternatively, the cyclical phosphorylation might be a vestigial remnant of an ancestor whose NE dispersed at mitosis.

Localization of nucleoporins within the NPC

The availability of specific antisera for single NPC proteins has enabled the sublocalization of some of the vertebrate nucleoporins within the greater structure of the NPC by high resolution immuno-electron microscopy (Pante and Aebi, 1994). It has become evident that individual XFXFG nucleoporins reside in different substructures of the NPC, including some peripheral structures. NUP153 is a constituent of the nuclear basket with at least one of its epitopes residing in its terminal ring (Cordes et al., 1993; Pante et al., 1994), whereas p250 is a constituent of the cytoplasmic filaments (Pante et al., 1994). The p62 protein appears to be distributed on both sides of the central plug domain (Pante et al., 1994). On the basis of the widely varied position within the NPC, these proteins must perform different roles. Perhaps their common sequence motifs are responsible for interactions with the same transport components as they travel through the NPC. Two non-repeat bearing proteins have also been sub-localized to specific pore domains. NUP180 is localized in the cytoplasmic ring and its associated fibrils (Wilken et al., 1993), whereas NUP155 is associated with both the nucleoplasmic and the

cytoplasmic aspect of the NPC and is therefore possibly a component of the symmetrically arranged NPC substructures. Unfortunately, an equivalent level of resolution has not been attained in preparations of yeast cells.

Other nuclear pore proteins

In addition to the nucleoporin proteins, which for the most part are confined to the interior, soluble part of the NPC, other classes of NPC components must exist. No constituents of the large ring structures of the NPC have been cloned or purified. Furthermore, there must be proteins that link the pore complex to the adjacent nuclear envelope membrane. Two proteins that apparently carry out this function have been cloned, one from rat and one from yeast. These proteins may constitute part of the projections into the lumenal space from the NPC that have been identified in ultrastructural studies. The gp210 protein was the first NPC component identified (Gerace et al., 1982). Unlike the nucleoporins, gp210 is modified with N-linked glycosylation in the endoplasmic reticulum. The sequence of this protein is unrelated to other NPC components (Wozniak et al., 1989). Ninety-five percent of the mass of gp210 is in the lumenal space (Greber et al., 1990). The single transmembrane domain of gp210 is sufficient to target heterologous proteins to the NPC membrane (Wozniak and Blobel, 1992). Interfering with gp210 function by the expression of anti-gp210 antibodies within the lumen of the NE by injection IgG mRNA into tissue culture cells leads to a cessation in nuclear import of proteins and passive diffusion of a dextran (Greber and Gerace, 1992).

The POM152 protein was identified as an integral membrane protein that binds to concanavalin A and co-fractionates with the purification of NPC complexes in yeast. Like gp210, most of the POM152 protein is in the ER cisternal space, with only a small domain protruding into the NPC. It may be that POM152 and gp210 serve equivalent functions in their respective organisms, however, there is no strong similarity between their coding sequences. POM152 is a non-essential gene which suggests there may be similar, redundant proteins within the yeast cell. Overexpression of this gene was toxic, but the cause of the toxicity is unknown. POM152 is sorted properly to the nuclear pore complex when expressed in mammalian cells, which

suggests a similarity in the targeting mechanisms for the membrane proteins of the NPC across kingdoms (Wozniak et al., 1994).

Two additional NPC components have been identified from mammalian cells that probably do not have any of the signature repeated domains of nucleoporins nor are they integral membrane proteins. NUP180 is a peripheral membrane protein that does not bind to WGA (Wilken et al., 1993). It was identified by its reactivity with an autoimmune serum from a patient with overlap connective tissue disease that was shown to react with NPC antigens by immunofluorescence. Addition of the anti-NUP180 antibody does not inhibit nuclear import *in vitro* or *in vivo*. The NUP155 protein was isolated as non-WGA binding protein from urea extracted rat liver nuclear envelopes. The gene was cloned and found to encode a protein with a unique sequence. The localization of the NUP155 protein in the NPC was confirmed by immuno-electron microscopy with a specific antibody. It is not yet known whether NUP155 has a direct role in nuclear import. The fraction from which NUP155 was purified also contains approximately 30 other peptides that are potentially NPC components.

All of the NPC constituents described above are thought to be general factors whose presence is expected in all the NPCs of an organism and in every organism. However, it is possible that there are NPC elements specific for the pores of a specific organism or tissue. Such structures could be used for regulation of transport substrate specificity, regulation of a variant mitosis, or as an organizing element for a unique internal nuclear structure. There is one example of a tissue specific NPC protein. *Germ cell-less* is a nuclear pore protein from *Drosophila* whose expression is limited to the pole cells at the posterior end of the developing embryo. The *gcl* gene was isolated in a screen for sterile flies: absence of the *gcl* gene product prevents the production of the pole cell tissue, thus leading to sterile adults (Jongens et al., 1994; Jongens et al., 1992). The *Germ cell-less* sequence is not related to any other known NPC constituents. It is unknown whether there are *Germ cell-less* homologs in other organisms, or *Germ cell-less* related proteins in other tissues of *Drosophila*.

Purification of nuclear pore complexes

Another promising approach to defining the constituents of the entire NPC is a direct purification of the entire organelle as a unit. Rout and coworkers have succeeded in isolating a very pure preparation of NPCs from the yeast *Saccharomyces uvarum* that largely maintain their characteristic morphology (Rout and Blobel, 1993). This preparation contains about eighty peptides that may all be NPC components. It was found that the nucleoporin proteins co-fractionated strictly with this preparation, which is good evidence for the utility of the procedure to isolate proteins that are important for the nuclear transport reaction. Therefore, given the advanced state of the Saccharomyces genome project, all of the NPC constituents relevant to nuclear transport may be isolated, cloned and sequenced within a short time. Once the entire composition of the NPC is known, the field can concentrate on the interactions between the subunits of the NPC and the mechanisms of pore biogenesis and nuclear transport.

1.5 Soluble factors required for nuclear import

In addition to the components of the NPC, there are other critical factors required for nuclear import, and presumably export as well. It was postulated early on that the receptors for NLS sequences might be free-floating in the cytoplasm and the targeting step of nuclear import may involve a receptor/substrate complex. Later, it was found that a cytosolic extract is required to supplement the NPC associated activities in an *in vitro* assay for nuclear import (Adam et al., 1990). Recently, great progress has been made towards identification of the nuclear import receptor as well as other soluble factors for import by fractionating the components of this *in vitro* assay. It is now evident that import of proteins requires a cycle of GTP hydrolysis mediated by a small cytoplasmic G protein, Ran/TC4. The remaining mysteries of the nuclear import reaction revolve around the interactions between these soluble factors and those fixed in the NPC.

***In vitro* assays for the import of proteins into the nucleus**

Nuclear import of proteins has been the area where most progress towards understanding the molecular mechanism of the transport process has been made. In addition to success with the *in vivo* microinjection

approach, several faithful *in vitro* systems for protein import have been developed (Adam et al., 1990; Newmeyer et al., 1986; Schlenstedt et al., 1993). These complementary approaches have illuminated some features of the transport mechanism and have allowed purification and cloning of some of the critical components of this system. There are two types of assays that have been developed to mimic nuclear import of proteins in vertebrates. The first system utilizes the capability of extracts from Xenopus oocytes to form import competent nuclei around exogenous DNA, such as phage λ (Forbes et al., 1983; Newmeyer and Forbes, 1988; Newmeyer et al., 1986). The other approach relies on the fact that the detergent digitonin preferentially permeabilizes the plasma membrane under conditions that leave the nuclear envelope intact (Adam et al., 1990). The nuclear reconstitution assay has the advantage of access to NPC constituents in a soluble state prior to assembly of the nucleus, therefore, it is possible to immunodeplete NPC components and assess the effects on nuclear import and other nuclear processes (Finlay and Forbes, 1990; Powers et al., 1995). Whereas the permeabilized tissue culture cell system has been more useful to purify the soluble components of cytosol that are required for nuclear import because of the ease of the assay (Moore and Blobel, 1992). There are several lines of evidence that indicate these *in vitro* assays accurately reflect the import process *in vivo*. In both cases, the nuclear envelope appears intact by electron microscopy (Adam et al., 1990; Newmeyer and Forbes, 1988). Also, gold labeled import intermediates bound to and inside the NPC can be observed under assay conditions (Newmeyer and Forbes, 1988). Furthermore, transport across the NPC *in vitro* requires ATP, is temperature dependent and signal sequence dependent, as mutant signal sequence bearing proteins fail to accumulate in the nucleus or bind to the NPC (Adam et al., 1990; Newmeyer and Forbes, 1988).

Using the nuclear reconstitution assay, it was shown that depletion of all three WGA binding nucleoporins or only p62 results in loss of import activity (Finlay and Forbes, 1990). Furthermore, addition of WGA binding proteins is sufficient to reconstitute the import activity. However, depletion of the nup97 GLFG nucleoporin has a minimal effect on nuclear import of the fluorescent substrate (Powers et al., 1995). Interestingly, these nup97Δ nuclei are not capable of executing DNA replication, an activity normally present in these nuclei formed *in vitro* from Xenopus oocyte cytosol.

Using the digitonin-permeabilized cell assay Moore and Blobel (1992) found there are two biochemically distinguishable classes of cytosolic factors required for import. Oocyte cytosol proteins were fractionated by anion exchange chromatography into two activities, "A" and "B". When added to permeabilized HeLa cells, fraction A is sufficient to target a fluorescent import substrate to the nuclear envelope surface. Therefore, it was concluded that this fraction contains the NLS recognition and targeting activity. This targeting activity is insensitive to cold and does not require ATP and so mimics the previous findings for the targeting step *in vivo* (Richardson et al., 1988). Fraction A was found to have a relative molecular mass of approximately 200 kilodaltons by gel-filtration chromatography and ultracentrifugation. The B fraction does not have any effect when added to the assay alone, but in the presence of fraction A, it stimulates the translocation of the substrate from the nuclear surface to the nuclear interior. These experiments indicate that there is a factor in the cytosol that is necessary for the translocation step of import. It appears that these two fractions contain all the factors necessary for import, because they stimulate uptake as efficiently as unfractionated cytosol. Subsequent work has focused on identifying and cloning the proteins in these two fractions that mediate the import reaction.

The limitation of the mammalian nuclear import assays is the lack of genetic tools to perturb individual components of the system. The only types of "genetics" that can be performed are the depletion of a component by biochemical means (e.g. Finlay and Forbes, 1990), or the introduction of a dominant mutant protein. However, it would be useful to test loss of function mutants in transport components for their contribution to the reaction. Therefore, the development of an *in vitro* assay in yeast that is similar to that developed for tissue culture cells was an important advance (Schlenstedt et al., 1993). To permeabilize the cells, yeast are treated with enzymes to digest the cell wall, then frozen and thawed. This treatment perforates the plasma membrane, but leaves the NE intact. The cytosol can then be washed away and replaced with defined components. The assay is performed with a fluorescent substrate and like the mammalian system requires ATP. Unlike the mammalian assay, the binding activity cannot be completely washed away from the permeabilized cells. This difference may reflect a difference in the affinity of the empty receptor for the NPC. Initially

two conditional mutations in nucleoporin genes were tested with this assay. It was found that a temperature sensitive *nsp1* mutant fails to import, but is competent for binding, whereas a *nup49* mutant neither binds nor imports. This assay has since been used to study the contributions of several soluble components of this system, including the import receptor (see Chapters 3 and 4).

Receptors for proteins targeted to the nucleus

The first approach that was applied to identification of the recognition element for nuclear import of proteins was to look for proteins that bound to NLS peptides *in vitro* (**N**LS **B**inding **P**roteins or NBPs). Adam and Gerace (1989) first showed by chemical cross-linking two proteins of approximately 60 and 70 kilodaltons found in rat liver cytosol bound to a synthetic SV40 T antigen peptide. This high affinity interaction was saturable and was not observed with a point mutant in the SV40 T antigen NLS. Similar strategies have also demonstrated a variety of NLS binding activities from budding yeast, flies, plants and mammals (Lee and Melese, 1989; Benditt et al., 1989; Pandey and Parnaik, 1991; Silver et al., 1989; Stochaj and Silver, 1992). It was also found that the approximately 70 kilodalton NBP conserved in many species is a phosphoprotein that requires phosphorylation for its NLS binding activity (Stochaj and Silver, 1992).

There are several lines of evidence that certain NBPs are required for nuclear import. One important issue is the expected intracellular localization of the *bona fide* NLS receptor. Because the NBP should be a factor that targets import substrate (synthesized in the cytoplasm) to the NPC, one might expect to find the appropriate NBP activity concentrated in the cytosol or the nuclear envelope. The approximately 60-70 kilodalton NBP is localized in the cytosol and the NE of mammalian cells (Adam et al., 1989; Li et al., 1992). The NBP70 from yeast has been shown to bind to at least three unrelated NLS peptides (Silver et al., 1989), which is expected to be a characteristic of an actual NLS receptor. The receptor should bind to many different NLS sequences, as different import substrates compete for recognition and uptake (Michaud and Goldfarb, 1993). In addition, it should be possible to interfere with nuclear import with neutralizing antibodies against an actual import component. Addition of an antibody raised against yeast NBP70 to a *Drosophila*

permeabilized cell system inhibits binding and uptake of synthetic nuclear import substrate (Stochaj and Silver, 1992), and antibodies directed against a mammalian nuclear envelope bound NBP inhibits import into permeabilized HeLa cells (Pandey et al., 1994).

The purified NLS receptor should have a stimulatory activity on import *in vitro*. This activity was first demonstrated for NLS binding proteins purified from bovine erythrocytes (Adam and Gerace, 1991). These NBPs were found to be of slightly smaller molecular weight than those of rat liver or yeast, however, after molecular cloning of the genes for these proteins it is apparent that the NLS receptors from many eukaryotes are highly conserved. The purified NBP54 and NBP56 proteins from bovine erythrocytes are sufficient to stimulate import several fold in the presence of limiting cytosol in the mammalian permeabilized cell assay. Furthermore, this fraction is competent to restore nuclear uptake to a reaction where the cytosol has been inactivated with the alkylating agent N-ethylmaleimide (NEM).

Some NLS binding proteins may be required for the import of a limited number of substrates or for their intranuclear transport. Nopp140 is another NLS binding protein that was isolated from rat liver nuclei. This highly phosphorylated protein is concentrated in the nucleolus but shuttles between the nucleolus and the cytoplasm. Immuno-electron microscopic localization of this protein revealed curved "tracks" leading between the nucleolus and the NPCs that may represent intranuclear transport intermediates (Meier and Blobel, 1990; Meier and Blobel, 1992). *NSR1* is a yeast gene encoding a 67 kilodalton NLS binding protein that also appears to be predominately located in the nucleolus (Lee et al., 1991). This protein is predicted to have a RNA binding domain, and in fact mutations in the gene affect the proper assembly of pre-ribosomal particles, possibly by mediating the association of rRNA and ribosomal proteins by its affinity for ribosomal proteins nuclear localization sequences and for rRNA via its multiple RNA recognition motifs (Lee et al., 1992). Neither Nopp140 nor Nsr1 is related to other proteins implicated in NLS recognition, nor have they been shown to have a role in nuclear import *in vitro*. However, they may be specific for a subset of import substrates, such as nucleolar proteins.

Cloning the NLS receptor

The nuclear import receptor was cloned by further fractionation of the cytosolic components that are required for the first step of nuclear import in the permeabilized cell assay system (Görlich et al., 1994). Görlich and colleagues fractionated Xenopus oocyte cytosol in such a way as to deplete only one essential factor for import. Purification of the depleted factor could then be monitored by adding back fractions to the depleted cytosol and performing the permeabilized cell *in vitro* assay for import. In this manner, a 60 kilodalton protein (importin 60) was purified that is sufficient to support binding of import substrate to the nuclear envelope in the absence of any other cytosolic factors. In this sense, importin 60 is equivalent to the activity of the fraction A of Moore and Blobel (1992). In fact, the fraction A activity can be immunodepleted by antibodies against a human homolog of importin (Moroianu et al., 1995). In addition to its requirement *in vitro* for targeting import substrate to the NE, it is also the major NLS binding protein found in Xenopus cytosol (Görlich et al., 1995). Srp1/importin is likely to recognize many protein NLS sequences because it has been shown that most nuclear proteins compete for transport receptors (Michaud and Goldfarb, 1993). It is now clear that importin 60 is equivalent to both the NBP70 protein of yeast and the NBP54-56 proteins of bovine erythrocytes (discussed in Chapter 4). The activity of the purified importin 60 protein is not sensitive to inhibition by NEM, suggesting that there is an accessory factor to importin that was inactivated in the earlier in vitro work. The identification of this factor is discussed below.

Two similar genes for importin 60 were cloned from a Xenopus cDNA library by hybridization with degenerate oligonucleotide probes based on microsequence of purified importin 60. The identity of these clones as importin 60 was confirmed by expressing these genes in *E. coli*, then purifying recombinant protein active for importin function. The sequence of importin 60 was found to be highly homologous to a number of genes in the database, notably *SRP1* from yeast. The *SRP1* gene was first cloned as a suppressor of conditional mutations in the zinc-binding domain of the large subunit (A190) of RNA polymerase I of Saccharomyces (Yano et al., 1992). The same mutant allele also suppressed mutations in the PolI A135 subunit. The Srp1 protein product is essential for viability and was shown by indirect immunofluorescence to be located primarily in the nuclear envelope of yeast

cells. Srp1 is the functional equivalent of Xenopus importin (Chapters 3 and 4). The *SRP1* gene encodes a protein of 542 amino acids consisting of three domains: the central domain, which is composed of eight degenerate 42-amino-acid tandem repeats, and the surrounding N-terminal and C-terminal domains, both of which contain clusters of acidic and basic amino acids and are very hydrophilic.

The *SRP1* gene was subsequently identified in a number of other genetic screens. The *SRP1* gene is a multicopy suppressor of a cold sensitive mutation in *CSE1,* which was isolated as a mutant with an elevated rate of chromosome loss. Furthermore, *SRP1* was isolated as a synthetic lethal mutant with a deletion of the *BIK1* gene, a mitotic spindle associated protein (Berlin et al., 1990; Pellman et al., 1995) and Chapter 3. Most importantly, *SRP1* was also isolated as a synthetic lethal mutation with a non-lethal deletion of the *NUP1* gene (Belanger et al., 1994). The genetic relationship between *SRP1* and *NUP1* is strong evidence for a functional interaction between the NPC and the nuclear import receptor. Further evidence was provided by the finding that Srp1 can be copurified with Nup1 or Nup2 from yeast extracts. The synthesis of the genetic and physical interactions between SRP1 and XFXFG nucleoporins suggests a model where nuclear import substrate/receptor complexes dock at the pore with nucleoporins prior to their translocation.

Given the diversity of the genetic interactions of *SRP1,* and its role as the nuclear import receptor in yeast, it is not altogether surprising that mutations in this gene lead to pleiotropic phenotypes. Depletion of the Srp1 protein by repression of its expression from an inducible promoter leads to a gradual cessation of growth, defects in nucleolar structure and rRNA transcription (Yano et al., 1994). Temperature sensitive alleles of *SRP1* exhibit defects in nuclear division and mitotic spindle structure (Yano et al., 1994) and Chapter 3. Interestingly, a *srp1 nup2* double mutant has a disorganized nuclear envelope phenotype reminiscent of that observed for temperature sensitive *nup1* mutants (Chapter 3).

Homologs of importin/Srp1 were cloned in three yeast interaction trap (two-hybrid) screens. Two screens were based on searches for proteins that interact with the immunoglobulin recombination protein RAG-1 (Cortes et al., 1994; Cuomo et al., 1994). One of these proteins, Rch1, is capable of replacing importin 60 in the in vitro assay (Görlich et al., 1995). Another

homolog, NPI-1 was isolated in a two hybrid screen employing the nucleoprotein from influenza A virus as a bait (O'Neill and Palese, 1995). In all three of these screens it was possible to co-immunoprecipitate Srp1 and the bait protein. Therefore, these two hybrid interactions within yeast probably represent *bona fide* high affinity interactions within the mammalian cells from which the baits were derived. It may be that these interactions reflect the role of Srp1 as the nuclear import receptor. Perhaps any NLS containing protein would be capable of a two hybrid interaction with Srp1. Cortes *et al.* (1994) found that the repeated domain of Srp1 was necessary for the two hybrid interaction. Therefore, it may be that this is the domain of this protein that is required for interaction with nuclear import substrates.

The 42 amino acid repeated domain from Srp1/importin is similar to a repeating motif found in a variety of other proteins (Peifer et al., 1994). Srp1, the *Drosophila* segment polarity gene armadillo, the adhesive junction proteins β-catenin, plakoglobin and p120, the APC tumor suppressor gene and the small GTPase exchange factor smgGDS all contain more than six iterations of this sequence which has been labeled the arm motif. The first four are constituents of both the actin and intermediate filament cytoskeletons (Gumbiner and McCrea, 1993; Kemler, 1993). The general mechanism proposed for these proteins is as linkers or adaptors from cytoskeletal elements to membrane bound proteins, such as cadherins. Their relationship to the function of Srp1 is unclear. However, the similarity between Srp1 and smgGDS is more tantalizing, because smgGDS acts on the small GTPases Rac, Rho, Ras and Rap1, which are all related to Ran, the nuclear GTPase essential for nuclear import (described in detail below). Therefore, it is possible that the repeated region of Srp1 mediates an interaction with Ran similar to that between smgGDS and its targets.

The second subunit of the nuclear import receptor

There is a second protein in the fraction A preparation that is required to facilitate binding of import substrates to the nuclear envelope. The p97 protein is the NEM sensitive component of the receptor (Adam and Adam, 1994). It must be added to purified bovine importin to execute the docking reaction (Adam and Adam, 1994), whereas Xenopus importin has some basal activity in the absence of p97. The p97 protein has no NE binding activity in the absence of importin (Adam and Adam, 1994; Görlich et al., 1995). *In vitro*,

recombinant importin 60 is sufficient to bind NLS peptide in the absence of other proteins (Görlich et al., 1995). Therefore p97 must act as an accessory factor whose interaction with importin does not involve direct binding to NLS sequences. The cloned mammalian p97 gene (also known as karyopherin β) is not closely related to any other known protein in the sequence data bases (Görlich et al., 1995; Radu et al., 1995) However, this sequence is homologous to anonymous cDNAs from human, rice, *Caenorhabditis elegans* and *Plasmodium falciparum.* Furthermore, human p97 is 34% identical to a budding yeast open reading frame that is predicted to encode a 95 kilodalton protein.

In Xenopus cytosol, the p97 protein forms a tight complex with importin (Görlich et al., 1995). Interestingly, it was also found that the importin/p97/substrate complex binds to at least three rat liver nucleoporins. When partially purified rat liver NE is immobilized on nitrocellulose and subsequently probed with fraction A previously incubated with NLS-conjugated human serum albumin, three major proteins are recognized that correspond to nup97, nup153 and nup214 (Radu et al., 1995). Furthermore, the p97 import factor can be purified from fraction A by passage over a column of immobilized nup97. Interestingly, nup97 is a GLFG nucleoporin that has been shown not to be required for nuclear import in the Xenopus nuclear reconstitution *in vitro* assay (Powers et al., 1995). The conflict between these results may be a consequence of the redundancy of importin/p97 binding sites within the pore as suggested by the three NLS receptor binding nucleoporins in rat liver NE and the capability of both Nup1 and Nup2 to bind Srp1 protein independently in yeast.

The small GTPase Ran/TC4 is required for the second step of protein import

It has been shown that the NLS receptor complex is not sufficient to drive import of nuclear proteins bound to the nuclear envelope into the nuclear interior in the *in vitro* permeabilized assay system (Moore and Blobel, 1992). The "fraction B" activity is required to stimulate the translocation of substrate across the nuclear pore. When further purified, it was found that the major active protein in fraction B was the small Ras related GTPase, Ran/TC4 (Moore and Blobel, 1993). Ran/TC4 was originally cloned on the basis of its homology to Ras (Drivas, et al., 1990). However, the

homology between Ras and Ran is limited to the region of the proteins required for guanine nucleotide binding. Ran is an extremely abundant protein that is present in the cytoplasm but is mostly concentrated in the nucleus. In HeLa cells 0.36% of total protein is Ran (Bischoff, et al., 1991). Unlike Ras, Ran is not farnesylated and is therefore not membrane associated. Highly similar homologs of Ran have been isolated from both *S. cerevisiae* and *S. pombe*. *S. cerevisiae* has two Ran homologs, Gsp1 and Gsp2 (Belhumeur et al., 1993). Gsp1 is essential for growth whereas Gsp2 is not.

The GTPase activity of Ran is required for its nuclear import function. Addition of the non-hydrolyzable analogs GTPγS or GMP-PNP to the permeabilized cell assay using Xenopus cytosol inhibits nuclear import (Melchior et al., 1993; Moore and Blobel, 1994), which suggests that the interconversion of Ran from the GTP bound form to the GDP bound form is necessary at some point of the import reaction. However, non-hydrolyzable analogs of GTP do not interfere with the permeabilized cell assay supplemented with bovine erythrocyte cytosol (Adam and Adam, 1994). At this stage, it is unclear whether the hydrolysis of GTP by Ran is the only source of energy required for the import reaction, or whether there are also other unidentified GTPases or ATPases involved.

Ran mutants

It appears that Ran and its associated proteins are involved in several nuclear processes. The variety of phenotypes associated with mutations in Ran and its associated proteins has led to a confusing picture. In addition to its role in the nuclear import of proteins, it has also been suggested that Ran plays a direct role in RNA export, intranuclear traffic of RNA and protein, and as sensor of DNA replication. As with any phenotypic study, determination of the primary defect of a mutant is difficult. In the case of the nuclear GTPase field, it seems each investigator claims that his or her avenue represents the primary function of the system. In reality, Ran is almost certainly a multifunctional enzyme.

The loss of function phenotypes of the Ran GTPase are unknown, because no conditional mutants are available. In lieu of a conditional mutant, researchers have employed dominant mutants that affect the GTPase function that were designed by analogy to well studied point mutations in Ras. For example, expression of the dominant G21V mutant of Gsp1 that

increases the stability of the GTP bound form of the protein is lethal to yeast cells (Belhumeur et al., 1993). The lethality of this mutant appears to be due to the inhibition of nuclear transport, as nuclear protein reporters are rapidly mislocalized upon induction of the mutant gene (Schlenstedt et al., 1995). On the other hand, transient expression in tissue culture cells of another Ran variant expected to persist in the GTP bound state disrupts host cell DNA synthesis and also perturbs the cell cycle (Ren et al., 1994; Ren et al., 1993). Furthermore, the T24N mutant of Ran that also favors the GDP-bound state does not inhibit import *in vitro* when added to wildtype cytosol in the Xenopus nuclear reconstitution system (Dasso et al., 1994; Kornbluth et al., 1994). However, this mutant does affect chromatin structure and inhibits $p34^{cdc2}$ kinase activity in the absence of nuclei, which suggests a direct role for Ran in modulating regulators of the cell cycle.

Mediators and effectors of Ran function

Because non-hydrolyzable analogs of GTP interfere with the function of Ran *in vitro*, and mutations in Ran and its homologs that affect its GTPase activity are defective for some of its functions *in vivo*, other proteins that modulate the GTPase cycle of Ran must also be involved in nuclear transport processes. Four classes of proteins have been identified that interact with a Ran family GTPase. Like Ras, the intrinsic rate of hydrolysis of GTP by Ran is slow and is modulated by a guanine nucleotide exchange factor or GEF, and a GTPase activating protein or GAP. In addition, two novel proteins have been identified that are unique to Ran and also appear to affect or mediate its activities.

RCC1 is the guanine nucleotide exchange factor for Ran in mammalian cells (Bischoff and Ponstingl, 1991; Bischoff and Ponstingl, 1991). It is an abundant nuclear protein that binds to chromatin *in vivo* and DNA *in vitro* (Bischoff et al., 1990; Lee et al., 1993; Ohtsubo et al., 1989). The gene for RCC1 was originally identified by a temperature sensitive cell cycle mutation of a hamster tissue culture cell line called tsBN2 (Nishimoto et al., 1978). When a randomly cycling tsBN2 culture is shifted to the non-permissive temperature, cells arrest in the G1 stage of the cell cycle. Furthermore, when tsBN2 cells are synchronized in early S phase prior to temperature shift, they prematurely enter mitosis as evidenced by the precocious condensation of their chromosomes. tsBN2 cells are insensitive to the S phase checkpoint in that

they will enter mitosis in the presence of hydroxyurea, a potent inhibitor of DNA replication (Nishitani et al., 1991). Further evidence that RCC1 has a direct role in DNA replication is the observation that Xenopus oocyte extracts are incapable of supporting DNA replication if RCC1 is immunodepleted (Dasso et al., 1992).

RCC1 is also required for nuclear protein import. However, the effect on nuclear uptake is dependent on the import substrate examined. One group has shown an effect on nuclear protein localization by the injection of a fluorescent substrate protein bearing a small number of SV40 T antigen NLSs into the cytoplasm of tsBN2 cells under permissive and non-permissive conditions (Tachibana et al., 1994). Moreover, they were also able to demonstrate the incompetence of tsBN2 nuclei to sustain import *in vitro* at the non-permissive temperature, even in the presence of wild-type cytosol. This is consistent with the location of RCC1 within the nucleus. However, another group has shown no effect on the import of a microinjected substrate conjugated with multiple E1a NLSs (Kadowaki et al., 1993). Therefore, the RCC1 mutant probably affects the kinetics of import, but does not utterly block the reaction.

The RCC1 enzyme has homologs in both *S. pombe* and *S. cerevisiae*. These enzymes are likely to be very similar in function to RCC1 because they can replace each other to varying extents and because mutants in each gene have related phenotypes. Mutants in the *S. cerevisiae* gene are partially complemented by the mammalian gene (Clark et al., 1991; Fleischmann et al., 1991) and the hamster mutant cell line is complemented by the budding yeast gene, as well as a *Drosophila* homolog (Ohtsubo et al., 1991). In *S. pombe*, the RCC1 homolog is encoded by the *pim1* gene, and a mutation in this gene displays a similar premature initiation of mitosis phenotype to that originally observed in the tsBN2 hamster cell mutant (Matsumoto and Beach, 1991). One allele of the Saccharomyces homolog, *srm1*, arrests in the G1 phase of the cell cycle, another aspect of the tsBN2 phenotype (Clark and Sprague, 1989). Furthermore, another conditional allele of pim1 is defective in chromosome decondensation (Sazer and Nurse, 1994), which suggests that this GTPase cycle may participate in the regulation of more than one point in the cell cycle. The *pim1* mutation is suppressed by overexpression of *S. pombe* Ran homolog spi1, which serves as genetic evidence for the conservation of this system across kingdoms (Matsumoto and Beach, 1991). RCC1 and Ran form a

tight complex inside the nucleus (Bischoff and Ponstingl, 1991). One function of the complex may be to ensure Ran remains nuclear, as loss of RCC1 function releases Ran from the nucleus (Ren et al., 1993).

The Ran exchange factor has also been implicated in the export of mRNA from the nucleus (Tartakoff and Schneiter, 1995). The *Saccharomyces* homolog was first isolated as an RNA processing mutant, *prp20* (Forrester et al., 1992). This conditional mutant blocks the accumulation of mRNA in the cytoplasm as observed by *in situ* hybridization with an oligo(dT) probe (Amberg et al., 1993; Kadowaki et al., 1993). The equivalent mutation also blocks RNA trafficking in *S. pombe* and mammalian cells (Kadowaki et al., 1993). The block in RNA export may represent an early step in the process because mRNA does not accumulate at the nuclear periphery or NPCs.

Another activity that constitutes part of the GTPase cycle of Ran is its GTPase activating protein or GAP (Coutavas et al., 1993). Recently it was determined that the Ran-GAP is the product of the Rna1 protein (Bischoff et al., 1995). The *RNA1* gene was originally isolated as a mutant that failed to properly process and localize RNA (Hopper et al., 1990; Hutchinson et al., 1969; Traglia et al., 1989). The Rna1 protein is cytoplasmic, so it was quite mysterious how it interfered with RNA metabolism within the nucleus. Rna1 is also required for nuclear protein import *in vitro* in yeast (A. Corbett and P. Silver, personal communication). Cytosol prepared from the *rna1-1* mutant is completely inactive for the translocation, but not the binding step of import. The import activity can be restored by the addition of bacterially expressed Rna1. This suggests a direct role for Rna1 in import. The role of Rna1 in RNA export, despite its cytoplasmic localization, and its coincident role in protein import, suggest a linkage between the import and export mechanisms. Surprisingly, a *RNA1* homolog was cloned from mice as a mutant that does not properly execute embryonic development (De Gregori et al., 1994). Perhaps this protein is a developmentally regulated factor that modulates the GTPase cycle at a particular step in development.

RanBP1 is a 23 KD protein that binds to the GTP-bound form of Ran, but not the GDP-bound form (Coutavas et al., 1993). It stimulates the GTP hydrolysis activity of RanGAP1/Rna1 by an order of magnitude (Bischoff et al., 1995). The yeast homolog of RanBP1, *YRB1*, has been cloned by several labs by different means. By homology to rat RanBP1 (Butler and Wolfe, 1994), as a synthetic lethal mutation with *nup1Δ* (L. Davis, personal

communication), as a suppressor of a mutation of the *FUS1* gene (M. Kunzler, personal communication) and as a protein that binds specifically to the GTP bound form of Gsp1 (G. Schlenstedt, personal communication). RanBP1 is thought to interact with Ran through the carboxy terminal six residues of the GTPase. Strong evidence that RanBP1 is an import effector of Ran function is that the dominant effects of the GTP bound mutant are blocked by a truncation of these terminal residues (Ren et al., 1994). It is therefore surprising that the carboxy terminal RanBP1 binding tail of Ran is not necessary for protein import *in vitro* (Ren et al., 1995). It is possible that RanBP1 plays a more direct role in RNA processing or the cell cycle functions of the GTPase.

A fourth factor that may be involved in the Ran GTPase cycle has been purified as an activity required for the translocation step of nuclear protein import in addition to Ran. A 10 kilodalton protein was purified from fraction B that is required to stimulate import *in vitro* with purified Ran back to the levels observed with whole fraction B (Moore and Blobel, 1994). This activity runs on an SDS gel as a single peptide of 10 kilodaltons, but has an apparent mass of 30 kilodaltons by gel filtration chromatography. Part of this peptide was sequenced and it was found to be homologous to pp15, a human placental protein of unknown function. Furthermore p10 is similar to a yeast open reading frame. Therefore, p10 is probably a conserved component of the nuclear protein import system. It is not known whether this protein directly interacts with the Ran protein or whether it participates in any of the other nuclear processes in which Ran and RCC1 have been implicated.

Obviously there are a number of unanswered questions concerning the role of the nuclear GTPase cycle in nucleocytoplasmic transport. The central issue is the nature of the pleiotropic phenotypes and activities ascribed to these genes. There are two general models that could account for this observed pleiotropy of Ran in the cell: either Ran regulates a central process whose consequences can be observed in a variety of systems, or active Ran interacts with a variety of effectors serving differing functions. If the first possibility were correct, the primary role of Ran is unclear. Assuming that the function of Ran in nuclear uptake of proteins is relatively direct, it is difficult to correlate with the isolation of Ran as an essential cytoplasmic factor for import with the observation that both Ran and RCC1 are primarily nuclear proteins. One possibility is that Ran shuttles in and out of the

nucleus, perhaps in association with transport substrates. The import of a substrate bound at the NPC may be catalyzed by the hydrolysis of GTP on Ran. Ran may be cotransported with the substrate (and the import receptor) into the nucleus, where it may be recharged by RCC1. At any given time, the bulk of Ran protein is in the nucleus, but this may be an aspect of the equilibrium of the transport process and the affinity of Ran for nuclear proteins, specifically for its exchange factor, RCC1. One interesting hypothesis suggests that both directions of transport across the pore require GTP hydrolysis on Ran. In this model, there might be a reciprocal cytoplasmic GEF and a nucleoplasmic GAP for Ran in addition to RCC1 and Rna1. However, Ran specific guanine nucleotide exchange is almost inactive in tsBN2 cells at the non-permissive temperature (Bischoff et al., 1995), which argues against a second GEF.

To prove the direct role of Ran in nuclear import, direct physical interactions will have to be established between this protein and other import factors, particularly NPC components. One interesting observation is that a temperature sensitive mutant of the pim1 RCC1 homolog from *S. pombe* has similar NE breakdown phenotypes to those demonstrated for *nup1* conditional mutants and *nup2 srp1* double mutants (Demeter et al., 1995). Potentially these shared phenotypes represent an interaction on the molecular level.

Cytosolic heat shock proteins are also required for import

The final class of proteins that have been demonstrated to have a role in import of proteins in to the nucleus are cytosolic hsp70 cognates (Goldfarb, 1992). The hsc70 and hsp70 heat shock proteins bind to NLS peptides and are required for the import of proteins into the nucleus *in vitro* (Shi and Thomas, 1992) and *in vivo* (Imamoto et al., 1992). Injection of anti-hsc70 antibodies into tissue culture cells inhibited nuclear uptake of an import substrate (Imamoto et al., 1992). In addition, cytosol from HeLa cells depleted of Hsp70 by passage over ATP-agarose or anti-hsp70 antibodies is inactive for import *in vitro* (Shi and Thomas, 1992). Depleted cytosol can be complemented by the addition of bacterially expressed hsp70 or hsc70. hsp70 does not seem to be required for the import of every nuclear protein, because its depletion has no effect on the uptake of the glucocorticoid receptor (Yang

and De Franco, 1994). These heat shock proteins must be involved in the binding step of the import process, because binding of substrate to the envelope is not observed with the depleted cytosol *in vitro*. Whether recognition of the NLS sequence of targeted proteins by hsp70 is a step in the recognition process is unknown. It is interesting to note that hsp70 or its cognates are involved in almost every transmembrane transport reaction, including nuclear import, mitochondrial protein import and insertion of proteins into the endoplasmic reticulum.

1.6 Genetic screens in yeast for nuclear transport mutants

As an independent approach to isolate more components of the import machinery, several groups have designed genetic screens or selections in *Saccharomyces* for mutants that have abnormal transport capabilities. Two screens for mutants in protein import have been carried out that were based on an intracellular localization competition strategy. The concept was to express a mitochondrial enzyme essential for respiration engineered with two competing targeting signals, a NLS and a mitochondrial import signal. Because the NLS is dominant over the normal localization signal of these proteins, they are incorrectly targeted to the nucleus, and the cell cannot grow on non-fermentable carbon sources, such as glycerol. Conditional mutants were then selected that had the ability for growth on glycerol under semi-permissive conditions, with the idea that they would represent cells with reduced capacity for nuclear import.

The first screen employing this strategy was performed with a reporter consisting of the nuclear localization sequence from SV40 large T-antigen fused to the N-terminus of cytochrome c1, a mitochondrial inner membrane protein (Sadler et al., 1989). The *npl1* mutation was isolated and was found to be allelic to *sec63*, a mutation that affects the earliest step in the secretion pathway. The Sec63/Npl1 is a integral membrane protein that contains a domain that is homologous to DnaJ, an *E. coli* heat shock protein. The *npl1* mutant also affects the proper localization of other nuclear proteins, which supports the validity of this screen. However, the *npl1* mutant may affect the assembly or maintenance of NPC structure, rather than directly participating in the nucleocytoplasmic transport reaction. A similar screen was carried out by the same laboratory using a NLS bearing derivative of the mitochondrial

F1 β-ATPase (Bossie et al., 1992). The two screens together yielded mutations in at least five genes. The most thoroughly examined strain from these studies is the *npl3* mutant. The *npl3* mutant mislocalizes NLS-F1 β-ATPase and other nuclear proteins, such as histone H2B. The *NPL3* gene encodes a 45 kilodalton protein that contains a several motifs indicative of a RNA binding protein. While the bulk of Npl3 protein is present in the nucleus, it shuttles to the cytoplasm (Flach et al., 1994). Furthermore, deletion of the NPL3 gene is viable, and a strain lacking NPL3 does not mislocalize nuclear proteins. Therefore, the mechanism of nuclear protein mislocalization of the conditional *npl3* mutants may also be indirect. Perhaps Npl3 is involved in the export of RNA from the nucleus, and its effect on protein import is due to a coupling of import and export as suggested by the phenotypes of mutants in the nuclear GTPase cycle.

The third screen for nuclear import mutants based on a competition strategy was performed by Gu and Goldfarb (1992). As in the previous study, cytochrome c with a nuclear localization signal added at the amino terminus was mistargeted to the nucleus. Reversion of the respiratory defect of this strain allowed the isolation of conditional mutants defective in the nuclear uptake of the NLS-cytochrome fusion. One mutant was isolated and named *nip1*. The effect of the *nip1* mutant on protein import was confirmed by monitoring the nuclear accumulation of a β-Gal protein containing a nuclear localization signal of the yeast ribosomal protein L29. *NIP1* encodes a 93kD cytoplasmic protein with no clear homologies to known genes (Gu et al., 1992). It has yet to be determined whether Nip1 has a direct role in the nuclear import reaction. None of these selections yielded genes that have been isolated by biochemical means as participants in the nuclear import process. It may be that the competition between two essential processes, respiration and protein import, does not allow for enough dynamic range to enable most import mutants to survive the selection process.

RNA export mutants

Although many of the components of the protein import apparatus have been isolated by biochemical means, the RNA export machinery has been refractory to study. Therefore, the development of an assay in yeast for the export of poly(A)+ RNA to the cytoplasm that was amenable to a genetic

screen (Amberg et al., 1992) was met with great excitement. By screening a bank of temperature sensitive yeast strains by the *in situ* hybridization assay for aberrant poly(A)+ RNA localization in the nucleus, a number of ribonucleic acid trafficking (*rat*) mutants were isolated. Another group independently initiated a similar screen for mutants they named *mtr* (mRNA transport defective) mutants (Kadowaki et al., 1994; Kadowaki et al., 1992).

The first mutant cloned from a mRNA mislocalization screen, *rat1*, is allelic to *TAP1*, a mutant selected for increased transcription from a RNA polIII promoter (Aldrich et al., 1993; Di Segni et al., 1993). These mutants also appear to have pleiotropic RNA processing defects. Furthermore, the *RAT1/TAP1* gene product has extensive sequence similarity to a yeast DNA strand transfer protein that is also a riboexonuclease (variously known as *KEM1*, *XRN1*, *SEP1*, *DST2*, or *RAR5*) This protein was also isolated as a G4-DNA-dependent nuclease that recognizes a tetrastranded G4-DNA structure that may be involved in meiotic chromosome segregation (Liu and Gilbert, 1994). Therefore the *rat1* mutant may be involved in a step of preRNA processing that is obligatory for export.

As mentioned above, the *prp20* RCC1 homolog and the *rna1* RanGAP were also identified as mutants that mislocalize poly(A)+ mRNA into the nucleus (Amberg et al., 1993; Hutchinson et al., 1969; Kadowaki et al., 1993). Therefore, unlike the selections for protein import mutants, these screens have been successful in detecting mutations in genes that have been implicated in nucleocytoplasmic trafficking by other means. In addition, several new components of the NPC have recently been identified by this approach (C. Cole, personal communication).

1.7 Regulated nuclear import

Although it appears that the transport of most molecules across the NE is a constitutive process, it is clear that the regulated transport of proteins into the nucleus can be utilized to potentiate gene expression and perhaps other nuclear processes. Some transcription factors require an extracellular signal to stimulate their nuclear uptake, such as some steroid hormone receptors, the *Drosophila* morphogen dorsal, and the growth factor responsive transcriptional activator NF-κB. There are a variety of different mechanisms that have been identified that prevent the uptake of these

proteins into the nucleus until the appropriate stimulus. A general mechanistic theme is the masking of the regulated NLS until a signal is received, whereupon the NLS is uncovered and thereby activated. The localization of other nuclear proteins is regulated during the cell cycle. The yeast transcription factor Swi5 and the DNA replication factors of the Cdc46 family are nuclear in G1 cells but cytoplasmic during the S, G2, and M phases of the cell cycle (Nasmyth et al., 1990).

The related proteins dorsal and NF-κB are both found in the cytoplasm, associated with an inhibitory subunit (cactus and I-κB, respectively), that covers their NLS until the stimulatory signal is received, whereupon the inhibitor is phosphorylated and released from the transcription factor, which then enters the nucleus, presumably through the constitutive nuclear import pathway (Baeuerle and Baltimore, 1988; Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990; Roth et al., 1991; Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). In a somewhat different manner, the control of the localization of the glucocorticoid receptor is achieved by masking the NLS with cytoplasmic Hsp90, which anchors the complex in the cytoplasm until displaced by ligand (Picard and Yamamoto, 1987; Sanchez et al., 1985).

Swi5 is a regulator of transcription of the HO endonuclease. To control the timing of mating type switching, HO is only induced during the G1 phase. This regulation is achieved by regulated entry of Swi5 into the nucleus (Nasmyth et al., 1990). It has been shown that the dephosphorylation of three serine residues near the NLS, which are phosphorylated by the Cdc28 kinase, is necessary for nuclear entry (Moll et al., 1991). In this case there is no evidence for an anchoring protein, rather the phosphorylated residues may directly prevent recognition of the Swi5 by the receptor. Therefore, the entry of Swi5 is prevented by active mitotic kinase until the end of mitosis, when phosphatases can act on Swi5 and prevent its nuclear entry. The Cdc46 protein family, whose functions are required for DNA replication, have essentially the same pattern of localization during the cell cycle as Swi5 (Hennessy et al., 1990; Hennessy et al., 1991; Yan et al., 1993). Therefore, the regulation of nuclear entry of these proteins is probably similar to that of Swi5.

Regulated import of nuclear proteins is likely to be a common form of gene regulation. There are probably proteins that respond to other stimuli by nuclear entry. Furthermore, regulated nuclear import may play an important

role in the cell cycle, particularly in lower eukaryotes with persistent nuclear envelopes such as yeast. For example, the nuclear entry of the Cdc46 protein is an important signal for the initiation of S phase. Furthermore, yeast may use regulated nuclear import of regulatory factors to supplant the need for NE breakdown to initiate mitosis, as discussed in Chapter 4.

1.8 Summary

Nucleocytoplasmic exchange is one of the major transport processes in eukaryotic cells. An enormous flux of proteins, RNAs and RNPs traverses the nuclear pore complex throughout the life of every cell. The molecular mechanism of the nuclear import of proteins is beginning to be elucidated by integrating a combination of biochemical and genetic approaches derived from both the vertebrate and fungal experimental systems. The signals for nuclear import, their recognition system and many components of the translocation system have been identified. A possible model for the current data concerning the mechanism of nuclear protein import is presented in Figure 1.3. On the other hand, the reciprocal traffic of RNA from the nucleus is less well understood. However recent results have suggested that the two processes may be linked, functioning perhaps as a direct exchange.

The nuclear import system can be viewed as two parts: soluble cytoplasmic components and the components of the NPC proper. There are at least six cytosolic proteins that are required for nuclear import of proteins *in vitro*: importin-60/Srp1, importin-90/karyopherinβ, Ran/TC4/Gsp1, RanGAP1/Rna1, p10, and Hsp70. These factors probably represent most of the cytoplasmic import system, as they can be combined in a defined *in vitro* system that supports efficient transport. They constitute the recognition system for nuclear targeting signals and a GTPase that is necessary to translocate proteins across the NPC. Importantly, it has not been established that the *in vitro* assays as currently executed faithfully reproduce the GTPase cycle as it occurs in cells. In addition, it may be that import *in vivo* has more levels of regulation that are not reflected in the *in vitro* assays. Therefore, there may be cytoplasmic components of nuclear protein import that remain to be identified.

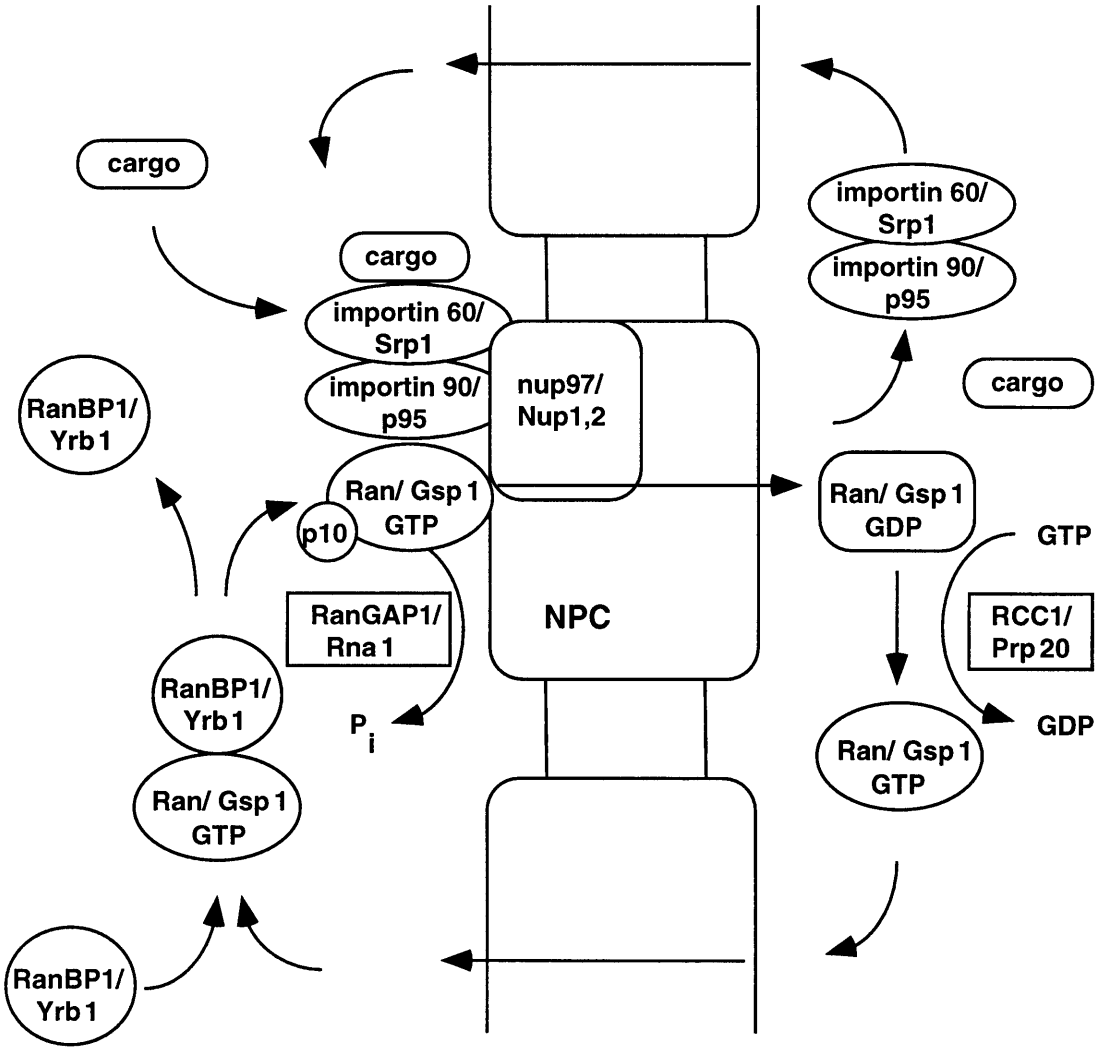
A large number of proteins (nucleoporins) have been characterized that are components of the NPC. Some of these proteins have been implicated in the nuclear import processes, either by the failure of mutants to

execute transport, or by the interaction between a particular pore component and a soluble nuclear import factor. Other NPC components have been implicated in other nuclear processes, such as maintenance of nuclear structure and DNA replication. Therefore, it has been proposed that in addition to its role in transport, the NPC is a structural organizer for the nuclear envelope/endoplasmic reticulum membrane, which might entail serving as a point of attachment for skeletal elements on both sides of the envelope as well as specifying subdomains of this complicated membrane system. Most of the NPC components that have been cloned fall into two classes of proteins that are characterized by repeated sequence motifs. The roles of these repeated sequences are not yet understood. Interestingly, members of one class of NPC components, the XFXFG nucleoporins, has been implicated in both realms of NPC function.

How are XFXFG nucleoporins in yeast involved in two different processes? While Nup1 and Nup2 appear to be directly involved in the import reaction as docking sites for Srp1/substrate complexes, Nsp1 interacts with an entirely different complement of proteins whose function seems to be more involved with the integrity and organization of NPCs within the NE. This may represent a division of labor within the NPC or it might be a limitation of the experimental approaches utilized thus far. For example, there is an apparent contradiction in the role of the nup97 protein as studies in the Xenopus reconstitution system suggest that it does not participate in import while other experiments have shown it to directly interact *in vitro* with the NLS receptor complex. Results such as these may reflect the redundancy of components of the NPC, as discussed in Chapter 2. Furthermore, it may be that the interpretation of the structural phenotypes of some nucleoporin mutants have been misinterpreted. It might be that all phenotypes of nucleoporin mutants are defects in either nuclear import or export, but that some may only affect a limited number of substrates. Chapters 3 and 4 discuss the phenotypes of mutations in the nuclear import receptor in yeast that are likely to fall into this category.

Figure 1.4 A model for the mechanism of nuclear import

Soluble components of nuclear import and some components of the NPC that are implicated directly in protein import are depicted. The name of the mammalian protein is shown on top, with the name of the yeast homolog below. "Cargo" is any NLS-bearing protein.


cargo
cargo
importin 60/
Srp1
importin 90/
p95
RanBP1/
Yrb1
nup97/
Nup1,2
Ran/ Gsp1
GTP
p10
RanGAP1/
Rna1
NPC
RanBP1/
Yrb1
Ran/ Gsp1
GTP
RanBP1/
Yrb1
P
i
importin 60/
Srp1
importin 90/
p95
cargo
Ran/ Gsp1
GDP
GTP
RCC1/
Prp20
GDP
Ran/ Gsp1
GTP
cytoplasm
nucleoplasm

Chapter 2

Nup2, a novel yeast nucleoporin, has functional overlap with other proteins of the nuclear pore complex

Preface

The work described in this chapter was published as "Nup2, a novel yeast nucleoporin, has functional overlap with other proteins of the nuclear pore complex" *Molecular Biology of the Cell* **4**:209-222. It is the product of a collaboration between myself and Laura Davis when she was a post-doc in the Fink lab. Laura had developed the monoclonal antibodies against mammalian NPC components during her gradute studies in Gunter Blobel's lab at the Rockefeller University. Upon arriving at MIT, she did the original screen of the yeast expression library to clone yeast nucleoporins, then she characterized the first product of that screen: *NUP1*. At this point I joined the laboratory and I proceeded to characterize the rest of the potential clones from Laura's screen. I performed all of the experiments described in this chapter, but I benefited from much advice and technical expertise from Laura.

2.1 Introduction

Since the nuclear envelope acts as a barrier to separate nuclear metabolism (e.g. transcription) from cytoplasmic metabolism (e.g. translation), transit of molecules between these compartments must occur via a specialized organelle, the nuclear pore complex (NPC). The bidirectional traffic of macromolecules through the NPC has been shown to be a specific, concerted process (for review see: Silver, (1991); Garcia-Bustos et al., (1991)). Conservation of the ultrastructure of the NPC among many species (Newport and Forbes, 1987), suggests that this organelle operates via the same mechanism in all eukaryotes. The prevailing model for nuclear import of proteins and snRNPs involves recognition by cytoplasmic receptors, receptor/substrate docking at the NPC, and either transport of the complex or release of the substrate to the pore apparatus for transport. Substrate recognition occurs via multiple cytoplasmic receptors (Fischer et al., 1991; Michaud and Goldfarb, 1991), some candidates for which have been identified (Adam and Gerace, 1991; Adam et al., 1989; Silver et al., 1989). The translocation step is an ATP dependent process (Newmeyer and Forbes, 1988; Richardson et al., 1988), but the ATP binding and hydrolyzing components have not yet been identified. The reciprocal transport of mRNA and ribosomes from the nucleus to the cytoplasm is less well understood. It appears that transport to the cytoplasm is intertwined with RNA processing steps within the nucleus, and that the transport apparatus itself might not be responsible for specificity (reviewed in (Davis, (1992)).

The NPC is a proteinaceous assembly of over 10^8 daltons, and it has been estimated to be composed of over 100 polypeptides (Reichelt et al., 1990). The majority of the NPC constituent proteins are yet to be identified. Most NPC proteins which have been characterized thus far belong to a family of immunologically related proteins (nucleoporins) first identified by monoclonal antibodies raised against mammalian NPC antigens (Davis and Blobel, 1986; Davis and Blobel, 1987; Park et al., 1987; Snow et al., 1987). Some nucleoporins have been shown by immunoelectron microscopy to be limited to the central, "transporter" region of the NPC, the site where the transport mechanism is suspected to reside (Akey and Goldfarb, 1989). In vertebrates, three nucleoporin proteins have been shown to exist as a complex of ~500kD (Finlay et al., 1991). Genes for three nucleoporins have been isolated: p62

from mammals, and *NUP1* and *NSP1* from budding yeast (Davis and Fink, 1990; Nehrbass et al., 1990; Starr et al., 1990). The immunological cross reactivity of these proteins is probably due to a repetitive sequence motif. This motif, which is not identical in each protein, consists of a series of a degenerate nonapeptides each separated by a less conserved, highly charged spacer sequence. Many, if not all, of these proteins are also recognized by the lectin wheat germ agglutinin (WGA) by the virtue of the fact that they contain multiple O-linked N-acetyl glucosamine residues.

Several lines of evidence suggest that nucleoporins are directly involved with nuclear transport. WGA inhibits the translocation step of protein import both *in vitro* and *in vivo* (Dabauvalle et al., 1988; Finlay et al., 1987). Furthermore, depletion of the WGA binding proteins from *Xenopus* cytosolic extracts prior to nuclear envelope reconstitution prevents even the initial binding of a transport substrate to the NPC, but does not affect passive diffusion (Finlay and Forbes, 1990). In addition, WGA binding proteins appear to bind a cytoplasmic factor that is required for the transport of proteins through the nuclear pore in an *in vitro* assay, suggesting that nucleoporins might serve as "docks" for receptor-substrate complexes (Sterne-Marr et al., 1992). Furthermore, the yeast Nsp1 protein has been implicated in the transport of proteins across the nuclear envelope, since a conditional allele mislocalizes nuclear proteins at the non-permissive temperature (Nehrbass et al., 1990).

The sequences of *NUP1* and *NSP1* suggest a common tripartite pattern for yeast nucleoporins: Each protein consists of unique terminal sequences flanking the central repeated motif recognized by the monoclonal antibodies. Not all of these sequence segments are required for viability. For example, a strain containing an *NSP1* disruption is viable if supplied with a construct containing the 217 amino acid carboxy-terminal region of *NSP1*, lacking the central repeated motif. This carboxy terminal domain is sufficient to target a heterologous protein to the nuclear envelope (Hurt, 1990). The dispensability of the repeated domain of *NSP1* indicates that the repeated motif of nucleoporins might have a common or redundant function, whereas the requirement for the unique carboxy terminal region suggests that each nucleoporin may have a unique role in NPC function.

To address the role of nucleoporins in nuclear transport, and to pursue the significance of the unusual domain structure of the nucleoporin gene

family, we have sought additional genes that contain the repetitive domain common to *NUP1* and *NSP1*. This search led to the isolation of a third related gene from yeast, *NUP2*. In contrast to *NUP1* and *NSP1*, *NUP2* is not essential for growth. However, in conjunction with mutations in either the *NUP1* or *NSP1* genes, *NUP2* is required. We have utilized the synthetic phenotypes of combinations of mutations in these genes to assess the significance of the conserved and divergent domains of this gene family.

2.2 Materials and Methods

Reagents

Enzymes for molecular biology were purchased from New England Biolabs, Pharmacia, and Promega. Yeast spheroplasts were prepared using oxalyticase (Enzogenetics) for immunofluorescence, and zymolyase 100T (Kirin Brewing Co., Ltd.) was used to prepare asci for tetrad dissection. Secondary antibodies were obtained from Jackson Immunoresearch. Radiochemicals were purchased from Amersham, and all other chemicals were obtained from Sigma.

DNA techniques

DNA subcloning was done by standard techniques outlined in (Sambrook et al., 1989). A complete list of plasmids employed in this study is shown in Table 1.

Cloning potential nucleoporin genes

In previous studies, a yeast genomic expression library was screened with MAb350 (Davis and Fink, 1990). 13 unique positive clones (including *NUP1*) were identified. Protein was prepared from lysogenic *E. coli* strains harboring the positive lambda clones, and subsequently immunoblotted with a series of monoclonal antibodies to mammalian NPC antigens, in order to identify the clones that express authentic nucleoporin epitopes. As a second test, Southern blots of plasmid DNA subcloned from each positive lambda clone were probed at low stringency with a degenerate oligonucleotide (deNSP1) complementary to all possible nucleotide sequences that can encode the peptide sequence (KPAFSFGAK), precisely conserved in 12 of 22 of the repeated units in *NSP1* (sequence of deNSP1: $5'$(A/T)TT IGC ICC (A/G)AA IGA (A/G)AA IGC IGG (C/T)TT$3'$ where positions in parentheses are equal mixtures of indicated nucleotides, and I indicates inosine). Hybridization conditions were as follows: 5.5X SSC (825mM NaCl, 83mM sodium citrate), 5X Denhardts solution, 1% sodium dodecyl sulfate, 0.2mg/ml calf thymus DNA, 1mM EDTA. Blots were incubated with end-labeled oligonucleotide probe for 18 hr at temperatures of either 40°C, 50°C or 65°C, then briefly washed at room temperature in 6X SSC, 1% sodium dodecyl sulfate, dried, and exposed to X-Ray film.

Full length clones of four genes which were positive in either the immunoblotting or the hybridization tests were isolated from a genomic plasmid library in the yeast shuttle vector YEp24 (Carlson and Botstein, 1982)

Table 1 Plasmids used in this study

Yeast Plasmid	relevant markers	comments	source
pB2201	2μ *LEU2*	derived from pRS305 by insertion of 2μ sequences at AatII site	D. Miller
pB2204	*CEN LEU2*	*GAL1-10* promoter inserted into pRS315	"
pJON35	2μ *URA3 NUP2*	13kB fragment containing *NUP2* from YEp24 library	This study
pJON37	2μ *LEU2 NUP2*	6.2kB BamH1 fragment containing *NUP2* in pB2201	"
pJON73	*CEN LEU2 NUP2*	6.2kB BamH1 fragment containing *NUP2* in pRS315	"
pJON80	*CEN LEU2 NUP2::HA*	HA epitope inserted at aa. 428 by oligo mutagenesis.of pJON73	"
pJON86	*CEN LEU2 GAL::NUP2*	*NUP2* coding sequence under GAL promoter in pB2204	"
pB2487	*CEN URA3 NUP1*	*NUP1* wildtype inserted into pRS316	"
pB2289	*CEN TRP1 NUP1*	*NUP1* wildtype in pRS314	"
pB2291	*CEN TRP1 nup1-8* (Δ 4-141)	internal deletion of pB2289 created by exoIII digestion	"
pB2300	*CEN TRP1 nup1-21* (Δ1042-1076)	"	"
pJON139	*CEN TRP1 nup1-8 NUP2*	wildtype *NUP2* inserted into pB2289	"
pJON146	*CEN TRP1 nup1-8 NUP2::HA*	*NUP2::HA* inserted into pB2289	"
pJON164	*CEN TRP1 nup1-8 nup2-9*	*nup2-9* (Δ86-720) inserted into pB2289	"
pJON149	*CEN TRP1 nup1-8 nup2-10*	*nup2-10* (Δ175-720) inserted into pB2289	"
pJON165	*CEN TRP1 nup1-8 nup2-11*	*nup2-11* (Δ556-720) inserted into pB2289	"
pB2486	*2μ LEU2 NSP1*	wildtype *NSP1* in YEp13	Nehrbass et al., 1990
pB2485	*CEN LEU2 C-NSP1*	*NSP1* carboxy terminus under ADH promoter	"
pB2483	*2μ LEU2 NSP1* (Δ173-605)	derived from pB2486	"

E.coli plasmids	description	source
pJB107	pUC118 with HindIII site destroyed by filllng in and religation	J. Brill
pLD15.1	a 3 kb EcoRI fragment from λgt11 clone 15.1 subcloned into pGEM7	this study
pJON12	*NUP2::URA3* : 2kB SacI fragment of pLD15.1 in pRS306	"
pJON53,54	*NUP2* 6.2 kB BamHI fragment pBluescript ks(+) inserted in both orientations	"
pJON107	NUP2 4kB PstI to BamHI in pJB107 mutagenized as per text	"
pJON113	*nup2-4::URA3* derived from pJON107	"
pJON115	*nup2-5::HIS3* "	"
pJON133	*nup2-7:TRP1* "	"
pJON166	*nup2-12::TRP1* created by replacing internal ApaI fragment of *NUP2* with *TRP1*	"
pB2337	*nup1-2::LEU2* created by replacing region from SnaBI to BspMI of *NUP1* with *LEU2*	"
pB2482	*nsp1::URA3* disruption construct	Nehrbass et al., 1990

notes: pRS vectors are from Sikorski and Heiter, 1989. All plasmids are amp^r.

by colony hybridization(Sambrook et al., 1989) with RNA probes synthesized *in vitro* from the plasmid subclones. These four plasmids were tested by the overexpression assay detailed in RESULTS. One clone (pJON35) was identified as *NUP2*. The three other lambda clones tested by the overexpression assay failed to show overexpression of a polypeptide recognized by the monoclonal antibodies. Since this approach depends upon isolation of the full length gene and its subsequent overexpression, either the YEp24 isolates identified by these lambda clones did not contain the full length gene or expression was modulated by unknown means in the constructs assayed.

DNA sequencing

Nested series of deletions were made from plasmids pJON53 and pJON54 by a modification of the ExoIII protocol of Henikoff (Henikoff, 1984), substituting ExoVII nuclease for S1 nuclease. Double stranded dideoxy sequencing with Sequenase was performed as per manufacturer's (US Biochemicals) instructions. The sequence was edited using the Lasergene package (DNASTAR inc.), analyzed using UWGCG programs (Devereux et al., 1984) and sequence searches were performed using the NCBI BLAST network service. We sequenced through two neighboring genes upstream of the *NUP2* coding sequence. 180 nucleotides 5' from the first potential start site for *NUP2*, is the tRNA $^{GLU}3$ CYH82 with its associated TY δ element distal to *NUP2* (Hauber et al., 1988). Further upstream is an ORF with very high homology to the S31 ribosomal protein of *Saccharomyces carlsbergensis* (Nieuwint et al., 1985).

Site-directed mutagenesis

Mutants were made by the single stranded mutagenesis technique of Kunkel. dut$^-$ung$^-$ strain RZ1032 was used to isolate single stranded plasmid DNA, synthesis was performed as described (Kunkel et al., 1987), and the reaction mixture was transformed by the $CaCl_2$ technique into *E.coli* strain DH5α.

The complete disruption constructs of *NUP2* were made by mutagenizing plasmid pJON73 with two oligonucleotides to introduce a

series of restriction sites just outside of the initiation and termination codons of Nup2. The 5' oligo was of sequence: CTCAAAAAAATCATTAACGAGGA TATCTAGACTAGTATGGCCAAAAGAGTTGCCG and the 3' oligo was of sequence: GCTAAAAAAGAAATGAAATAAACTAGTCTAGATATCG AGTAAATACAGTTAACAATTTTAAATAG. A BamHI/PstI fragment containing the mutagenized *NUP2* was subcloned into pJB107, then the *NUP2* coding region of the resulting plasmid, pJON107, was replaced by selectable markers by standard cloning techniques to yield pJON113, pJON115 and pJON133. The HA antigen was introduced into *NUP2* by mutagenesis of pJON73 with a oligo of sequence: GGTACCGAGAAAGAGAAGTACCC ATACGACGTCCCAGACTACGCTGAAAGTAAAAAGCACTCA. The C-terminal truncations of Nup2, *nup2-9*, *nup2-10* and *nup2-11* were created by introducing stop codons in plasmid pJON139 at the positions indicated in the text with the following oligos: GCTCAGGTTGATAATAGCCCTTACCCATAC GACGTCCCAGACTACGCTATGAAATAATAGTGACCGCGGACCACAGAA AGCAATTCCAGA, GATGAGGTTAAGGTGGAGGGGTACCCATACGACG TCCCAGACTACGCTTAATAGTGACCCAAGTTCACAATAGATGCT, and GGGTTCAAATTTTCTTTGCCATACCCATACGACGTCCCAGACTACG CTATGAAATAATAGTGACCGCGGTTTGAACAAAAAGGTAGTCAA respectively.

Yeast growth conditions and strain construction

Yeast cell culture and crosses were performed essentially as outlined in Sherman et al., 1986(Sherman et al., 1986). Yeast plasmids and linear DNA fragments for integration were transformed into yeast by lithium acetate transformation (Ito et al., 1983). Selection against Ura$^+$ strains was done by culture on solid synthetic media containing 1mg/ml 5-fluoro-orotic acid as described in Boeke et al., 1984(Boeke et al., 1984). A complete list of strains employed in this study is shown in Table 2.

Construction of the strain (JLY25) bearing the *NUP2* gene marked with *URA3* for mapping was performed as follows: Strain L2612 was transformed with a plasmid (pJON12) that contains approximately one half of *NUP2*, from the internal SacII fragment to beyond the 3′ end of the coding sequence in the vector pRS305 (Sikorski and Heiter, 1989). This plasmid contains the yeast *URA3* gene but lacks a yeast origin of replication. pJON12 was linearized in

the *NUP2* coding sequence with HpaI, and selected integrants on synthetic media lacking uracil. The positive transformants bear *URA3* flanked by a partial duplication of *NUP2* on one side and wild-type *NUP2* on the other.

Table 2.2 Yeast strains

Strain name	genotype	source
L2612	*MATα ura3-52 leu2-3,112 trp1-289*	Fink collection
L3212	*MATa cdc25-1 ura3-52*	"
L3288	*MATα rna1-1 met trp1^1 ade2 ura3-52*	"
WLY401	*MATα nsr1-1::HIS3 lys2-801 ade2-101 trp1^1 his3^200 ura3-52*	Lee et al, 1991
LDY53	*MATa ura3-52 leu2-3,112 trp1-289*	this study
JLY25	*MATα NUP2::URA3 ura3-52 leu2-3,112 trp1-289*	"
JLY48	*MATα nup2-4::URA3 ura3-52 leu2-3,112 trp1-289*	"
JLY242	*MATα nsp1::URA3 ura3-52 leu2-3,112 trp1-289* {pB2486}	"
L4745	*MATα nup1-2::LEU2 his3^200 trp1^1 ura3-52 leu2-3,112* {pB2487}	"
JLY119	*MATα nup1-2::LEU2 his3^200 trp1^1 ura3-52 leu2-3,112 nup2-5::HIS3* {pB2487}	"
JLY54	*MATα nup2-12::TRP1 ura3-52 leu2-3,112 trp1-289*	"
JLX19	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289*	"
JLX48	*MATa/MATα NSP1/nsp1-URA3 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289*	"
JLX49	*MATa/MATα NUP1/nup1-2::LEU2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289*	"
JLX53	*MATa/MATα nsp1::URA3/NSP1 nup2-7::TRP1/NUP2*	"
JLX78	*MATa/MATα nup2-4::URA3/nup2-4::URA3 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289*	"

All strains are of S288C background. Strains LDY53,JLY25, 48, 54,242, and JLX19-JLX78 are isogenic to L2612. The L2612 isogenic series is also *can1* and *cyh1*. Strains JLY119 is isogenic to L4745. Brackets indicate resident yeast plasmids.

2.3 Results

Isolation of the gene for a novel yeast nucleoporin

A previous screen for yeast nucleoporins of a yeast λgt11 phage expression library with the monoclonal antibody 350 yielded twelve independent positive clones in addition to *NUP1* (Davis and Fink, 1990). Four were chosen for further analysis on the basis of the reactivity of their β-galactosidase fusion proteins expressed in *E. coli* with our panel of monoclonal antibodies to nuclear pore proteins (Davis and Blobel, 1986; Davis and Fink, 1990), and also by their homology to the cloned nucleoporins as assessed by low stringency Southern hybridization. One phage clone, λ15, was very strongly recognized by a degenerate oligonucleotide probe (deNSP1: MATERIALS AND METHODS) the sequence of which was designed to identify the repeated element of the *NSP1* gene. This probe also hybridizes to a *NUP1* plasmid, but none of the other phage encoding putative nucleoporins.

We further examined these four clones to identify those that expressed proteins in yeast corresponding to novel nuclear proteins recognized by our panel of MAbs. Our test was as follows: High copy plasmids containing sequences corresponding to the promising lambda clones were isolated from a library of yeast genomic DNA in the high copy yeast shuttle vector YEp24 (Carlson and Botstein, 1982) by hybridization. These YEp plasmids were then introduced in the haploid yeast strain L2612 and total protein was immunoblotted with MAbs 306, 350 and 414 (Figure 1A,B,C; respectively). The 2μ origin of replication of YEp24 specifies very high copy number, therefore a cloned gene under control of its own promoter should lead to enhanced expression of its protein relative to a vector control. Three major nuclear proteins react with our MAbs (Davis and Fink, 1990). Nup1 migrates at 130kDa, and is detectable in whole cell extracts only with the 306 antibody. All three MAbs recognize a major doublet at 100kDa which is comprised of Nsp1 and an uncharacterized protein. Strains bearing plasmid (pJON35) clearly overexpress this 95 kilodalton protein, which is distinct from Nsp1 (Figure 1, lanes 2 and 3). The pJON35 plasmid was isolated using the λ15 clone as a probe. The presence of this protein in a strain bearing a truncated version of *NSP1*(Δ 173-605; (Nehrbass et al., 1990)) provides further evidence that this protein is not Nsp1 or its degradation product (Figure 1, lane 4). The

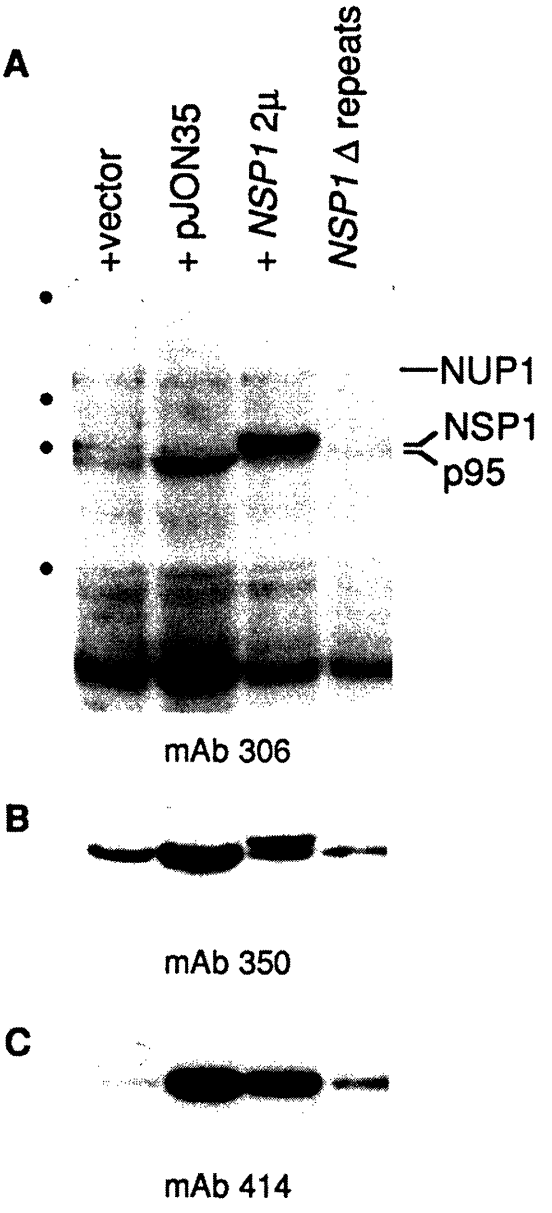
truncated *NSP1* gene lacks the central repeated domain, and is unreactive with our MAbs. Furthermore, we tested our overexpressing extracts with the 414 antibody (Figure 1C). Thereby we have shown that *NSP1* and the newly identified gene must encode the two yeast nucleoporin proteins originally characterized on the basis of their reactivity with this antibody (Aris and Blobel, 1989). *NSP1* encodes the protein Aris and Blobel refer to as p110, and the new gene encodes the p95 protein. It is also probable that this new gene encodes the protein previously referred to as NSP2 (Nehrbass et al., 1990).

The 95 kilodalton protein is encoded by *NUP2*

Various fragments of the 13 kilobase insert of pJON35 were subcloned and assayed for overexpression of p95 by Western blot. Plasmid pJON37 contains a 6.1 kilobase BamHI insert that maintains the overexpression phenotype. This BamHI fragment was mapped and sequenced (Figures 2,3). The longest open reading frame in the sequence can encode a 77,954 dalton, 720 amino acid protein, assuming the first ATG is the initiator codon. This ORF shows similarity to *NUP1*, *NSP1* and rat p62 (in order of similarity score when compared to the PIR, SWISS-PROT and translated GenBank protein databases). On the basis of this homology and other evidence presented in subsequent sections, this gene was named *NUP2*. The only other significant matches appear to be a result of the serine rich nature of the predicted *NUP2* sequence (13.5% serine as well as 12.3% lysine). Interestingly, the best match among serine rich proteins was with the *NSR1* gene, which encodes another yeast protein implicated in nuclear protein trafficking (Lee et al., 1991). Though the predicted Nup2 polypeptide is highly charged throughout its length, the predicted pI is 7.1. There are no putative transmembrane domains nor are there any regions particularly acidic or basic. No informative motifs were found when the sequence was compared against a library of approximately 400 well characterized protein motif consensus sequences (Fuchs, 1991).

Figure 2.1 Overexpression of a novel nucleoporin gene.

Western blots of total protein from the indicated strains were probed with anti-nucleoporin monoclonal antibodies as indicated. Lane 1; wild type strain L2612, transformed with the YEp24 vector, lane 2; L2612 bearing the pJON35 plasmid, lane 3; L2612 bearing pB2486 (YEp13 *NSP1*, (Nehrbass et al., 1990)), lane 4; JLY241, a *nsp1::URA3* strain bearing pB2483 (YEp13 *NSP1* D173-605). The ~50 kd polypeptide recognized by the antibodies has been shown to be cytoplasmic (Davis and Fink, 1990). The dots at left correspond to the following molecular weight standards: myosin 220 kDa, β-galactosidase, 116 kDa, Phosphorylase b, 97 kDa, Bovine Serum Albumin, 66 kDa. Yeast whole cell extracts were prepared by glass bead lysis directly into trichloroacetic acid (Ohashi et al., 1982) Western transfer and antibody probing were done as described by Davis and Fink (1990) using 5% non fat milk instead of 2% BSA.


A
+vector
+ pJON35
+ NSP1 2μ
NSP1 Δ repeats
NUP1
NSP1
p95
mAb 306
B
mAb 350
C
mAb 414

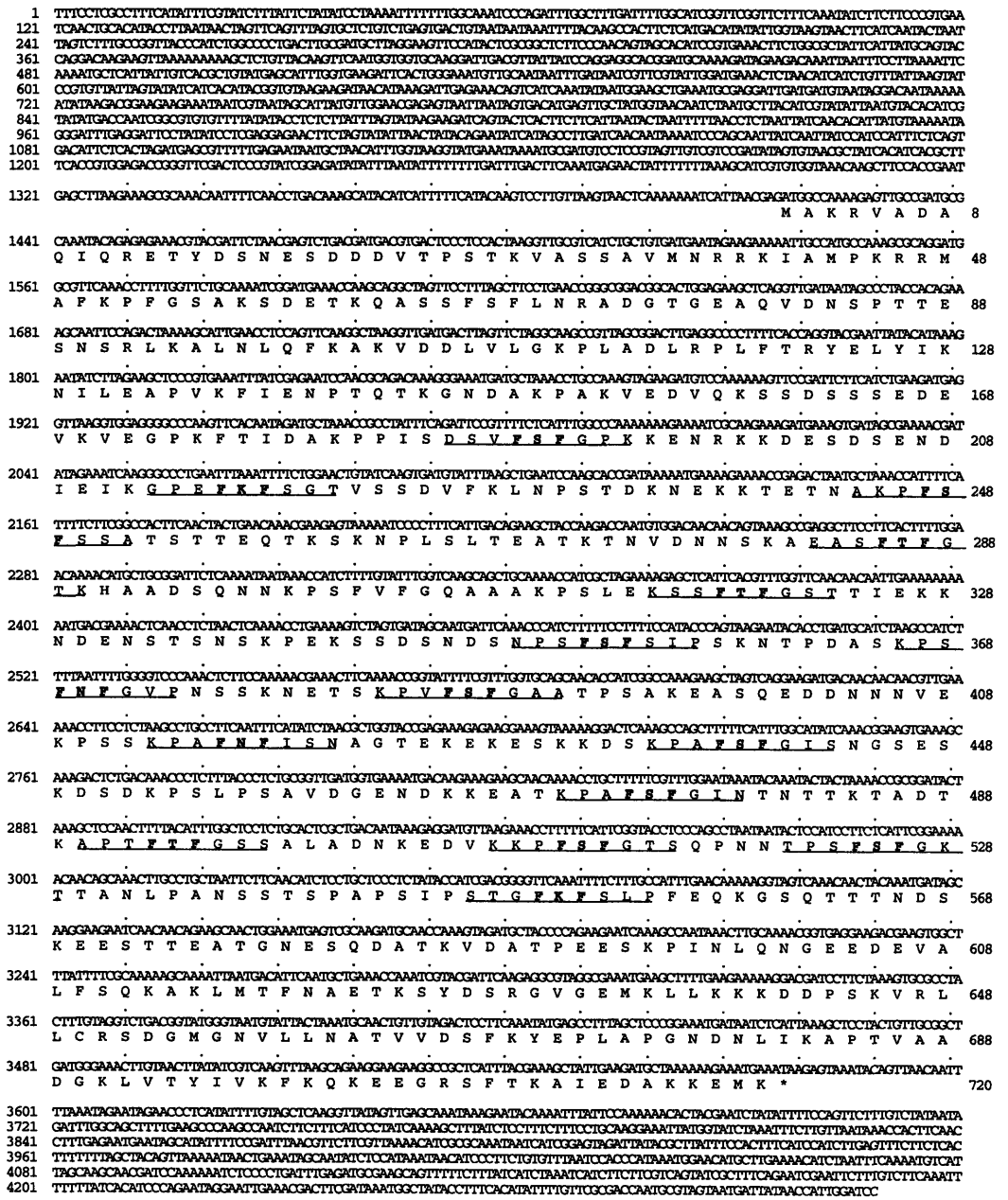
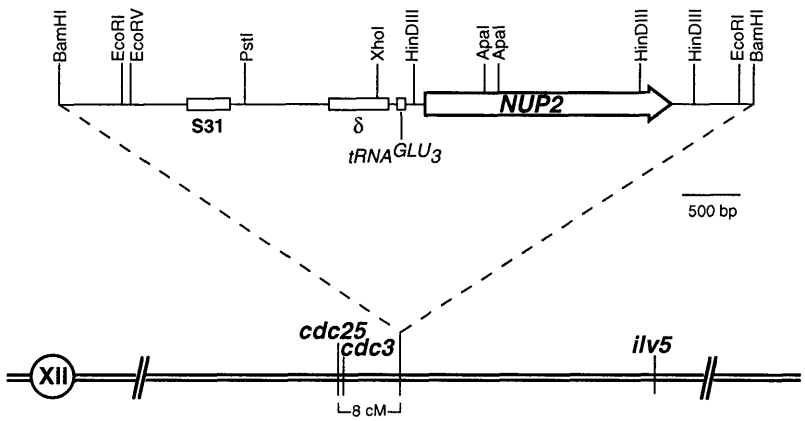


Figure 2.2 Nucleotide and predicted protein sequence of the *NUP2* gene.

Nucleoporin repeated motifs are underlined and the central phenylalanine-polar-phenylalanine stretch is in bold face. The EMBL accession number for the *NUP2* nucleotide sequence is X69964

Figure 2.3 Physical and genetic map of *NUP2* locus.

Top: restriction map of *NUP2* region. The endpoints are equivalent to the ends of the insert in plasmid pJON39. Bottom: partial genetic map of chromosome XII indicating the position of *NUP2* relative to neighboring loci.


BamHI
EcoRI
EcoRV
PstI
XhoI
HindIII
ApaI
ApaI
HindIII
HindIII
EcoRI
BamHI
NUP2
S31
δ
tRNA^GLU3
500 bp
cdc25
cdc3
ilv5
XII
8 cM

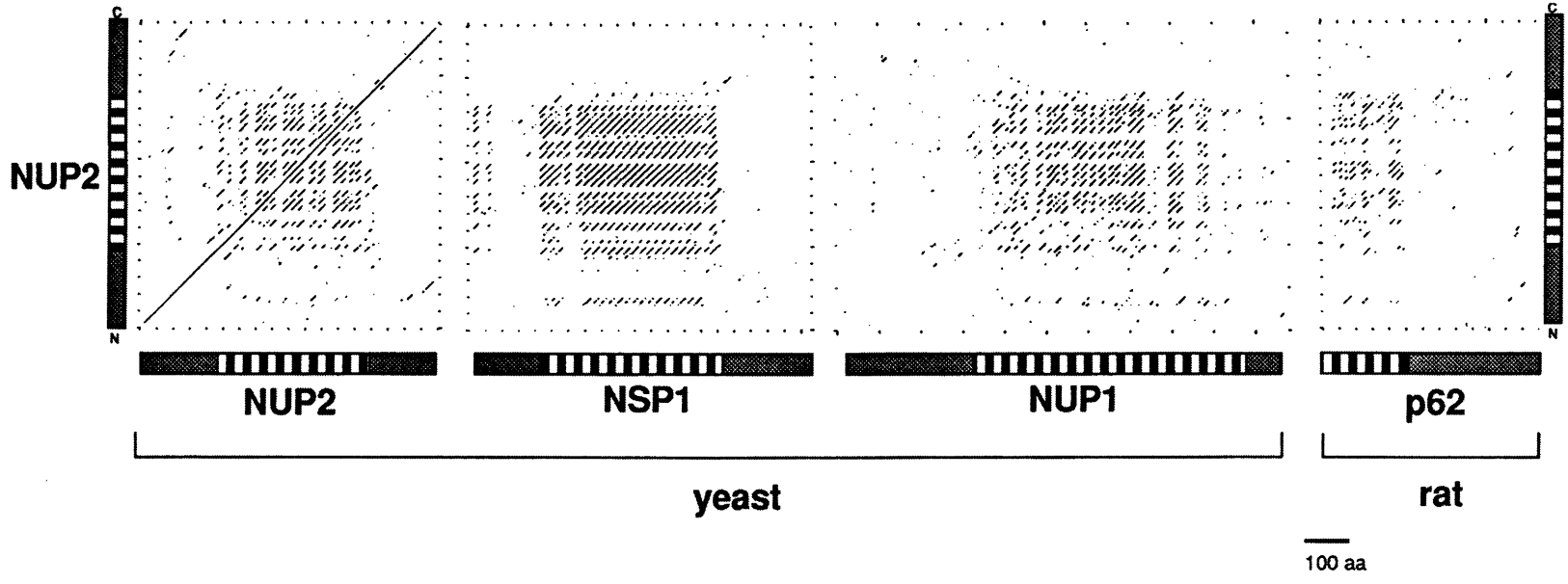
The position of *NUP2* on the physical and genetic maps of the yeast genome (Figure 3) was determined by hybridizing a labelled probe to a set of ordered λ phage clones (λ Prime filters; L. Riles and M. Olson). The *NUP2* probe hybridized to a single clone, λ6165, which is on chromosome XII and lies between the previously mapped genes *cdc3* and *ilv5*. To confirm this location, a haploid yeast strain (JLY25) containing the *NUP2* locus marked with the *URA3* gene was constructed. JLY25 was crossed with the *cdc25-1* strain L3212 (*cdc25* is just centromere proximal to *cdc3*) 56 tetrads were dissected, yielding 44 parental ditypes, 8 tetratypes and 0 nonparental ditypes, locating *NUP2::URA3* approximately 8cM from *cdc25-1*. The orientation of the coding region of *NUP2* relative to the centromere of chromosome XII was determined by comparing our restriction map and sequence to the EcoRI/HindIII physical map of Riles and Olson.

The repeated domain defines a family of nucleoporins

The homologies detected between the other nucleoporin protein sequences and the *NUP2* sequence are limited entirely to their repeated domains (Figure 4). We assigned the repeats of *NUP2* by plotting the regions of extreme hydrophobic moment in the β-sheet conformation using the algorithm of Eisenberg et al. (1984) and by analogy to the other nucleoporins. *NUP2* has 16 repeats (indicated by underline in Figure 2), in comparison to 28 in *NUP1* and 22 for *NSP1*. *NUP2* lacks an uncharged domain, which is present at the carboxy terminus of *NUP1* and at the amino terminus of *NSP1*. Both *NUP2* and *NSP1* have several sequences at their amino termini that are similar to the repeated motif. Since the only sequence similarity shared between all four proteins is the repeats, it is very likely that this sequence defines the common epitope between these proteins. The hallmark of the repeats is a pair of phenylalanines flanking a polar residue, most commonly serine (Figure 2 in boldface). This tripepetide sequence contributes most of the amphipathic nature to the repeats. Within *NUP1*, *NUP2* and p62 the repeats follow a loose primary sequence homology, whereas the repeating unit of *NSP1*, as well as its inter-repeat spacers, is well conserved from one unit to the next. *NSP1* and p62 have a second region of homology, residues 350-520 of p62 and residues 610-820 of *NSP1*. It has been shown that the repeats of

Figure 2.4 Sequence homology between *NUP2* and other nucleoporins is confined to the repeated domain.

Amino acid sequence matrix comparison derived from the UWGCG package COMPARE program: window 20, stringency 25. Vertical stripes indicate regions which have the canonical hydropathy profile of the repeated domain.

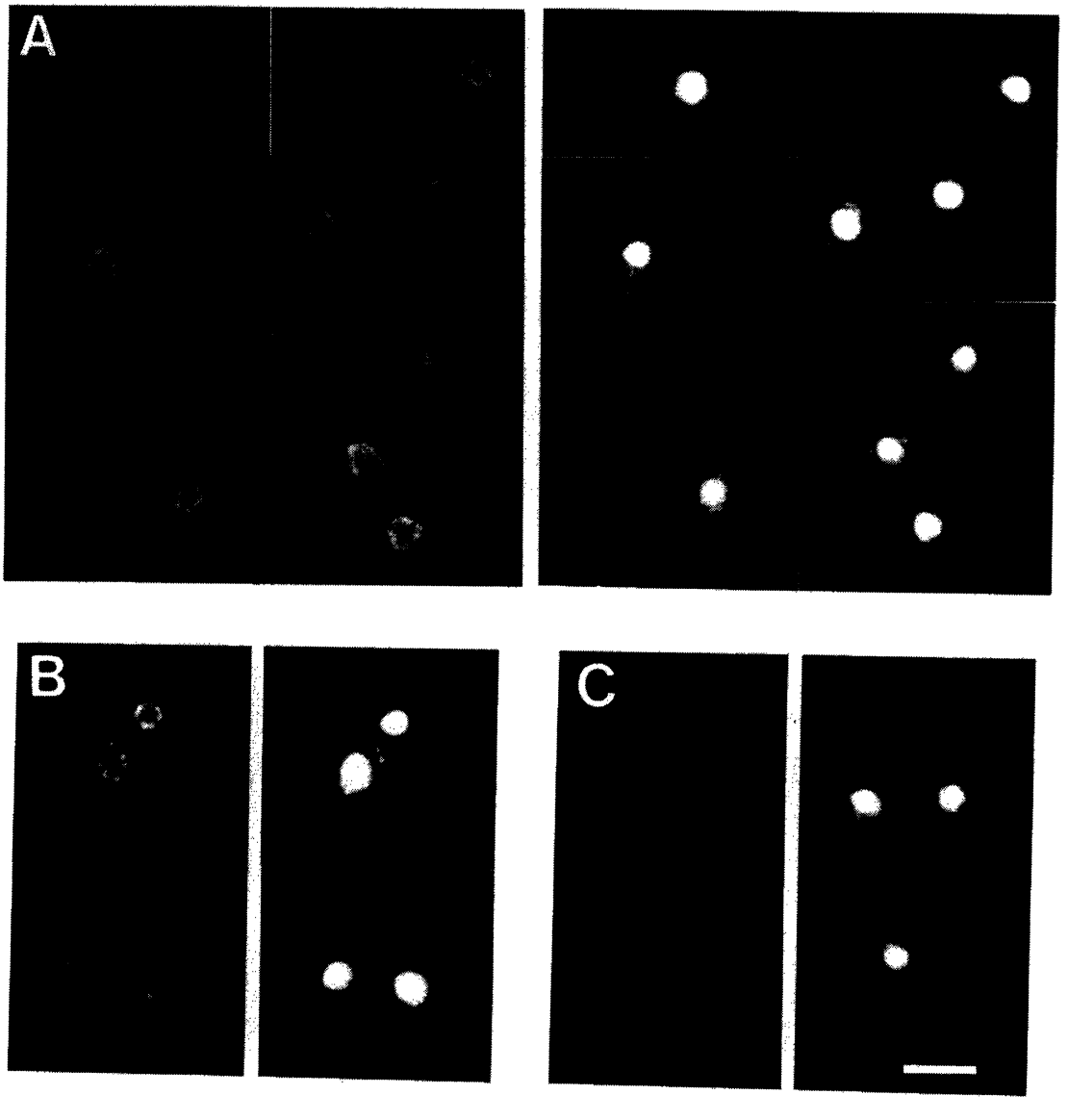

C
NUP2
N
C
N
NUP2
NSP1
NUP1
p62
yeast
rat
100 aa

NSP1 are dispensable for function (Nehrbass et al., 1990), whereas it is unknown whether the repeated domain of *NUP1* is required.

Nup2 is a component of the nuclear envelope

The intracellular localization of Nup2 was determined by indirect immunofluorescence. As none of our monoclonal antibodies is absolutely specific for Nup2, we constructed a *NUP2* derivative containing a DNA insert that encodes the nine amino acid influenza hemaglutinin (HA) antigen (Wilson et al., 1984). This foreign epitope was placed at position 428 in the spacer region between the ninth and tenth repeat, and results in a functional Nup2 protein (see subsequent section) that can be uniquely identified by the 12CA5 MAb directed against the HA epitope. This construct (pJON80) was expressed in a diploid strain lacking *NUP2* coding sequences (JLX78; described below), and the localization of the tagged protein was ascertained by indirect immunofluorescence (Figure 5A). Western blots of total protein from strains with epitope-tagged *NUP2* showed a specific 95kD band as well as a 60kD non-specific band which could also be detected in strains lacking the tagged construct . The cross-reactivity of the 12CA5 antibody with this native 60kD protein probably contributes to the cytoplasmic background shown in controls containing only the vector (Figure 5C). The fluorescence staining of Nup2 was reminiscent of that previously shown with an antibody specific for Nsp1 (Nehrbass et al., 1990) and for epitope tagged Nup1 (Davis and Fink, 1990) as well as anti-nucleoporin MAbs (Figure 5B). The antibody highlighted the nuclear envelope as a punctate, brightly staining rim that surrounded the less intensely staining intranuclear region. No staining above background was evident in the cytoplasm. Also, in some fields cells could be seen where staining was visible as the envelope extends into the bud neck early in mitosis and as a long process connecting daughter nuclei at a late stage in karyokinesis.

Figure 2.5 Immunofluorescence localization of the Nup2 protein.
In each panel, a collage of cells from several fields are shown stained with the indicated MAb in the left column, while the corresponding DAPI stained nuclei of these cells are shown on the right. A; *nup2Δ/nup2Δ* diploid strain JLX78 bearing the *NUP2::HA LEU2 CEN* plasmid pJON80 stained with MAb 12CA5 directed against hemaglutinin epitope. B; wild-type diploid strain JLX19 stained with anti-nucleoporin MAb 306. C; JLX78 bearing a *LEU2 CEN* vector plasmid, as a negative control for antibody specificity, stained with 12CA5. Immunofluorescence on yeast spheroplasts was performed as described in Davis and Fink (1990) except that 1% polyethyleneimine in H_2O was substituted for poly-lysine and filtered 5% nonfat dry milk was used in place of 2% BSA as the coating agent. (Bar=5μ)


A
B
C

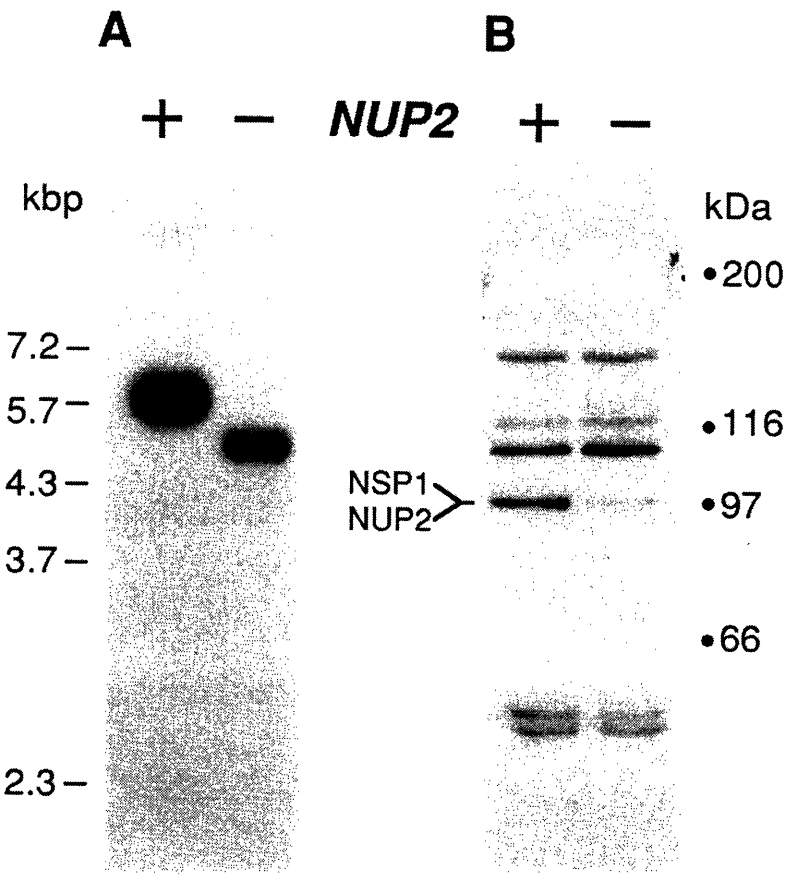
Strains containing a null mutation of *NUP2* are viable

Null mutations of *NUP2* were created by deleting the entire coding sequence of *NUP2* (MATERIALS AND METHODS) and replacing it with either *URA3*, *HIS3* or *TRP1* (*nup2-4::URA3*, *nup2-5::HIS3* and *nup2-7::TRP1*). The deletion/insertional alleles were integrated by transformation to replace the resident *NUP2* allele on one of the two copies of chromosome XII in the diploid strain JLX19 (derived by mating the isogenic strains L2612 and LDY53). Transformants of each construct were sporulated, and tetrads were dissected. In approximately 90% of the tetrads, all four spores germinated, and Nup2$^-$ spores were found at an equal frequency to Nup2$^+$ spores. Hybridization experiments with one construct showed directly (Figure 6A) that the transformants have the *NUP2* coding sequence replaced by *URA3*. Strains bearing the marked *nup2* deletion alleles appear to lack any detectable Nup2 protein, since levels of Nup1 and other uncharacterized cross reactive proteins remain constant, while the Nup2/Nsp1 doublet is dimished in intensity (Figure 6B).

The growth rate of the isogenic strains L2612 (*NUP2*) and JLY48 (*nup2-4::URA3*) on rich media at 30°C is identical. Since *nup2* deletion strains grow well under normal conditions, they were tested for other phenotypes such as temperature sensitivity, increased chromosome loss, inability to grow on non-fermentable carbon sources, and sensitivity to heat shock as well as carbon and nitrogen starvation. Strains carrying the *nup2* deletion showed no differences from an isogenic wild-type strain for any of the phenotypes tested. Nup2$^-$ strains mate with wild type efficiency and so a *nup2-4::URA3/nup2-4::URA3* diploid (JLX78) was constructed and used for the immunofluorescence experiments described in the preceding section. Examination of the *nup2* deletion strains by Nomarski microscopy failed to reveal any morphological abnormalities. Furthermore, no defects were detected in nuclear structure, as visualized by DAPI (4,6-diamidino-2-phenylindole) staining of DNA or in the nuclear envelope as visualized by immunofluorescence of NPC antigens with the 306 MAb. The viability of strains deleted for *NUP2* contrasts with the inviability of null mutants of *NUP1* and *NSP1* (Davis and Fink, 1990; Hurt, 1988).

Figure 2.6 Deletion of the *NUP2* coding sequence.

A. Southern blot of wild-type (+) and *nup2Δ* (-) strains. Genomic DNA from the parent strain (L2612) and from a strain transformed with the *nup2-4::URA3* construct (JLY48) was digested with EcoRI, electrophoresed, transferred to a nylon membrane, and then probed with a 0.4 kb HindIII fragment which contains the last 200 nucleotides of the *NUP2* coding sequence, as well as 200 nucleotides of 3' flanking sequence (see Figure 3). The alteration in mobility in the *nup2D* lane indicates a replacement of the 2.1 kb *NUP2* coding sequence with the 1.1 kb *URA3* gene. B. Western blot of total protein from the same strains as shown in A., probed with MAb 350.

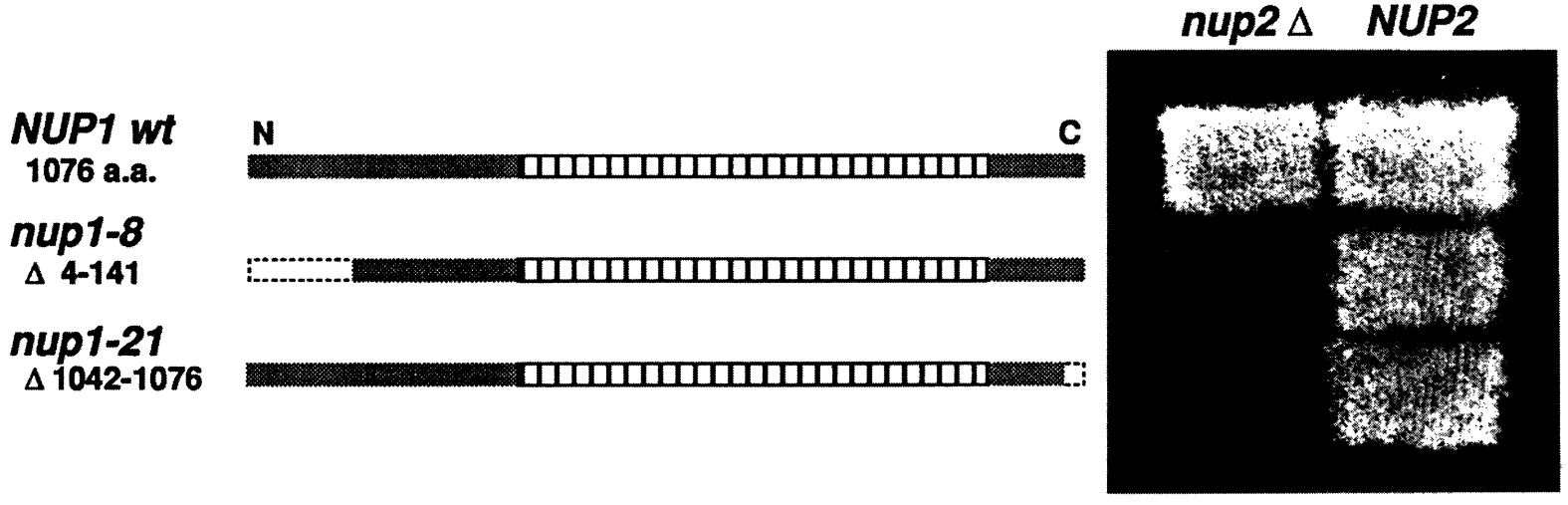

A
B
+
—
NUP2
+
—
kbp
kDa
7.2
5.7
4.3
3.7
2.3
200
116
97
66
NSP1
NUP2

To test the effects of *NUP2* overexpression, we constructed a transcriptional fusion of the *NUP2* coding sequence to the *GAL1-10* promoter (plasmid pJON86) and introduced this construct into a wild-type strain. When *NUP2* overexpression was induced by addition of galactose to the media, the cells continued to grow, but at an extremely reduced rate. The partial inhibition of growth upon overexpression of *NUP2* was also observed in strains bearing *NUP2* on 2μ plasmids. In contrast, overexpression of *NUP1* leads to complete cessation of growth (Davis and Fink, 1990). The failure of Nup2 to inhibit growth as dramatically as Nup1 upon overexpression could be due to the fact that Nup2 levels are not as high as Nup1 upon induction. Analysis of the level of Nup2 expression on glucose and galactose media by Western blotting showed that the Nup2 protein content was induced only 2-5 fold as compared to the >10 fold increase previously shown for the *GAL::NUP1* construct.

Nup2 function is required for viability in strains carrying truncations of Nup1

A series of genetic experiments was performed in order to identify functional overlap or redundancy between *NUP2* and the other yeast nucleoporins. The similarity in primary structure of *NUP2* to the other yeast genes suggested that segments of other nucleoporins might compensate for the absence of the Nup2 gene product in *nup2Δ* strains. Strains carrying an amino terminal truncation, *nup1-8* (deleted for amino acids 4-141), or a carboxy-terminal truncation, *nup1-21* (deleted for amino acids 1042-1076), on centromere containing plasmids covering a *nup1* null mutation grow somewhat more slowly than strains carrying the full length *NUP1* on a plasmid (L.D.; unpublished observations). However, these same *nup1* truncation alleles are inviable in a *nup2Δ* background. This result was obtained by a "plasmid shuffle" (Boeke et al., 1984) assay (Figure 7) using strains harboring two plasmids; one containing a wild type copy of the *NUP1* gene (*NUP1 URA3 CEN*) and the other, a *nup1* truncation (on a *CEN TRP1* vector). The chromosome contained a deletion of *nup1* marked with the *LEU2* gene (*nup1-2::LEU2*: L.D. unpublished) and either a wild type copy of *NUP2* (strain L4745) or a complete deletion of *NUP2* marked with *HIS3*

Figure 2.7 Synthetic lethality between truncations of *NUP1* and *nup2Δ*. *nup1-2::LEU2* yeast strains bearing the indicated *NUP1* allele on a *TRP1 CEN* plasmid, shown schematically, as well as wild-type *NUP1* on a *URA3 CEN* plasmid, and the indicated allele of *NUP2*, were patched onto synthetic complete medium lacking tryptophan, grown to confluency, then replica plated to synthetic complete medium containing 5-FOA. Photo was taken at 48 hours after transfer to 5-FOA media.


nup2 Δ
NUP2
NUP1 wt
1076 a.a.
N
C
nup1-8
Δ 4-141
nup1-21
Δ 1042-1076

(JLY119). These strains were grown on synthetic complete (SC) media lacking tryptophan but including uracil, which permits the growth of segregants that have lost the *NUP1 URA3* plasmid. Segregants that have lost *NUP1* could be identified by replica plating onto SC + 5-fluoroorotic acid (5-FOA), a medium that selects against strains that retain the *URA3 NUP1* plasmid.

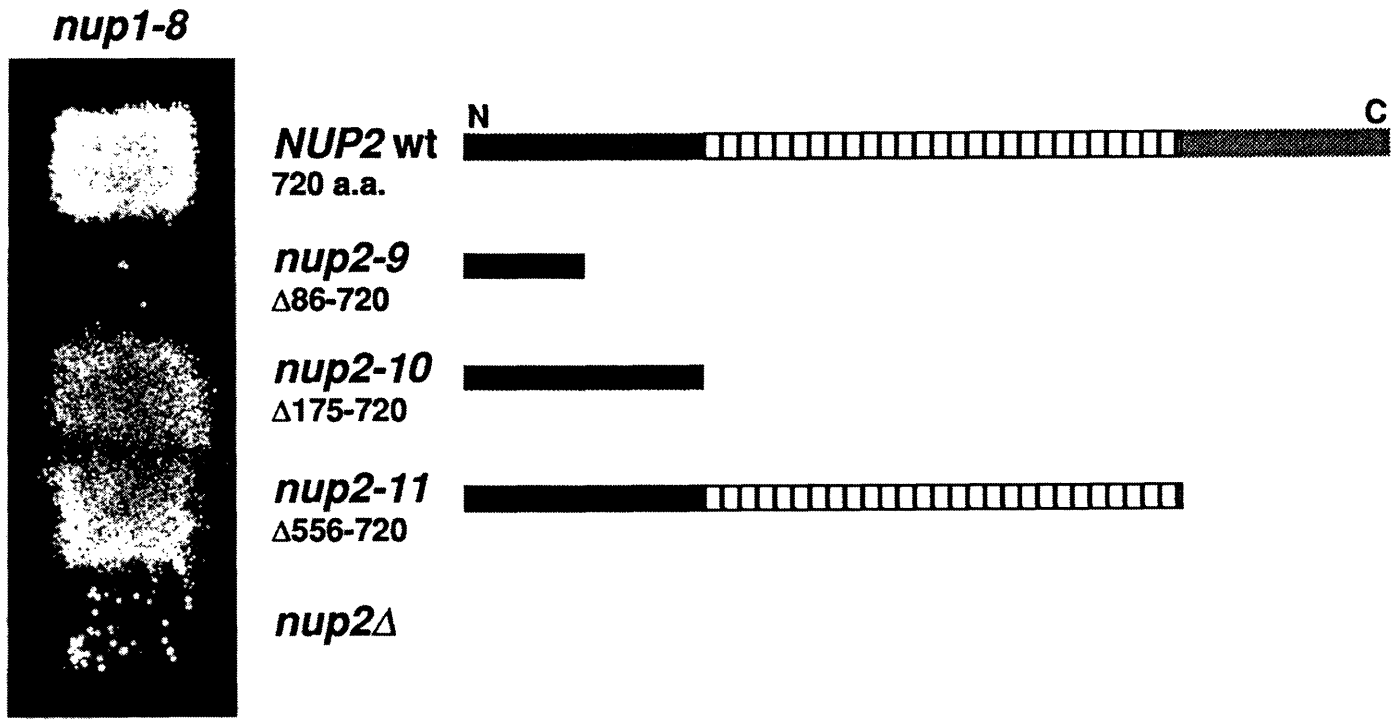
Strains containing a functional *NUP2* could lose the wild-type *NUP1* plasmid when provided with either Nup1 truncation mutant, and hence papillated on the medium containing 5-FOA. These *NUP2 nup1* (truncation) strains grow more slowly than *NUP2 NUP1* strains, but form colonies on standard medium. By contrast, strains deleted for *NUP2* cannot sustain growth with either *nup1* truncation, since they are unable to lose the *NUP1* wild-type plasmid and thus do not grow in the presence of 5-FOA. Therefore, strains bearing certain deletions of the amino and carboxy terminal regions of *NUP1* require a functional *NUP2* gene.

Either the amino terminus of Nup2 or an amino terminal region of Nup1 is required for growth

Since cells require *NUP2* in some *nup1* mutant backgrounds, we were able to develop an assay to test the ability of various *NUP2* constructs to provide the *NUP2* function required in these *nup1* strains. We modified the plasmid shuffle strategy described above by constructing a *TRP1 CEN* plasmid bearing both wild-type *NUP2* and the *nup1-8* amino terminal truncation of Nup1 (pJON139), and introducing it into the *nup1Δ nup2Δ* strain (JLY119) used previously. Upon loss of the wild-type *NUP1 URA3* plasmid, this construct must provide both the Nup1 specific function, as well as the overlapping function of Nup1 and Nup2. As predicted, the *nup1-8 NUP2* plasmid conferred viability to the *nup1Δ nup2Δ* strain (Figure 8, top row). One limitation of this assay is the appearance of fast growing colonies in patches of the negative control strain, which lacks active Nup2 (Figure 8, bottom row). These suppressed strains are probably the result of recombination between the *NUP1* wild-type gene on the *URA3* plasmid, and the mutant *nup1-8* gene on the second plasmid. The appearance of these colonies is distinguished from the growth observed in other patches by their low frequency and by their rapid rate of growth; therefore, even inefficient complementation can be detected by this assay.

Figure 2.8 Complementation of synthetic lethality by truncations of *NUP2*.

A *nup1-2::LEU2, nup2-5::HIS3* strain bearing wild-type *NUP1* on a *URA3 CEN* plasmid (JLY119) was transformed with a second plasmid bearing the *nup1-8* amino terminal truncation and the indicated allele of *NUP2*. The plasmid shuffle assay was then performed as in Figure 7. Photo was taken after 4 days at 30°C.


nup1-8
N
C
NUP2 wt
720 a.a.
nup2-9
Δ86-720
nup2-10
Δ175-720
nup2-11
Δ556-720
nup2Δ

We first used this assay to confirm that the modified version of Nup2 bearing the HA epitope is capable of providing the function shared between Nup1 and Nup2. The ability of *NUP2::HA* to complement is evidence that the tagged protein used for immunofluorescence localization experiments is functional, as well as localized at the nuclear envelope (as shown in Figure 5).

To determine which domain(s) of *NUP2* are genetically overlapping with the *NUP1* amino terminal truncation mutant, we constructed three carboxy terminal truncations: *nup2-9*, *nup2-10* and *nup2-11*, in the *nup1-8 NUP2* plasmid. These constructs were made by introducing the HA epitope followed by a series of 3 stop codons into *NUP2* after amino acids 85, 174 and 555, respectively. Surprisingly, the *nup2-10* and *nup2-11* plasmids were capable of partially complementing the synthetic lethal phenotype in the plasmid shuffle assay (Figure 8). After loss of the *NUP1* wild-type plasmid, both *nup1-8 nup2-10* and *nup1-8 nup2-11* strains grow slowly compared to the *nup1-8* strain with the wild-type *NUP2* allele. In contrast, the most extensive deletion, *nup2-9*, was completely incapable of complementing the *nup1-8 nup2Δ* synthetic lethal phenotype. The partial complementation of *nup1-8 nup2Δ* by carboxy terminal truncations of Nup2 implies that the amino terminal region, which precedes the repeated domain of Nup2, is able to provide the essential activity necessary in strains lacking the amino terminal 141 amino acids of Nup1.

The amino terminus of Nup2 is also required for the function of amino terminal truncations of Nsp1

Various mutations of *NSP1* and *NUP2* were tested in combination to determine whether these two genes also interact. A summary of these results is presented in Table 3. In strains carrying a functional copy of *NUP2* , the carboxy terminal 217 amino acids of Nsp1 (*C-NSP1*) are sufficient to complement the function of a null *nsp1* mutant (Nehrbass et al., 1990). To ask whether *C-NSP1* could suffice in the absence of *NUP2*, we examined the viability of the *nup2Δ C-NSP1* double mutant among spores arising from a diploid strain with the genotype *nsp1::URA3/NSP1, nup2-7::TRP1/NUP2* (JLX49), containing *C-NSP1* expressed from the *ADH* promoter on a *LEU2 CEN* plasmid. No *nup2Δ C-NSP1* double mutant spores (Ura^+ Trp^+ Leu^+) were ever recovered among the ascospore progeny (40 tetrads examined),

whereas both single mutants (*nup2Δ NSP1* and *NUP2 C-NSP1)* appeared at the expected frequency. In a control cross using an isogenic strain bearing an *NSP1 LEU2 CEN* wild-type plasmid, approximately 50% of Ura$^+$ Leu$^+$ spores contained *nup2-7::TRP1* deletion, as expected. These data suggest that the amino terminal and repetitive domains of Nsp1 are dispensable only in strains carrying a functional *NUP2* gene. In order to define the region of *NSP1* which is required in a *nup2Δ* background, we performed another cross using a construct in which only the repetitive domain of *NSP1* (amino acids 173-605 deleted, (Nehrbass et al., 1990) is deleted. In this case the double mutant (*nup2Δ nsp1Δ173-605)* is viable, but grows more slowly than either single mutant. This result indicates that the 172 N-terminal amino acids of *NSP1* must be vital for the shared function of *NSP1* and *NUP2*, and furthermore, that cells can tolerate the simultaneous loss of the repeated domains of *NSP1* and *NUP2*.

Table 3. Growth of *NSP1 NUP2* double mutants

NSP1 genotype	*NUP2* genotype	Ascospore growth
NSP1	*NUP2*	+++
nsp1Δ 173-605	*NUP2*	+++
nsp1Δ 173-605	*nup2Δ*[a]	+++
C-NSP1	*NUP2*	+++
C-NSP1	*nup2Δ*	-
C-NSP1	*nup2Δ* 175-720[b]	+

+++ is wild type growth. - means did not germinate.

[a]*nup2-7::TRP1*, a precise replacement of the *NUP2* coding sequence with the *TRP1*.

[b]*nup2-12::TRP1*, replacement of the internal ApaI fragment of *NUP2* with the *TRP1* gene. Adds the sequence EFHMLKstop after amino acid 172 of NUP2.

To test whether a similar relationship exists between the amino termini of Nup2 and Nsp1 as was shown for Nup2 and Nup1, a chromosomal truncation of *NUP2* (*nup2-12*, Δ175-720; pJON166 in MATERIALS AND METHODS), similar to the plasmid borne *nup2-10* was constructed, and crossed to the *C-NSP1* strain. This cross was analyzed, and viable *C-NSP1 nup2-12* double mutants were recovered. Since this double mutant is viable, whereas the *C-NSP1 nup2Δ* mutant is lethal, the amino-terminus of Nup2 must provide the shared function with the amino terminus of Nsp1.

The synthetic lethality between mutant alleles of *NSP1*, *NUP1* and *NUP2* appears to be specific because mutations in other genes implicated in nuclear transport do not show any interactions with *nup2Δ*. Double mutants between the *nup2* deletion and *nsr1-1::HIS3*, a null allele of a putative nuclear localization sequence receptor (Lee et al., 1991), were tested for viability. The *nsr1-1 nup2* double mutant grows at the same rate as the *nsr1-1 NUP2* strain, suggesting that there is no interaction between the two mutations. We also examined double mutants between *nup2Δ* and *rna1-1*, a temperature sensitive allele of a gene encoding a cytoplasmic protein required for the export of mRNA from the nucleus (Hopper et al., 1990; Hutchinson et al., 1969). Again, the double mutant, *nup2Δ rna1-1* fails to show any phenotype indicative of a *NUP2 RNA1* interaction.

2.4 Discussion

We have isolated a gene encoding a new nucleoporin, Nup2 (95kD). Like the two previously identified yeast nucleoporins, Nup1(130kD: (Davis and Fink, 1990)) and Nsp1(100kD: (Nehrbass et al., 1990)), Nup2 is recognized by a panel of monoclonal antibodies that cross-react with nucleoporins from many different eukaryotes. Several lines of evidence suggest that Nup2 is a component of the nuclear pore complex. The patchy, punctate staining of the nuclear envelope observed by indirect immunofluorescence of epitope tagged Nup2 protein is very similar to that shown for other NPC proteins in yeast (Davis and Fink, 1990; Nehrbass et al., 1990). Furthermore, Nup2 and Nsp1 are the only nuclear proteins which are recognized by MAb 414 (Aris and Blobel, 1989; Davis and Fink, 1990); this work) which has been shown to be specific for mammalian nuclear pore complexes by immunoelectron microscopy (Davis and Blobel, 1986). The three cloned yeast nucleoporins have a similar structure: Each can be divided into three domains defined by a centrally located segment consisting of a loosely conserved nine amino acid repeating unit (the FSF repeated motif) that is separated by highly charged spacers of variable length. The sequence homology between *NUP2* , *NUP1* and *NSP1* is restricted to the central domain. Several other proteins associated with yeast nuclei are recognized by the anti-nucleoporin MAbs. In particular, there is a group of three proteins of 45-65kD (Davis and Fink, 1990). Why did our screen fail to identify any of these proteins? Of 13 immunoreactive clones, only *NUP1* and *NUP2* hybridized strongly to the degenerate Nsp1 repeat oligonucleotide, suggesting that Nsp1, Nup1 and Nup2 may be the only proteins in yeast with similar repetitive domains. However we cannot rule out either that there are other nucleoporins with repetitive domains which were underepresented in our library, or that there is a class of nucleoporins which lacks the FSF repeat, but still is recognized by the anti-nucleoporin MAbs.

NUP2 , unlike *NUP1* or *NSP1*, is not essential for viability because deletion of the entire coding sequence of *NUP2* has no effect on growth. However, *NUP2* is required in strains containing certain mutations in either *NUP1* or *NSP1*. Small truncations from either end of *NUP1* are viable in a strain with a functional *NUP2* gene, but are lethal in combination with a *NUP2* deletion. Similarly, a strain expressing only the carboxy terminal

domain of Nsp1(a deletion of the amino terminal and repeated domains) is viable with a functional *NUP2* gene, but is inviable in conjunction with a *NUP2* deletion. This "synthetic lethality" among yeast nucleoporins provides the first evidence for functional interaction between these NPC components. These relationships suggest that the nucleoporins have similar activities, or that they interact physically. However, we have also found that Nup2 protein in high dosage is unable to supply the essential functions missing in *nup1Δ* or *nsp1Δ* strains, which underscores the fact that members of the family must also have unique activities. In addition, the inviability of these double mutants also rules out the existence of another protein which is completely equivalent to Nup2, although there might be more proteins which are partially redundant, or interact in a similar mode. In light of the genetic interactions identified between *NUP2* and both *NSP1* and *NUP1*, it would be informative to construct double mutations between *NUP1* and *NSP1*, which might provide further clues as to which regions of the nucleoporins specify unique functions, and which regions are genetically overlapping.

Our data suggest an important role for the amino terminal domain of each of the three nucleoporin genes. Amino terminal truncations of Nup1 have progressively more severe phenotypes and truncations with mild phenotypes are exacerbated by deletion of *NUP2*. Furthermore, although the amino terminus of Nsp1 is dispensable in strains carrying a functional *NUP2* gene, it becomes essential in the absence of Nup2 protein. What is remarkable is that the amino terminal domain of Nup2 is, expressed by itself, able to to confer viability to strains carrying amino terminal truncations of either Nup1 or Nsp1. These data suggest that, in a genetic sense, the amino terminal domains of these three proteins are functionally redundant; in other words, either one polypeptide or the other is required. Strikingly, their amino acid sequences contain no significant similarities. Despite the fact that we have shown an important role for the amino terminus of all three yeast nucleoporins, the only mammalian nucleoporin cloned, p62, which has extensive homology to *NSP1*, does not have an analogous domain amino terminal to its repeated domain, implying there are functionally significant differences between the nucleoporins in yeast and their metazoan counterparts.

It is likely that the central repeated domain, common to all three yeast nucleoporins, has a similar function in each protein. The conservation of the

nucleoporin repeated motif in widely divergent species suggests its importance in NPC function. However, we have been unable to create a situation where it has an essential role *in vivo* for any of the yeast nucleoporins. Deletion of the repeats individually or simultaneously from Nsp1 or from Nup2 does not affect viability. One explanation for the dispensability of the repeats could be that the role of this domain is critical, but it can be supplied by any one of the repeat bearing nucleoporins. If this were the case, then it implies that the repeated domain can function independently of any of the specific functions of the termini of these proteins. The fact that the terminal segments of Nup2 and Nsp1 are active when expressed by themselves further suggests that the repeated domains could not be directly involved in assembling or positioning the terminal domains into their active form. Furthermore, this model predicts that simultaneous deletion of the central domain from each repeat bearing nucleoporin would be lethal.

We propose that the synthetic lethality in these double mutant strains (*nsp1nup2* or *nup1nup2*) reflects an underlying similarity in action among these proteins. Two general models have been suggested to explain synthetic lethality. One is that it reflects protein-protein interactions within a multi-subunit complex: a complex may function if one constituent is defective, but may not function if multiple constituents are defective. For example, temperature sensitive mutations in *SEC17* and *SEC18*, yeast homologs of the mammalian proteins NSF and SNAP, known to be part of a protein complex involved in membrane fusion, are lethal in combination (Kaiser and Schekman, 1990; Clary et al., 1990). An alternative explanation for synthetic lethality is that two or more proteins have redundant activities that carry out some essential function. Loss of function of one protein can be compensated by the activity of the other; when neither is available the cell, unable to carry out the necessary activity, is inviable. Most instances of this type of relationship involve families of highly similar genes, such as tubulin (Huffaker et al., 1987) or protein kinases (Toda et al., 1987). Our genetic experiments cannot distinguish between these two possibilities. Although the yeast genes are superficially similar in sequence organization, the sequence similarity is restricted to the central domains. Moreover, it is the nonhomologous terminal domains that exhibit functional overlap. The partial sequence similarity and partial redundancy of function is reminiscent

of the yeast G1 cyclin family (Richardson et al., 1989). Perhaps, like cyclins, nucleoporins operate as units of a modular system, where each of these molecules physically interacts with the same complement of accessory proteins, and complexes including different sets of nucleoporins serve similar but distinct roles in NPC function. Biochemical experiments underway in our laboratories could provide evidence for the postulated complexes and their function in nuclear transport.

Acknowledgments

J.D.J.L. would like to dedicate this work in memory of his father, the late Joseph H. Loeb. We would like to acknowledge all the members of the Fink lab for many useful discussions and technical advice, and particularly thank B. Bartel, J. Celenza, K. Cunningham, P. Ljungdahl, and D. Miller for their assistance. Furthermore, we appreciate critical comments on the manuscript by G. Marcus, P. McCaw and D. Pellman. We thank D. Miller for constructing the GAL and 2μ derivatives of pRS plasmids and J. Brill for providing pJB107. l Prime mapping filters were kindly provided by Linda Riles and Maynard Olson. Furthermore, we thank E. Hurt for *NSP1* constructs. L.I.D. was a Lucille P. Markey Scholar during a portion of this work. G.R.F. is American Cancer Society Professor of Genetics. This work was supported by NIH grant GM-40266.

Chapter 3

SRP1 and nucleoporins are required for maintenance of nuclear envelope structure and nuclear transport

Preface to Chapters 3 & 4

My work on the *SRP1* gene described in Chapters 3 and 4 is an amalgam of many people's ideas and efforts. I originally became interested in *SRP1* after David Pellman, then in the Fink laboratory, isolated a mutation in *SRP1* in a screen for genes that are synthetically lethal with the microtubule associated protein, Bik1. It had already been shown that Srp1 is a component of the nuclear envelope by Masayasu Nomura and his collaborators at the University of California, Irvine. Therefore, I decided to determine whether the *SRP1* gene interacted with nucleoporin genes, the subject of my work to that time. David initiated the collaboration with Nomura's group to obtain the temperature sensitive mutations of *SRP1* that are the cornerstone of this work. Professor Nomura and his student Michelle Tabb were extremely helpful and provided me with advice and several other reagents. With these mutants in hand, I was able to show a variety of genetic interactions between SRP1 and nucleoporins, and I observed several interesting phenotypes that constitute the bulk of these chapters. When I began to realize that Srp1 might be directly involved in nuclear import, I approached Prof. Pam Silver of Harvard Medical School to learn the *in vitro* assay for nuclear transport that she and Gabriel Schlenstedt had developed. Gabriel and I performed the *in vitro* experiments described here together. Gabriel's unending patience made these painful experiments possible, and almost fun. Lastly, Daniel Kornitzer performed the pulse-chase experiment that demonstrated the accumulation of cyclin proteins in the *srp1-31* mutant was due to defects in cyclin degradation. I performed all the other experiments described in these chapters with the expert advice of all those mentioned above.

Chapter 4 has been submitted to PNAS.

3.1 Introduction

In all eukaryotes, the nuclear envelope (NE) serves to separate the nucleus and cytoplasm into distinct compartments. It does so by acting as a selectively permeable barrier to macromolecules, therefore maintaining the unique constitutions of the nucleus and cytoplasm. The nuclear pore complex (NPC) is the sole site of this bi-directional transport of macromolecules across the NE (Dingwall and Laskey, 1992; Newmeyer, 1993; Silver, 1991). In addition to its central role in nucleocytoplasmic transport, the NPC may have additional functions. It has been postulated that the NPC might serve as an organizing center for the nuclear contents (Blobel, 1985). Chromatin is arranged around the NPC such that channels in the heterochromatin lead between the nuclear interior and the pores. "Tracks" of specific RNAs have been shown to travel from sites of transcription to the NPC through these channels (Lawrence et al., 1989; Rosbash and Singer, 1993). Furthermore, both nuclear localization sequence binding proteins and components of the RNA processing machinery are localized to these regions (Carter et al., 1993; Meier and Blobel, 1992; Xing et al., 1993). The NPC is likely to be the structure responsible for the tight apposition of the inner and outer NE membranes because it is the major structure known to span them. Furthermore, the NPC might be a site of connection between the NE and the cytoskeleton, as suggested by numerous reports describing filaments emanating from both sides of the NPC e.g.: (Goldberg and Allen, 1992; Jarnik and Aebi, 1991). Such a linkage might be important for the maintenance of the regular structure of interphase nuclei, as well as defining domains in the outer NE in order to differentiate it from the endoplasmic reticulum with which it is contiguous.

The NPC is a complicated organellar assembly of as many as 80 different polypeptides (Reichelt et al., 1990; Rout and Blobel, 1993). Few of these proteins have been characterized on the molecular level. Most of the well studied NPC components belong to a class of immunologically related proteins (nucleoporins) first identified by monoclonal antibodies raised against mammalian NPC antigens (Davis and Blobel, 1986; Davis and Blobel, 1987; Park et al., 1987; Snow et al., 1987). Some nucleoporins have been shown by immunoelectron microscopy to be limited to the central "transporter" region of the NPC, or the inner nucleoplasmic "basket", sites

where the transport mechanism is suspected to reside (Akey, 1989; Sukegawa and Blobel, 1993). The nucleoporin proteins that have been cloned so far fall into two broad classes based upon signature repeated elements. The FXF class is represented by the *NUP1*, *NUP2* and *NSP1* genes in the budding yeast, *Saccharomyces cerevisiae* and the nup62, nup153 and pom121 proteins in mammals (Davis and Fink, 1990; Hallberg et al., 1993; Hurt, 1988; Loeb et al., 1993; Starr et al., 1990; Sukegawa and Blobel, 1993), and the GLFG class is comprised of *NUP49*, *NUP100* and *NUP116* genes in yeast (Wente et al., 1992; Wimmer et al., 1992). No vertebrate members of the GLFG family have been reported thus far.

Conditional mutations in the yeast nucleoporin genes have been useful in determining the role of nucleoporins in the function of the NPC. The requirement of nucleoporins for transport of mRNA and proteins has been firmly established *in vivo* and *in vitro* by analyzing the phenotypes of temperature sensitive mutants under non-permissive conditions. The Nsp1, Nup49 and Nup1 proteins are required for the proper localization of nuclear proteins (Bogerd et al., 1994; Nehrbass et al., 1993; Schlenstedt et al., 1993), while both *nup116* and *nup1* mutants have been shown to retain mRNA within the nucleus (Bogerd et al., 1994; Wente and Blobel, 1993). Expression of *NSP1* is required for the normal accumulation of NPCs during growth (Mutvei et al., 1992). A role for nucleoporins in the regulation of NE growth was recently suggested by the finding that a *nup116* deletion mutant that is conditional for growth exhibits defects in the bilayer structure of the NE in the vicinity of the NPC (Wente and Blobel, 1993).

If indeed the NPC in yeast is required to maintain nuclear structure, and/or organize the nuclear contents, one might expect to find mutations in NPC constituents with defects in many nuclear functions. Many nuclear processes have structural constraints that might depend on NPC function. For example, if the NPCs are linked in some way to chromatin, then any mutations that disrupt this linkage might affect the maintenance or segregation of chromosomes, manifested as increased frequency of chromosome disjunction. Alternatively, disruption of connections to nucleoskeletal elements might perturb organized intranuclear domains, such as splicing foci or nucleoli. Furthermore, in light of the persistence in yeast of the NE throughout mitosis, mutations that abrogate a linkage between the NPC and cytoskeletal elements might lead to improper segregation of the

nucleus to daughter cells. Identifying phenotypes such as these caused by mutants of known NPC constituents would be an important step in verifying the postulated roles of the NPC in nuclear structure.

A new protein that has been suggested to be an NPC constituent in yeast has recently been identified. In this paper we report that mutational analysis of this gene, *SRP1*, has revealed phenotypes consistent with some of the predictions made above for a protein that links the NPC to structural elements in the nucleoplasm and/or cytoplasm. *SRP1* is an essential gene that was originally isolated as a suppressor of temperature sensitive mutations in the largest subunit of RNA polymerase I (Yano et al., 1992). The Srp1 sequence contains a repetitive domain structure (the ARM repeat) that is related to a diverse family of eukaryotic proteins including β-catenins and the APC recessive oncogene (Peifer et al., 1994). Indirect immunofluorescence with anti-Srp1 sera suggested that the protein was localized to the NE of yeast cells. *SRP1* was also isolated by its synthetic lethality with the deletion of the *NUP1* gene (Bogerd et al., 1994) and furthermore shown to be in complex with both the Nup1 and Nup2 proteins (Belanger et al., 1994) and M.T., J.L. and M.N., unpublished observations). In addition, *SRP1* was found to be a multicopy suppressor of *cse1*, a mutation that leads to elevated levels of chromosome loss during mitosis (M. Fitzgerald-Hayes, personal communication). In this paper, we seek to unify the diverse phenotypes of *SRP1*. We demonstrate that Srp1 is a *bona fide* nuclear pore constituent, and provide further genetic evidence for interactions between *SRP1* and nucleoporins. Additionally, we have identified a conditional double mutant, *srp1-49 nup2Δ*, whose catastrophic NE and nuclear segregation phenotypes reveal the requirement for NPC proteins to maintain NE structure and dynamics. These results suggest that the pleiotropic phenotypes associated with *SRP1* are due to the involvement of the NPC in multiple aspects of nuclear metabolism.

3.2 Materials and Methods

Reagents

Enzymes for molecular biology were purchased from New England Biolabs, Pharmacia, and Promega. Yeast spheroplasts were prepared using oxalyticase (Enzogenetics) for immunofluorescence, glusulase for electron microscopy (NEN) and zymolyase 100T (Kirin Brewing Co., Ltd.) was used to prepare asci for tetrad dissection. Secondary antibodies for immunofluorescence and immunoelectron microscopy were obtained from Jackson Immunoresearch and Biocell Sciences. All other chemicals were obtained from Sigma.

Table 3.1 Plasmids used in this study

Yeast Plasmid	relevant markers	comments
pNOY172	*URA3 srp1-31*	*srp1* mutant allele cloned into pRS306 as a KpnI-BamHI fragment
pNOY175	*URA3 srp1-49*	"
pDP168	*CEN URA3 SRP1*	functional 2.2 kB fragment of *SRP1* cloned into pRS316
pDP58	*2μ URA3 ADE3 BIK1*	NheI/BamHI fragment of *ADE3* cloned into pVB20 (Berlin et al., 1990) cut with NheI/BamHI
pB2976	*CEN URA3 GAL1-10::GFP*	NheI-EcoRI fragment of TU#58 (Chalfie et al., 1994) cloned into the XbaI and EagI sites of pRS316 *GAL* (Liu and Bretscher, 1992)
pJON280	*CEN URA3 GAL1-10::H2B1::GFP*	PCR fragment of the entire coding region of *H2B1* inserted in frame into the BamHI site of pB2796

DNA techniques

DNA subcloning was done by standard techniques outlined as in (Sambrook et al., 1989). A complete list of plasmids employed in this study is shown in Table 1.

Yeast growth conditions and strain construction

Yeast cell culture and crosses were performed essentially as outlined in Sherman *et al.*, (1986) and Guthrie and Fink, (1992). Yeast plasmids and linear DNA fragments for integration were transformed into yeast by lithium acetate transformation (Ito et al., 1983). Selection against Ura^+ strains was done by culture on solid synthetic complete media containing 1 mg/ml 5-fluoro-orotic acid as described in Boeke *et al.*, 1984. A complete list and description of strains employed in this study is shown in Table 2.

Integration of *srp1^{ts}* alleles into the chromosome

We found that the plasmid borne temperature sensitive alleles of *SRP1* that were constructed by *in vitro* mutagenesis (Yano et al., 1994) were difficult to use for our experiments because their phenotypes were variable and suppressors occurred with high frequency. Therefore we integrated these previously characterized mutations into the *SRP1* chromosomal locus by a pop in/pop out strategy (Winston et al., 1983). First, the *srp1^{ts}* alleles were cloned into the pRS306 vector (YIp *URA3;* (Sikorski and Heiter, 1989)) linearized at the internal EcoR1 site, then transformed into a haploid isogenic derivative of W303 (L4884). Ura^+ transformants were analyzed by southern blotting to confirm the expected structure of the duplicated *SRP1* locus. For each mutation, two independent strains with duplications were then grown non-selectively in liquid YPAD medium (YPD with 0.3 mM added adenine) to saturation, then plated on 5-FOA plates to select for recombination to remove the duplication at *SRP1*. 96 Ura^- colonies from each duplication were then tested for growth at 37°C on YPAD plates and 25°C on YPA plates with 3% glycerol and 3% ethanol to differentiate petite strains from *srp1^{ts}* strains. Putant *srp1^{ts}* strains were then transformed with wild-type *SRP1* on a plasmid to show rescue by the wild-type gene. At least four independent strains of both *srp1-31* and *srp1-49* were isolated, and each isolate had identical

growth properties. Isolates of *srp1-31* mutants isolated in this manner were semi-dominant relative to *SRP1* on a *CEN* plasmid.

Table 3.2 Yeast Strains

Strain name	genotype	source
isogenic to W303-1a		
L4852	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*	Fink collection
L4884	*MATα ade2-1 ura3-1 his3-11 trp1^63 leu2-3,112 can1-100*	"
JLY379	*MATa nup2-4::URA3 ade2-1 ura3-1 his3-11 trp1^63 leu2-3,112 can1-100*	this study
JLY417	*MATa nup1-2::LEU2 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*	"
JLY517	*MATa nup2-4::ura3::HIS3 ade2-1 ura3-1 his3-11 trp1^63 leu2-3,112 can1-100*	"
JLY543	*MATα srp1-31 ade2-1 ura3-1 his3-11 trp1^63 leu2-3,112 can1-100*	"
JLY555	*MATα srp1-49 ade2-1 ura3-1 his3-11 trp1^63 leu2-3,112 can1-100*	"
JLY616	*MATα srp1-49 nup2-4::ura3::HIS3 ade2-1 ura3-1 his3-11 trp1^63 leu2-3,112 can1-100*	"
SWY29	*MATa nup116Δ::HIS3 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*	S. Wente
isogenic to YPH877		
YPH877	*Mata ura3-52 lys2-801 ade2-101 his3^200 trp1^1* CFVII (*RAD2*.d.YPH877) *TRP1 SUP11*	P. Hieter
PY766	*Mata ura3-52 lys2-801 ade2-101 his3^200 trp1^1 srp1-31* CFVII (*RAD2*.d.YPH877) *TRP1 SUP11*	this study
JLY747	*Matα ura3-52 lys2-801 ade2-101 his3^200 trp1^1 srp1-31* CFVII (*RAD2*.d.YPH877) *TRP1 SUP11*	"
isogenic to PY434		
PY434	*Matα bik1Δ::TRP1 ura3 leu2 trp1 ade2 ade3*	this study
PY703	*Matα bik1Δ::TRP1 ura3 leu2 trp1 ade2 ade3 srp1-101 {BIK1 ADE3 2μ}*	"
ABYS1	*MATa pra1 prb1 prc1 cps1 ade⁻*	G. Schlenstedt

Immunofluorescence

Immunofluorescence on yeast spheroplasts was performed as described in Davis and Fink (1990) except that 1% polyethyleneimine in H_2O was substituted for poly-lysine and filtered 5% nonfat dry milk was used in place of 2% bovine serum albumin as the coating agent. The YOL1/34 hybridoma supernatant was obtained from Accurate Biochemicals and used at a 1/10 dilution. The secondary antibody was donkey anti-rat IgG conjugated to Texas Red (Jackson Immunoresearch).

Electron Microscopy

For immunoelectron microscopy, wild type *S. cerevisiae* cells were fixed with 2.5% paraformaldehyde and 0.2% glutaraldehyde in PBS (pH 7.2) for two hours at room temperature. After quenching and washing the cells, they were dehydrated with dimethyl formamide and embedded in Lowicryl K4M resin. Thin sections were then incubated with affinity purified sheep anti-Srp1 polyclonal serum (Yano et al., 1992). To detect the localization of these antibodies, the sections were then incubated with donkey anti-sheep IgG-gold (5 nm) secondary antibodies (Biocell Sciences).

The protocol to simultaneously visualize cellular membranes and proteins was derived from Byers and Goetsch (1991) and is described in Wente and Blobel (1993). Briefly, cells grown in rich medium were washed in 40 mM K_2HPO_4-KH_2PO_4 0.5 mM $MgCl_2$ pH 6.5 then fixed in 2% glutaraldehyde in the same buffer. The cells were then washed in 0.17 M KH_2PO_4 30 mM sodium citrate pH 5.8. Sphereoplasting was performed in the same buffer with 7 mM β-mercaptoethanol, 40μg/ml oxylyticase, and 1/10 volume glusulase at 30°C for 30 minutes. The cells were washed, then post-fixed in 2% Osmium tetroxide, 0.05 M NaAcetate for 15 minutes at room temperature. After dehydration in ethanol, the cells were embedded in Poly/Bed 812 resin (PolySciences, Inc.). Potassium permanganate staining of whole yeast cells was performed essentially as described in Kaiser and Schekman, (1990). Specific growth conditions are described in results. All electron microscopy was performed using a Phillips EM410 microscope at 80kV.

***In vitro* nuclear transport assay**

The assay for the uptake of fluorescent nuclear localization sequence bearing substrate protein into permeabilized yeast cells was performed essentially as described (Schlenstedt et al., 1993). *srp1^{ts}* mutants were grown as described in the legend of figure 8, harvested and spheroplasted at 30°C, then frozen slowly over liquid N_2. *In vitro* import was performed by combining the permeabilized cells, wild type cytosol (prepared from yeast strain ABYS1), ATP regenerating mix and rhodamine labeled NLS peptide conjugated human serum albumin on ice, then the reaction was allowed to proceed at room temperature for ten minutes, then DAPI was added to 0.25 μg/ml and the reaction mix was mounted on a glass slide for microscopy. Transport efficiency was scored by counting the percentage of DAPI staining nuclei that bound and/or imported substrate.

Histone::GFP fusion as an *in vivo* assay for nuclear transport

The histone::GFP expression plasmid was constructed by cloning a PCR derived fragment of histone H2B1 (Wallis et al., 1980) as an amino-terminal fusion to GFP in the yeast galactose inducible GFP expression vector B2976 constructed by H. Mosch. Wild-type and mutant cells were transformed with the resulting histone::GFP plasmid (pJON280) and cultured in synthetic complete glucose media lacking uracil to maintain the reporter plasmid while repressing the expression of histone::GFP. Prior to the experiment cultures were grown in synthetic complete raffinose -uracil in order to derepress the GAL1-10 promoter. In order to induce the expression of histone::GFP, galactose was added to the medium to 3%. Within two hours of induction by galactose, the histone::GFP fusion protein is detected entirely within the nucleus of wild-type cells when observed by fluorescence microscopy. Experimental conditions used to assay uptake of histone::GFP into mutant cells are described in the legend of figure 10. Expression of the histone::GFP fusion protein is somewhat toxic: expressing cells grown on solid galactose media grow more slowly than controls. Independent of GFP expression, occasional cells (<5%) in culture are highly fluorescent throughout their cytoplasm. Therefore when observing GFP localization, there is a background of cells with cytoplasmic staining. However, this can readily be distinguished from authentic GFP signal because it is also visible in the rhodamine channel,

whereas the GFP fluorescence is limited to shorter wavelengths. GFP fluorescence was photographed on a Ziess axioskop equipped with an epifluorescence filter set designed for fluorescein fluorescence (high Q FITC; Chroma Technology Corp.)

3.3 Results

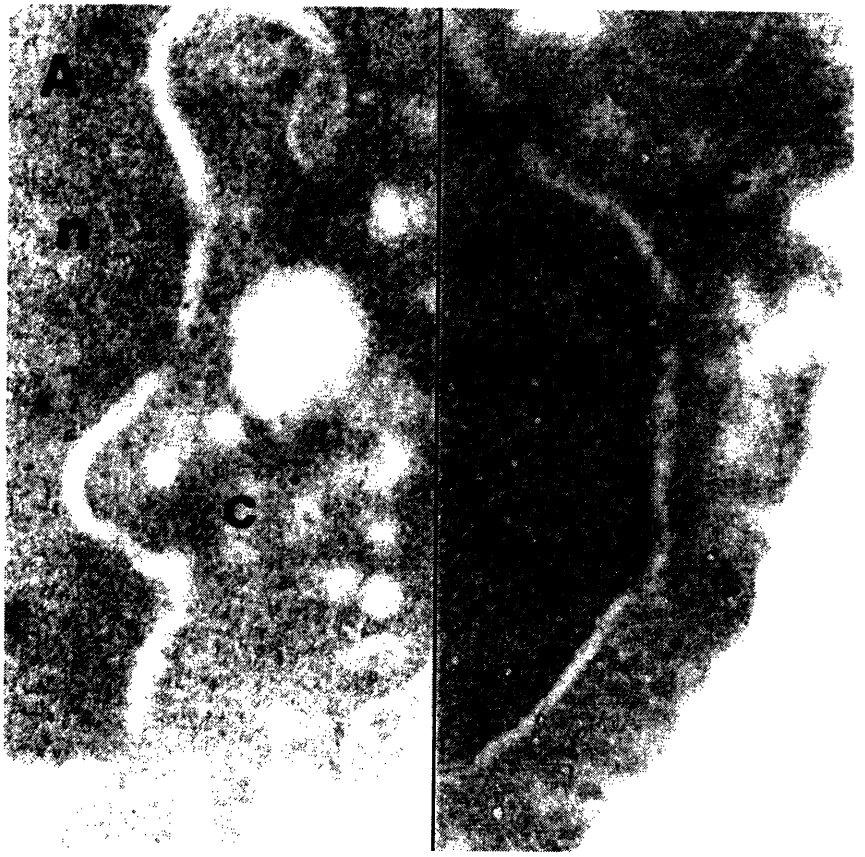
SRP1 is a component of the nuclear pore complex in yeast

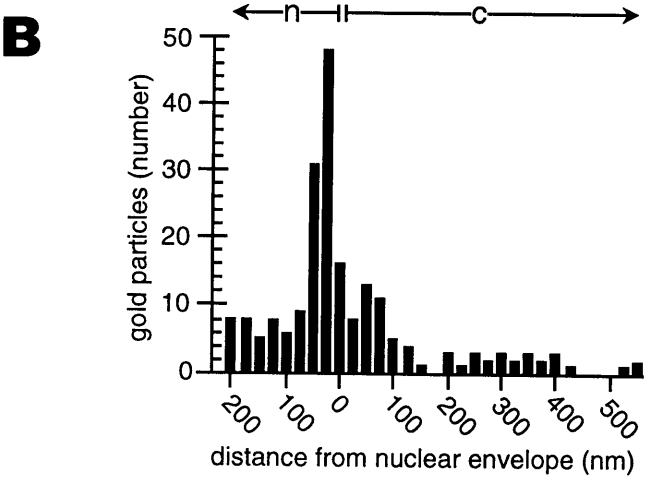
In order to determine whether the punctate staining of the NE described in Yano *et al.* (1992) indicated the localization of Srp1 to the NPC, we sought to localize Srp1 employing the superior resolution of immunoelectron microscopy. On immunoblots, the affinity purified sheep anti-Srp1 antisera (Yano et al., 1992) recognizes a single band of approximately 70 kilodaltons, which corresponds to the predicted molecular weight for Srp1. Wild-type *S. cerevisiae* cells were prepared for immuno-electron microscopy and stained with anti-Srp1 sera, then detected by secondary antibody conjugated to colloidal gold particles. As shown in figure 1A, the majority of gold particles decorate a region comprised of the NE and the nucleoplasm just inside the envelope surface in these sections. Regions decorated by multiple particles (>3) are rare, and always coincide with the NE, occasionally in gaps in the NE that correspond to nuclear pores (see figure 1A left side). A histogram of gold particles versus distance from the NE confirms the observation that most Srp1 is localized to the inner surface of the NE (figure 1B). However, this quantitation suggests significant amounts of Srp1 might be dispersed throughout the nucleoplasm as well as just outside the NE. Therefore, immuno-electron microscopy confirms the preliminary localization of Srp1 to the NE of yeast and further suggests that a significant fraction of Srp1 is contained within large structures at the NE that probably corresponds to nuclear pores.

SRP1 and nucleoporins genetically interact

Because Srp1 colocalizes with the nucleoporins, we tested whether constructing double mutations between $srp1^{ts}$ alleles and various nucleoporin mutants genes leads to synthetic phenotypes. There are many precedents for mutations in proteins that act together in a complex having synergistic defects. It has previously been shown that constituents of nucleoporin protein complexes can interact genetically as well as physically (Grandi et al., 1993; Wimmer et al., 1992). In order to study the effects of loss of the *SRP1* gene function, two temperature sensitive alleles of *SRP1* were constructed *in vitro* by a plasmid shuffle technique (Yano et al., 1994). We

Figure 3.1 Immuno-electron microscopic localization of Srp1 in yeast . (a) Micrographs of wild type yeast cells thin sectioned and stained with affinity purified anti-Srp1 polyclonal serum as described in MATERIALS AND METHODS. Nucleoplasm (n) and cytoplasm (c) are indicated. (b) Quantitation of Srp1 localization in yeast cells. Distance of each gold particle to the nearest NE was measured in enlarged photographs of 39 cell sections. A total of 207 particles were counted, and the results tabulated.


A
n
c


B
n
c
50
40
30
20
10
0
gold particles (number)
200
100
0
100
200
300
400
500
distance from nuclear envelope (nm)

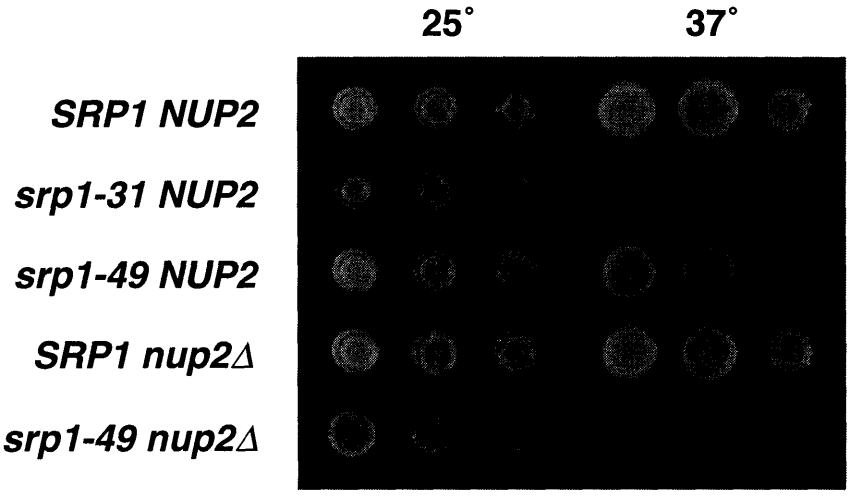
replaced the wild-type *SRP1* allele with these mutant alleles by a pop-in/pop-out strategy (Winston et al., 1983) into a haploid strain isogenic to the W303 background (Thomas and Rothstein, 1989). The *srp1-31* allele is a change of serine 116 to phenylalanine while the *srp1-49* allele is a change of glutamic acid 145 to lysine. The *srp1-31* mutation is in the amino-terminal unique region of *SRP1* while *srp1-49* is located at the beginning of the first repeated region (Figure 2A). Figure 2B illustrates the growth characteristics of these $srp1^{ts}$ strains. Note that *srp1-31* is a strong temperature sensitive mutation at 37°C, while *srp1-49* is considerably less severe.

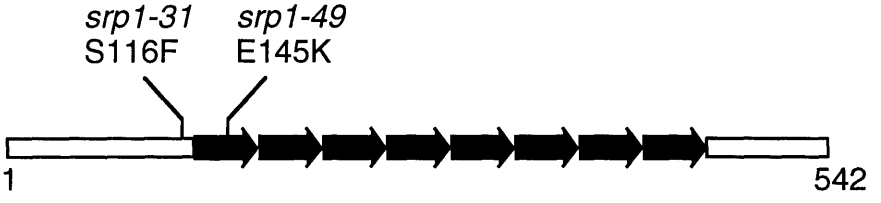
We then crossed strains containing one of these two integrated $srp1^{ts}$ alleles to strains bearing deletions of three previously described nucleoporin genes. Diploids heterozygous for a $srp1^{ts}$ mutation and a *nup* deletion were sporulated, tetrads dissected, and ascospores analyzed for their genotype and phenotype. The results of this series of crosses are summarized in Table 3. $srp1^{ts}$ mutants interact genetically with both classes of nucleoporins; the FXF repeat family, represented by *nup1Δ* and *nup2Δ*, as well as the GLFG repeat family, represented by *nup116Δ* (Wente et al., 1992) Note in this genetic background, *NUP1* is not essential for viability, in contrast to the finding reported for the same deletion introduced into the S288C genetic background (Davis and Fink, 1990). With the exception of *nup2Δ srp1-49*, double mutant spores were not recovered. This indicates that together these pairs of genes are required for germination. To ascertain whether the double mutants are capable of vegetative growth, similar crosses were performed where a wild-type *SRP1* gene was introduced on a vector containing the *URA3* gene, double mutants were then recovered where the required *SRP1* function was covered by the wild-type gene on the plasmid. These strains were then challenged by growth on 5-FOA medium which selects for loss of the *URA3* plasmid. Each double mutant strain that did not germinate also did not grow on 5-FOA medium at 25°C which indicates that the shared function of these genes is also required for vegetative growth.

While *srp1-31 nup2Δ* is inviable, *nup2Δ* exacerbates the temperature sensitive growth phenotype of *srp1-49* (shown in figure 2B). *srp1-49 nup2Δ* strains grow slowly at 25°C and do not grow at 37°C. Note that both single mutants grow well at 37°C: *srp1-49* has a slight growth defect, while *nup2Δ* is indistinguishable from wild type (as previously shown in Loeb *et al.*, 1993). Thus, the stronger allele, *srp1-31*, is lethal in combination with *nup2Δ*, while

Figure 3.2 Growth characteristics of *srp1^{ts}* strains.

A) Schematic diagram of Srp1, indicating the location of the amino acid change in mutant alleles. Boxes indicate the 42 amino acid ARM repeats B) Log phase cultures of strains with the indicated genotypes grown in YPAD liquid at 25°C were counted, then diluted to equal densities into a 96 well dish. Five-fold serial dilutions were made, and the resulting cultures were spotted onto YPAD agar plates, then incubated at the indicated temperatures. Photographs were taken after two days of incubation.


25°
37°
SRP1 NUP2
srp1-31 NUP2
srp1-49 NUP2
SRP1 nup2Δ
srp1-49 nup2Δ


srp1-31
S116F
srp1-49
E145K
1
542

the double mutant with the weaker allele, *srp1-49*, is viable, but considerably more temperature sensitive than *srp1-49* alone. The nucleolar unfolding phenomenon described by Yano *et al.* (1994) is specific to the *srp1-49* allele, suggesting the defect caused by each mutant is different. This is consistent with data provided below that differentiates the terminal phenotypes of the *srp1-31* single mutant from the *srp1-49 nup2Δ* double mutant, although they are both inviable at 37°C.

Table 3.3 Genetic interactions between *SRP1* and nucleoporins

nucleoporin mutant	*SRP1* allele		
	SRP1	*srp1-31*	*srp1-49*
nup1Δ	slow growth	inviable	inviable
nup2Δ	wt[a]	inviable	slow growth/ts
nup116Δ	ts[b]	inviable	inviable

[a] indicates growth rate indistinguishable from *SRP1 NUP2* parent strain.
[b] temperature sensitive: growth at 25°C, no growth at 37°C.

SRP1 and _NUP2_ are required for proper nuclear segregation and chromatin structure

Morphological analysis of *SRP1* mutant cells showed several abnormal morphological features, particularly in *srp1-49 nup2Δ* strains. At the permissive temperature, double mutant cells are much larger than wild-type, and many cells have unusual extended buds (figure 3C). Staining nuclei with the DNA binding dye DAPI (4'6-diamidino-2-phenylindole dihydrochloride) revealed multinucleate and anucleate cells as well as cells with indistinct, irregular regions of DAPI staining material. Specific "unraveling" of nucleolar DNA has been previously observed for the *srp1-49* mutation (Yano *et al.*, (1994) and figure 3B). At 37°C the effects on nuclear segregation are more severe (figure 3D). Many cells are observed where the

Figure 3.3 Nuclear morphology of mutant strains visualized by DAPI staining.

Strains were grown as described below, then fixed by the addition of an equal volume of 100% ethanol, incubated for at least 30 minutes at -20°C, washed in 50 mM Tris pH 7.5, stained with 0.2 μg/ml DAPI in the same buffer at 25°C for 15 minutes, washed, then mounted for microscopy by adding an equal volume of 1% low melting point agarose (FMC). (a). wild type grown at 37°C for 6 hours. (b). *srp1-49*; 37°C, 6 hours, (c). *srp1-49 nup2Δ*; 25°C, (d) *srp1-49 nup2Δ* ; 37°C 3 hours. Scale bar is 5μ.

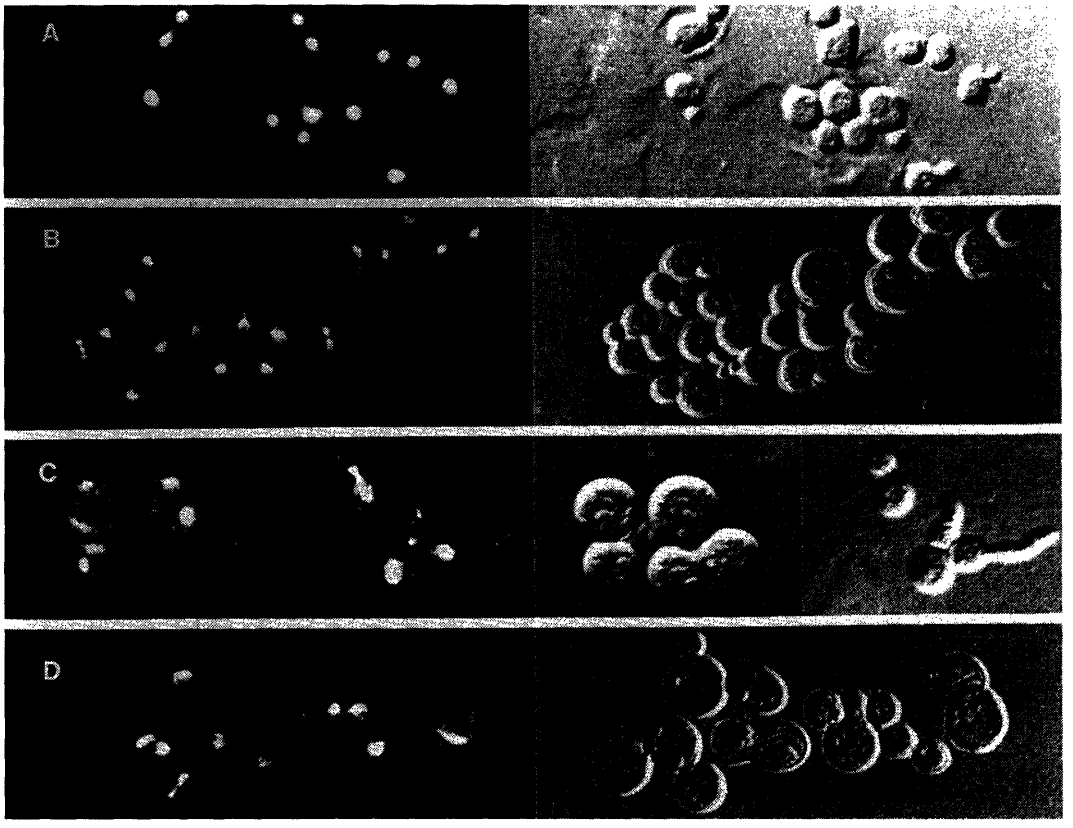
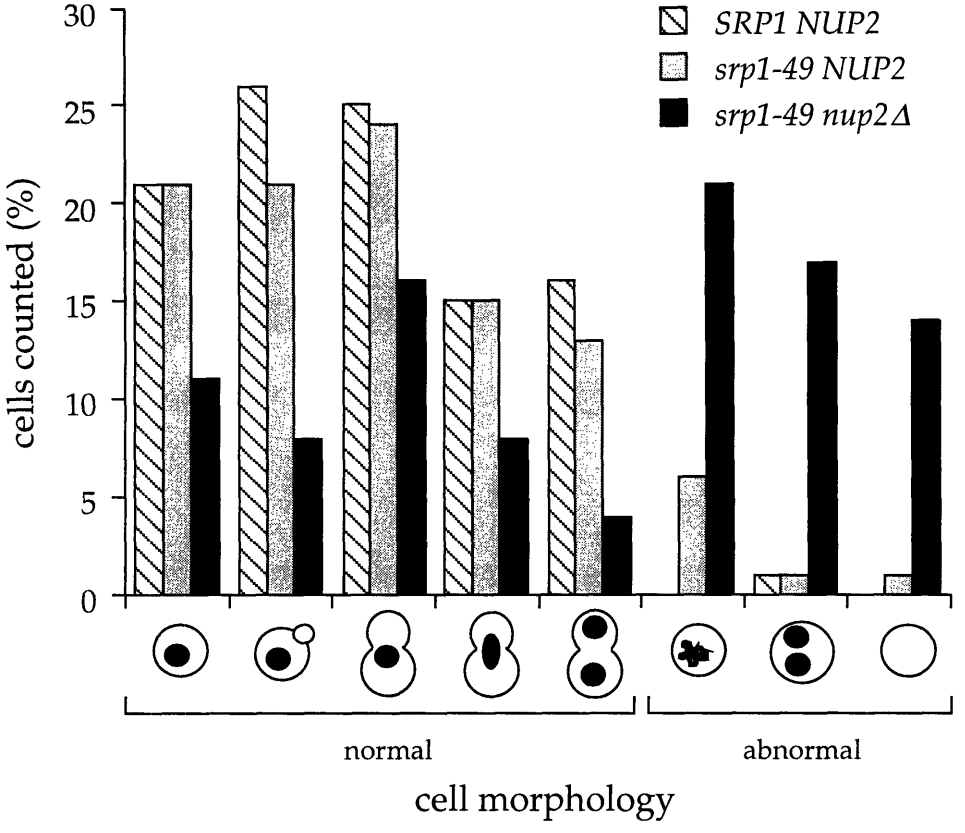

A
B
C
D

Figure 3.4 Quantitation of cell morphology at the non-permissive temperature.

The three indicated strains were grown, prepared for microscopy and observed as described in figure 3, except each culture was fixed three hours after temperature shift. A minimum of 150 cells were counted from each culture into the indicated classes.


30
25
20
15
10
5
0
cells counted (%)
SRP1 NUP2
srp1-49 NUP2
srp1-49 nup2Δ
normal
abnormal
cell morphology

chromatin appears diffuse or missing. Quantitation of the effects of these mutations on nuclear segregation and structure as assayed by DAPI staining is summarized in figure 4. *SRP1 NUP2*, *srp1-49 NUP2* and *srp1-49 nup2Δ* strains were grown to early log phase at 25°C, shifted for three hours to the non-permissive temperature, then fixed and stained. Cells from each culture were then counted into major classes as shown. Note that 52% of the *srp1-49 nup2Δ* cells fall into the three abnormal categories (anucleate cells, multinucleate cells and cells with diffuse "unfolded" DAPI staining), while the *srp1-49* strain contains 6% "unfolded" nuclei and the wild type does not significantly populate any abnormal category. In contrast, no DAPI phenotype was observed for the *srp1-31* mutant after three hours at 37°C, despite the severe growth defect of this allele. The *srp1-49 nup2Δ* strain appears to be equally depleted of all the normal morphological classes, suggesting that abnormal cells are terminal phenotypes derived from all points in the cell cycle. In order to confirm that this mutant strain has no specific defect in the cell cycle, DNA content of the mutants and wild-type cells at the non-permissive temperature was measured by propidium iodide staining and flow cytometry. In mutant cells the proportions of cells in the various cell cycle stages was normal, with the exception of accumulations of anucleate and polyploid cells that probably correspond to the multinucleate fraction.

SRP1 mutants affect chromosome segregation

In addition to *SRP1* mutations isolated as suppressors of RNA polymerase I, *SRP1* was also isolated as a multi-copy suppressor of *cse1*, a gene required for normal mitotic segregation of chromosomes (Z. Xiao and M. Fitzgerald-Hays, personal communication). Therefore, we tested *srp1-31*, our most severe temperature sensitive allele of *SRP1*, to ascertain whether a defect in Srp1 also affects the fidelity of chromosome transmission (Table 4). We tested the rate of chromosome loss by a red/white sectoring assay that measures the loss of a minichromosome containing the *sup11* gene (Gerring et al., 1990). Both chromosome loss and gain (non-disjunction) are measured in this assay. We found the *srp1-31* mutant strain grown at 30°C has elevated rates of both types of chromosome mis-segregation: 28 times *SRP1* for non-disjunction and 8 times *SRP1* for loss.

Table 3.4 Chromosome loss in a *srp1* mutant

	nondisjunction 2:0 (red/white)	loss 1:0 (red/pink)
SRP1	0.03%	0.03%
srp1-31	0.84%	0.25%
FOLD	28	8

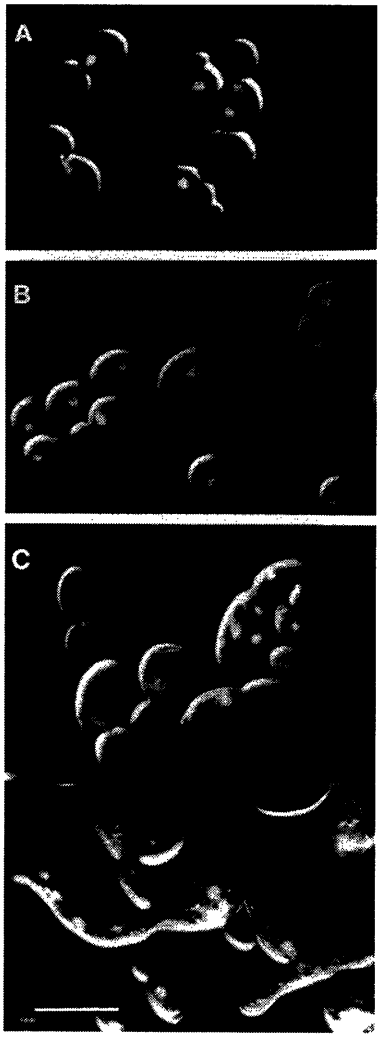
We have also found that *SRP1* interacts genetically with another mutant that affects chromosome segregation. We isolated *SRP1* mutants in a screen designed to find genes that are synthetically lethal with a deletion of the *BIK1* gene, a non-essential microtubule binding protein that is required for nuclear fusion during mating (Berlin et al., 1990). The *bik1* deletion strain also has ten-fold elevated levels of chromosome loss, presumably due to the malfunction of mitotic spindles. The complete results of this screen will be presented elsewhere (Pellman et al., 1995). The screen was performed using a colony sectoring assay to monitor the loss of a wild-type *BIK1* gene on a plasmid (Bender and Pringle, 1991). Two alleles of *SRP1* were isolated as non-sectoring strains in this screen (*srp1-101* and *srp1-102*). The *srp1-101 bik1Δ* double mutant is not entirely inviable, but has a pronounced growth disadvantage compared to the *srp1-101* single mutant. Therefore, double mutant cells could be obtained by screening for loss of the wild type *BIK1* plasmid which results in slow growing white colonies. When the double mutant strain was examined by DAPI staining, we observed a dramatic multinucleate phenotype in many cells (Figure 5C). *bik1Δ* mutants have been previously reported to have an slightly elevated frequency of multinucleate cells (Berlin et al., 1990), as shown in figure 5A, whereas the *srp1-101* mutants grow normally (figure 5B).

Aberrant microtubule structures accumulate in *srp1^{ts}* and *srp1^{ts} nup2Δ* strains

Mutant and wild-type cells were labeled with antibodies against tubulin to observe the mitotic spindle in order to study further the effects of *srp1-49 nup2Δ* mutations on nuclear segregation (figure 6). *SRP1 NUP2* cells grown

Figure 3.5 Double mutants between *BIK1* and *SRP1* are highly multinucleate.

Strains were culture on YPAD plates at 30°C, scraped into buffer then stained with DAPI as described in Figure 4. Micrographs are simultaneous DAPI fluorescence and nomarski images A) *bik1Δ::TRP1 SRP1*. B) *BIK1 srp1-101*. C) *bik1Δ::TRP1 srp1-101*.


A
B
C

at 37°C are shown in figure 6A. A selection of normal microtubule arrays at several stages of the cell cycle are visible. Figure 6B shows the *srp1-49* single mutant strain grown at 37°C for six hours. A significant fraction of these cells exhibit the unusual diffuse DAPI staining characteristic of this mutant, furthermore, as is demonstrated in Yano *et al.* (1994), cells with both aberrant spindles and abnormal, elaborate cytoplasmic microtubules are observed. As shown in figure 6C, the *srp1-49 nup2Δ* strain examined three hours after temperature shift exhibits a variety of abnormal microtubule structures consistent with the defect in nuclear segregation observed by DAPI staining. All stages of the cell cycle are observed, as indicated by the presence of monopolar spindles as well as short and long bipolar spindles. Mutant cells are clearly competent to make and extend spindle structures although they are frequently improperly oriented, resulting in nuclear division within the mother cell. Multiple spindle pole bodies (6C; upper right) as well as multiple spindles (6C; lower left) are frequently observed. Most, but not all concentrations of nuclear DNA coincide with microtubule structures, suggesting that nuclear fragmentation is mediated by spindle forces. In addition to the defects observed for spindle microtubules, hypertrophied cytoplasmic microtubule arrays are also observed in many cells.

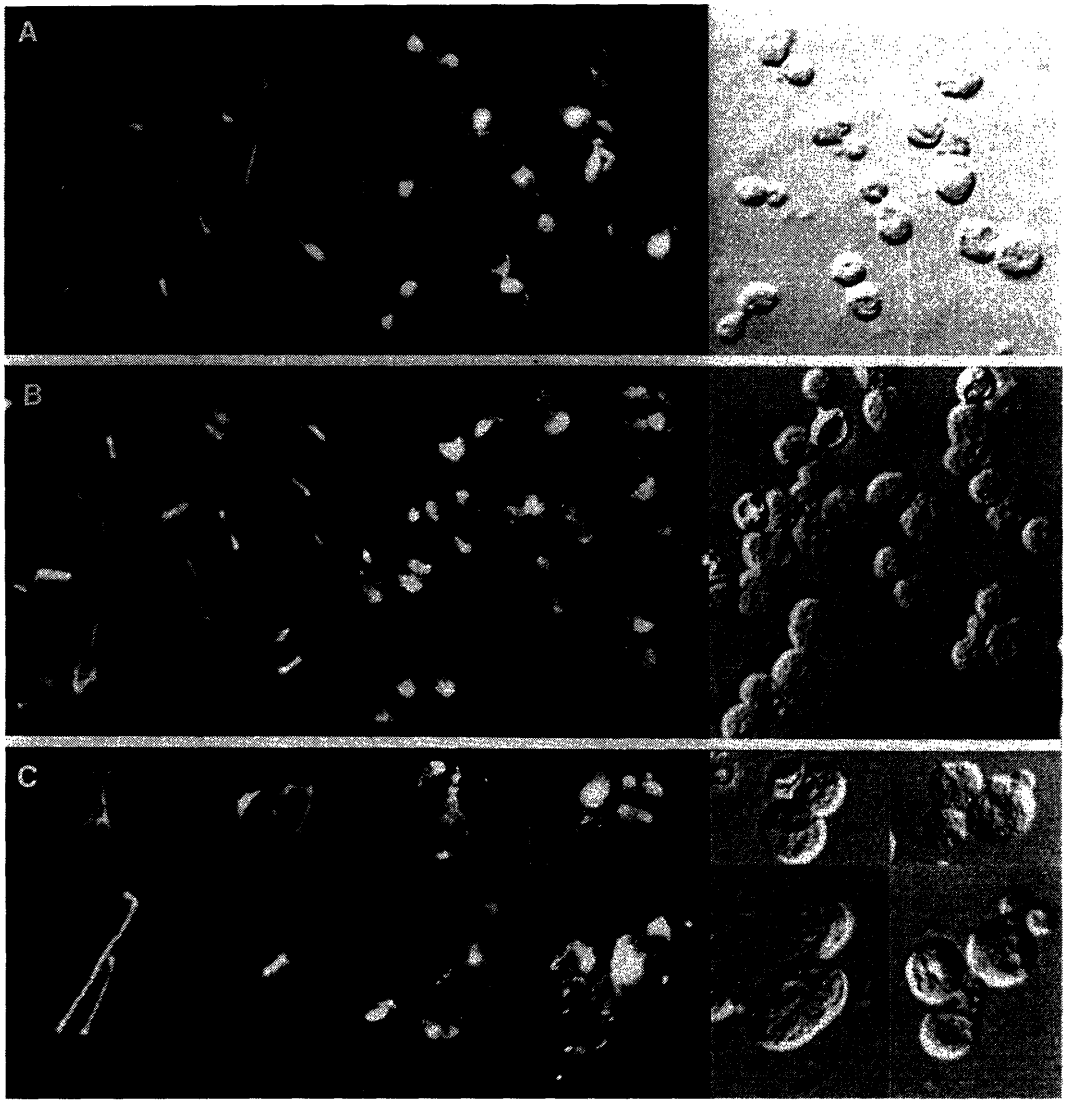
Electron microscopy reveals disorganization of NE membrane

Based upon the DAPI staining and tubulin immunofluorescence phenotypes, and in consideration of the localization of Srp1 to the NPC, we suspected that the morphological defects in the *srp1-49 nup2Δ* mutants might be a result of some structural change in the NE itself. Therefore we performed transmission electron microscopy on thin sections the mutant cells with two fixation and staining regimens that accentuate the NE and NPCs to see whether determine if these strains have any structural defects of the NE (figure 7). A typical wild-type large budded cell is shown in figure 7A. Note that the spindle pole body aligned towards the bud and the NPCs are seen as electron dense regions spanning the NE. The *srp1-49* single mutant cells shifted to 37°C for six hours rarely show any identifiable NE phenotype, however, a small percentage of cell sections display abnormal inclusions of cytoplasm within the nucleus (figure 7B). Surprisingly, no consistent nucleolar staining phenotype was observed in this strain. By contrast, despite

the fact that growth ceases completely and rapidly at the non-permissive temperature, *srp1-31* did not exhibit any detectable NE phenotype when

Figure 3.6 Microtubule structures in *srp1^{ts}* strains.

Indirect immunofluorescence microscopy of the indicated strains grown as described, then stained with the YOL1/34 anti-tubulin antibody. Each strain was grown to early log phase at 25°C, then shifted to 37°C and fixed after the indicated period of time. (a) wildtype; 6 hours, (b) *srp1-49*; 3 hours (c) *srp1-49 nup2Δ*; 3 hours. Each panel is a montage of the anti-tubulin staining, the DAPI staining and a nomarski/DIC image of the same field, respectively.


A
B
C

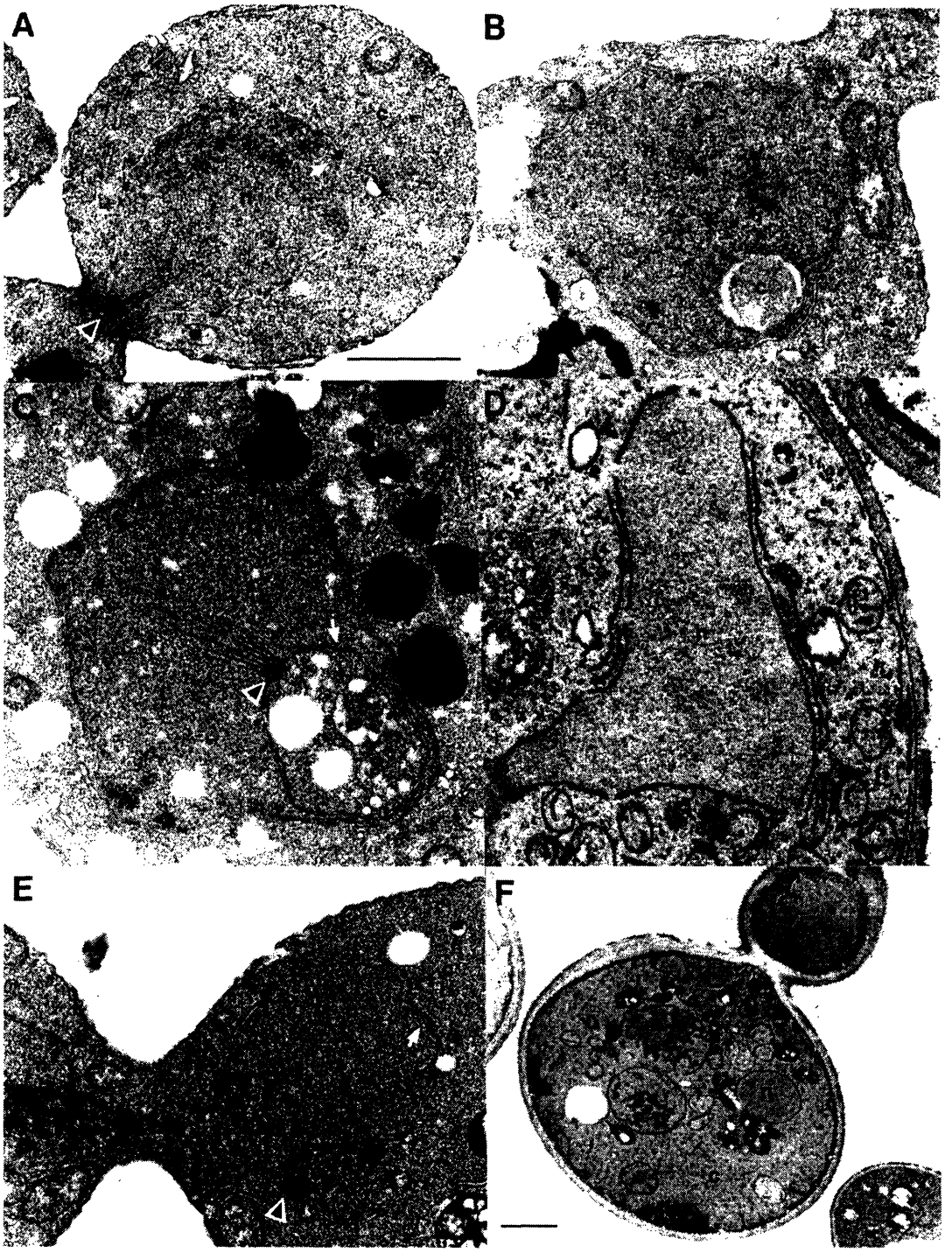
observed under these conditions. The *srp1-49 nup2Δ* double mutant shifted to 37°C for three hours exhibits a variety of abnormal NE phenotypes (figure 7C-F). Very large inclusions of cytoplasm are common, which may represent irregularities in the NE surface, tubes of cytoplasm transecting the nucleus or membrane bound compartments enclosed within the nucleus (figure 7C). Note that there are pores leading into this inclusion, as well as on the outer surface of the surrounding envelope, demonstrating that these inclusions are comprised of NE. In addition, the cell section depicted in figure 7C also shows a spindle pole body embedded into the wall of one of these inclusions. Strikingly, a large number of *srp1-49 nup2Δ* cells have accumulations of extra sheets or tubules of NE membrane which are parallel to the main NE, either just inside or outside (figure 7D). These extra stacks are certainly NE because they contain NPCs. As many as three extra layers have been observed. The spindle pole bodies are morphologically normal in the mutants, but the abnormal spindle phenotypes are seen in occasional sections such as figure 7E, where a large budded cell is shown that has already partitioned some of its nucleus into the daughter, but has two spindle pole bodies in the mother cell. Figure 7F shows another mutant cell at a lower magnification, where the multinucleate phenotype observed by DAPI staining is apparent. This suggests that at least in some cases the dispersion of chromatin into several sites throughout the cell is coincident with partitioning of the NE. In all cases the localization and morphology of the pores themselves is normal. No examples of nuclear envelope herniation or NPC clustering were observed.

srp1-49 nup2Δ is defective for nuclear import *in vitro*

We examined whether the *srp1-49 nup2Δ* double mutant is defective for the import of nuclear proteins, because there is evidence from experiments performed with both mammalian and yeast cells that nucleoporins are required for nuclear import of proteins and export of RNAs. We employed two complementary approaches to study the import of proteins into $srp1^{ts}$ *nup2Δ* mutant nuclei. We used a recently developed *in vitro* assay for measuring the import of a fluorescently labeled protein bearing multiple nuclear localization sequences (Schlenstedt et al., 1993). Also, to provide independent verification for our results *in vitro*, we developed a

Figure 3.7 Structure of the NE in *srp1^{ts}* strains as visualized by electron microscopy.

(A) logarithmic phase culture grown at 37°C of wild type strain L4884 fixed and prepared for EM microscopy by the osmium-Poly/Bed technique as described in the MATERIALS AND METHODS. (B) *srp1-49* log culture at 25°C was shifted to 37°C and grown for 6 hours, then prepared by the osmium-Poly/Bed technique. (c,e) *srp1-49 nup2Δ*; 3 hours osmium-Poly/Bed (d,f) *srp1-49 nup2Δ*; 3 hours prepared by the KMnO4 technique. Scale bar in (a) is for (a-e) and represents 1 μ. Scale bar in (f) represents 1 μ.


A
B
C
D
E
F

novel method to monitor the import of a nuclear targeted fluorescent reporter protein *in vivo.*

Analysis of temperature sensitive mutants by the *in vitro* assay is performed by growing the cells under non-permissive conditions (37°C) for varying lengths of time, preparing permeabilized spheroplasts at 30°C, then the assay itself is performed at room temperature. Therefore, phenotypes of mutant proteins that are able to recover by return to permissive conditions might not be detected. However, mutants that are defective for synthesis or are irreversibly inactivated at the non-permissive temperature should fail to import the substrate. Figure 8 depicts import into wild-type and mutant cells, while figure 9 summarizes quantitation of percent import from each strain. Import of the transport substrate into wild-type nuclei is shown in figure 8A. Greater than 50% of the wild-type spheroplasts imported the substrate. The *srp1-49* single mutant displays a significant decrease in nuclear import activity after six hours at the non-permissive temperature (figure 8B), despite this strain's ability to continue to grow under these conditions. When grown under permissive conditions, the *srp1-49 nup2Δ* double mutant has significantly reduced ability to transport the substrate, comparable to *srp1-49* under non-permissive conditions (figure 8C). At the non-permissive temperature, the double mutant has almost no transport activity (figure 8D). *nup2Δ* cells were also tested for nuclear import *in vitro* and it was found that the loss of *NUP2* activity has negligible effect on import. As shown in figure 9, binding of substrate to the surface of the nuclear envelope appears to be unaffected in *srp1-49 nup2Δ* mutants. Quantitation of import for double mutant at the permissive temperature is probably an over estimation of the amount of import due to the difficulty in scoring binding to the surface of extremely abnormally shaped nuclei. An effort was made to err towards scoring cells as import competent. The double mutant was also tested after a shorter temperature shift of 3 hours. At this time point we observed an intermediate level of import compared with the permissive temperature and 37°C for six hours.

Figure 3.8 An *in vitro* assay indicates nuclear import is blocked in *srp1-49 nup2Δ* cells at the non-permissive temperature.

Strains were grown as described, then spheroplasted and permeabilized. The assay was performed in the presence of wild-type cytosol and ATP at room temperature. The left side of each panel shows the rhodamine conjugated import substrate while the right side is DAPI staining of DNA. (A) *SRP1 NUP2* cells grown at 37°C for 6 hours. (B) *srp1-49* cells grown at 37°C for 6 hours. (C) *srp1-49 nup2Δ* cells grown at 25°C. (D) *srp1-49 nup2Δ* cells grown at 37°C for 6 hours.

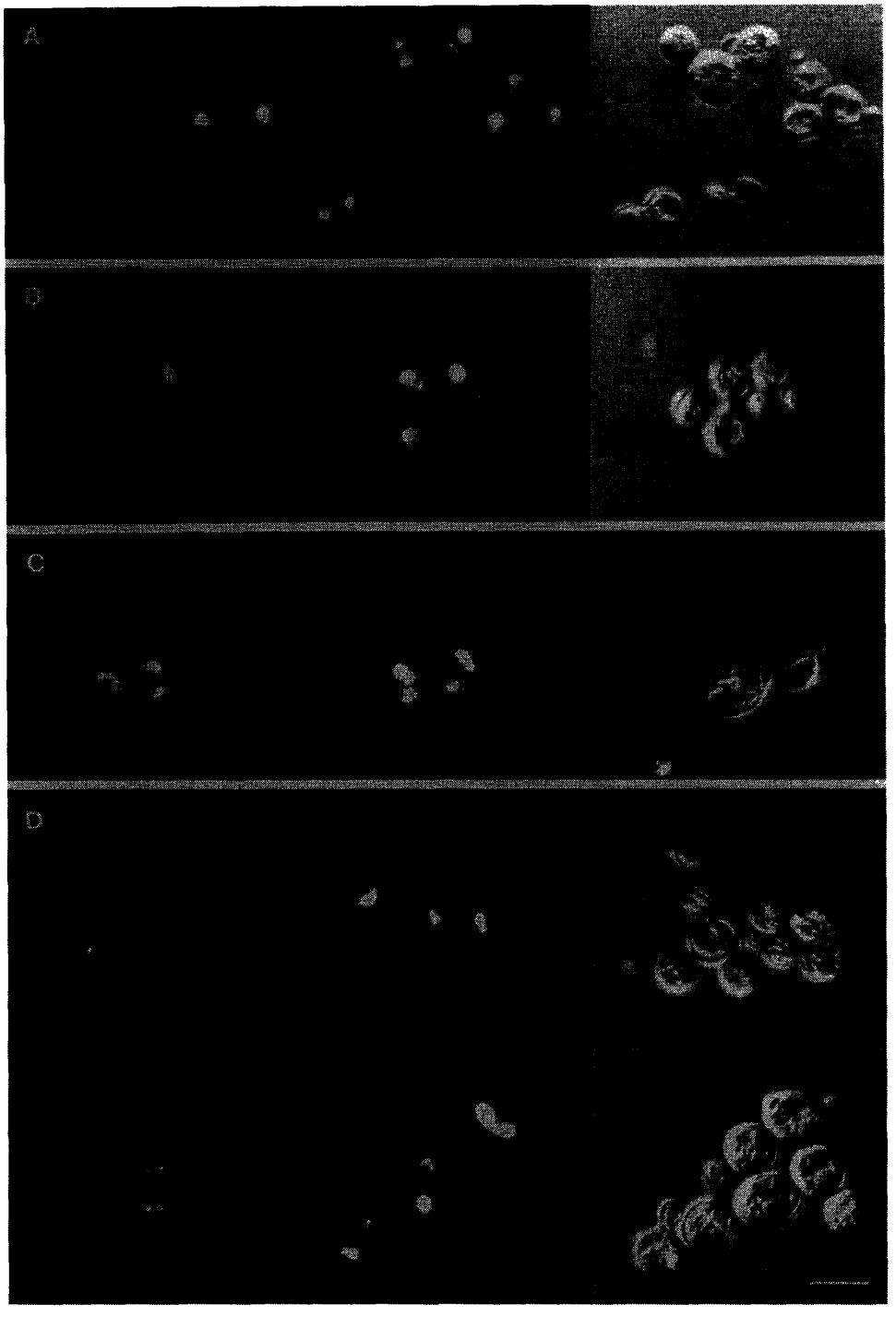
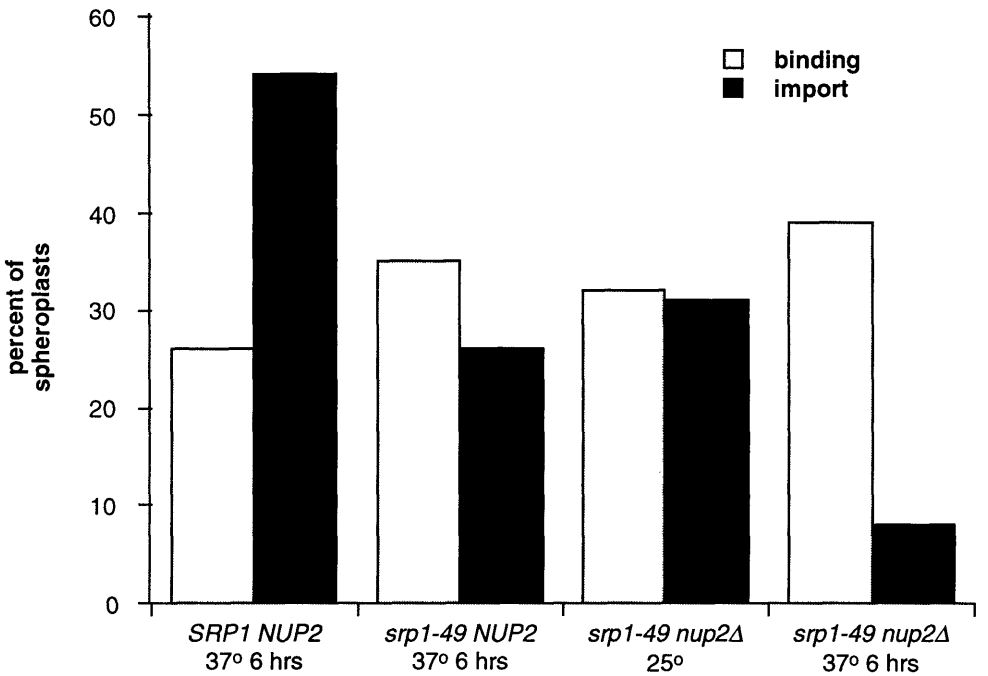

A
B
C
D

Figure 3.9 Quantitation of *in vitro* nuclear import defect.
Assay was performed as described in figure 8. At least 150 DAPI staining spheroplasts were scored for accumulation of the import substrate as either no staining, binding of substrate to the NE or import of substrate into the nuclear interior.

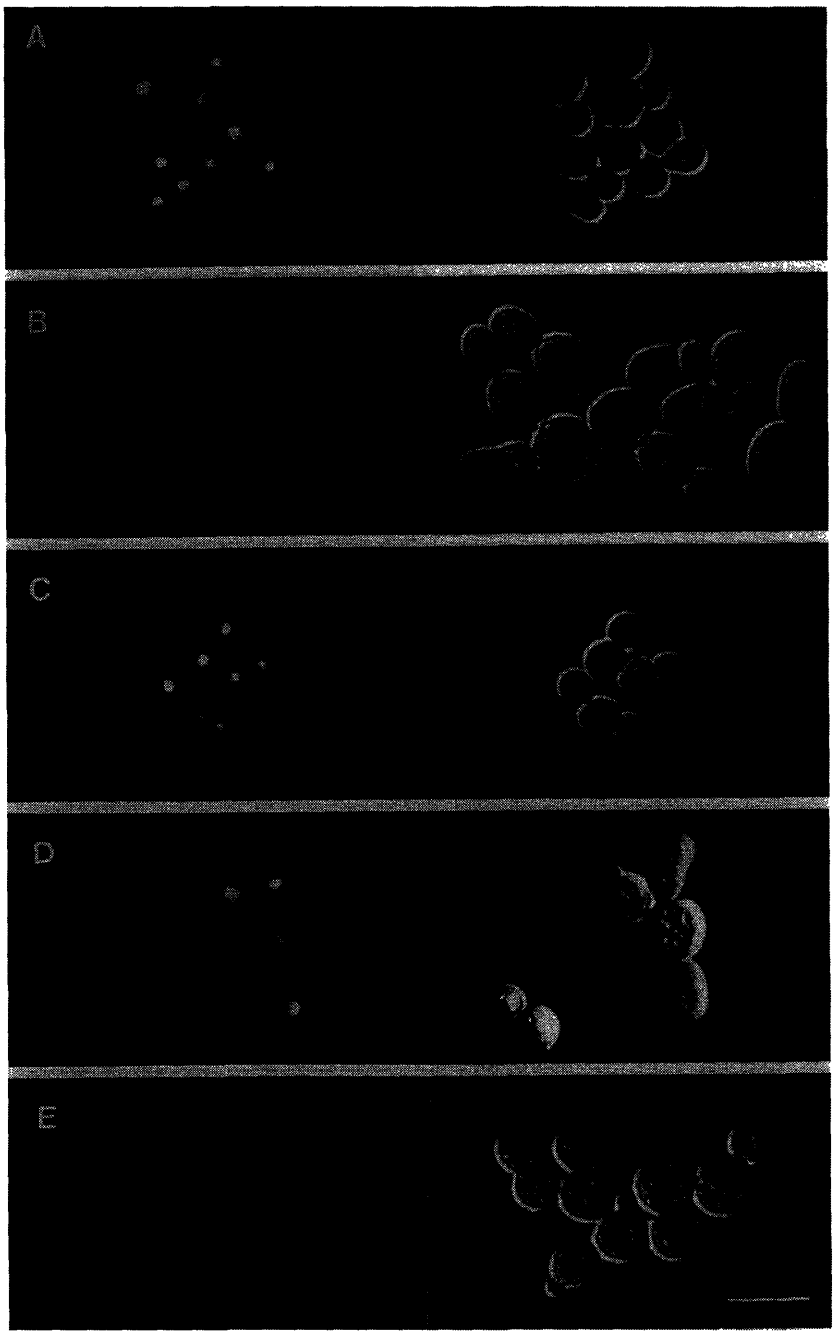

60
50
40
30
20
10
0
percent of spheroplasts
binding
import
SRP1 NUP2
37° 6 hrs
srp1-49 NUP2
37° 6 hrs
srp1-49 nup2Δ
25°
srp1-49 nup2Δ
37° 6 hrs

A novel assay for nucleocytoplasmic transport *in vivo* confirms a requirement for *SRP1* in import

We utilized the gene for the naturally occurring green fluorescent protein (GFP) of *Aequora victoria* (Chalfie et al., 1994; Prasher et al., 1992) as an *in vivo* indicator for nuclear protein localization as follows. GFP is fluorescent *in vivo* when expressed in many heterologous cell types by virtue of the cyclization of an internal chromophore consisting of the tripeptide Ser-dehydroTyr-Gly (Chalfie et al., 1994; Cody et al., 1993). We fused the entire histone *H2B-1* coding sequence from *S. cerevisiae* (Wallis et al., 1980) to the amino-terminus of GFP in a *URA3 CEN* plasmid bearing the *GAL1-10* inducible promoter (kindly provided by H. Mosch). The efficient nuclear import of *H2B-1* fused to the amino terminus of β-galactosidase has been shown previously (Moreland et al., 1987). When transformed into wild-type cells and induced by growth in synthetic media with galactose as a carbon source, the histone::GFP fusion protein is localized entirely to the nucleus in a pattern that is nearly indistinguishable to that of DAPI staining for nuclear DNA when observed by fluorescence microscopy (figure 10A).

To assay protein import *in vivo*, cells were grown in raffinose medium to derepress expression of the reporter, then induced by addition of galactose to the medium. Some cultures were simultaneously shifted to 37°C to monitor the effect of the conditional mutants on accumulation of the fluorescent protein in the nucleus. Wildtype cells accumulated histone::GFP when observed three hours after the temperature shift (figure 10A). As a control for a mutant that we would expect not to be competent for nuclear transport under these conditions, we used a *nup116Δ* strain. As shown in figure 10B, this strain does accumulate some histone::GFP in the nucleus of a fraction of cells after three hours at 37°C. This is somewhat surprising, since it has been shown that poly A^+ RNA accumulates in the nucleus after one hour at the non-permissive temperature and the nuclear pores appear completely sealed by membrane after three hours (Wente and Blobel, 1993). *srp1-49* mutants cultured at 37°C also accumulate histone::GFP in their nuclei efficiently after three hours (figure 10C). In contrast, we found no accumulation of histone::GFP into *srp1-49 nup2Δ* mutants (figure 10D). The *srp1-49 nup2Δ* strain did properly synthesize and transport histone::GFP when allowed to remain at the permissive temperature (figure 10E).

Figure 3.10 Nuclear transport is also defective *in vivo*.
Cells bearing the *H2B1*::GFP plasmid were grown overnight at 25°C in SC raffinose to mid-log phase, then *H2B1*::GFP expression was induced by the addition of galactose to 3%. After one hour, cultures were split, and half was shifted to 37°C. A) wildtype 37°C, 3 hours after shift from 25°C. B) *nup116Δ* 37°3 hours after shift. C) *srp1-49* 37°C, 3 hours. D) *srp1-49 nup2Δ* 25°C E) *srp1-49 nup2Δ* 37°C 3 hours.


A
B
C
D
E

Accumulation of histone::GFP in the nucleus was unaffected in other temperature sensitive mutants with similar growth arrest kinetics that affect unrelated processes such as the late step secretion mutant *sec6-4* (Novick et al., 1980) and the topoisomerase II mutant *top2-1* (Holm et al., 1985).

3.4 Discussion

Srp1 is a nuclear pore complex constituent

We have demonstrated by immunoelectron microscopy that the majority of Srp1 is associated with the NE and clusters of Srp1 staining are located at the nuclear pores. Furthermore, we and others have shown specific genetic interactions between several alleles of *SRP1* and nucleoporin mutants. These data correlate well with fractionation and immunoprecipitation studies of yeast cells that have shown Srp1 is physically associated with Nup1 and Nup2, both of which are well characterized NPC proteins (Belanger et al., 1994) and M.T., J.L. and M.N. unpublished results).

The immunoelectron microscopy experiment indicated that the majority of Srp1 is in the NPC just inside of the NE. Some nucleoporin proteins have been shown to be specifically localized to one face of the NPC (Sukegawa and Blobel, 1993; Wilken et al., 1993). Therefore it is possible that the main site of action of Srp1 is the inner surface of the NPC or the basket domain. Importantly, it has not been shown that all of the Srp1 protein is located in the NPC, a fact that might be relevant to explain the pleiotropic effects of *SRP1* mutants. Some of the Srp1 is distributed along both sides of the NE in regions that are not identifiable as pores. Furthermore, it is difficult to determine whether gold particles observed in the nucleoplasm and cytoplasm are non-specific background or whether they correspond to other structures containing Srp1. If a pool of Srp1 is present elsewhere besides the NPC, it is probably not concentrated in a large, recognizable structure like the nucleolus, but perhaps it is distributed along the nuclear envelope.

The genetic interactions we have discovered between $srp1^{ts}$ mutants and deletions of nucleoporin genes imply that these proteins are involved in the same cellular processes. Genetic interactions of this type do not require physical interaction, since similar synthetic lethality has been shown between nucleoporins that do not co-purify, for example *NUP2* with either *NUP1* or *NSP1* (Belanger et al., 1994; Loeb et al., 1993). However, because Nup1 and Nup2 can be co-immunoprecipitated with Srp1 and have been localized within the same organelle, we believe that these interactions are the consequence of Srp1 functioning in the same complex as these nucleoporins. We have also shown that $srp1^{ts}$ is synthetically lethal with the deletion of the GLFG nucleoporin *NUP116*, but we have not yet determined whether Srp1 is

physically associated with Nup116p. Synthesis of genetic, localization and association data suggest the presence of multiple nucleoporin complexes composed of similar components that are involved in overlapping, but distinct duties within the NPC.

We found that the *srp1-49 nup2Δ* double mutant fails to transport reporter proteins into the nucleus *in vitro* and *in vivo*. These mutations in *SRP1* do not affect the recognition of the import substrate or its targeting to the nuclear envelope, as evidenced by the appearance of the substrate protein around the nuclear periphery in the *in vitro* assay. However, the translocation step is disrupted. Therefore, the phenotype is more similar to $nsp1^{ts}$ mutants than to $nup49^{ts}$ or $npl3^{ts}$ mutants (Schlenstedt et al., 1993). The inability to translocate proteins into the nucleus may be due to some structural imposition in the transport channel, as is probably the case for mutants in *NUP116*, or it may be due to a more specific defect in the translocation apparatus.

We adapted an existing *in vivo* assay for nuclear transport to utilize the recently developed green fluorescent protein (GFP) as an *in situ* reporter for protein localization. The ability to visualize GFP in living cells by fluorescence microscopy is a major advantage over other techniques. The assay is far more rapid than immunofluorescence and artifacts of fixation are avoided. One drawback of the *in vivo* assay whether performed by immunofluorescence or by GFP localization is that it is not entirely specific for the import of newly translated proteins. Under non-permissive conditions, there is little accumulation of histone::GFP in the cytoplasm of *srp1-49 nup2Δ* mutants, which suggests a defect in the export of mRNA as well as import of protein. Furthermore, we have demonstrated the utility of employing GFP fusions as "epitope tags" to determine the subcellular localization of proteins in living yeast cells. This technology should provide a means to observe many cellular processes in real time.

The phenotypes of $srp1^{ts}$ *nup2Δ* mutants indicate that the NPC has an important role in nuclear envelope structure

Genetic interactions between $srp1^{ts}$ mutants and nucleoporin mutants have enabled us to establish a functional link between Srp1 and the NPC. The interaction that has been most useful in refining our understanding of

NPC function has been the combination of the *srp1-49* mutant with a deletion of the entire *NUP2* coding sequence. The elimination of Nup2 exacerbates the weak temperature sensitive phenotype of *srp1-49*. We presume that in this strain the mutant Srp1 must be performing its essential function at the permissive temperature in association with Nup1 or other unidentified factors. The conditional phenotypes of *srp1-31* and *srp1-49 nup2Δ* enable us to assess the physiological defects manifested upon loss of the Srp1 activity. These defects are consistent with the series of predictions we have made for mutant NPC components involved in maintaining nuclear structure. Srp1 does not appear to be necessary for the normal synthesis or maintenance of NPCs, as assayed by electron microscopy, as no defects were detected in NPC morphology or distribution. However, several profound aberrations in nuclear envelope structure and function were observed shortly after shift to the non-permissive temperature.

The *srp1-31* mutant has an increased level of chromosome loss that might indicate either defects in the mitotic spindle, loss of connections between chromosomes and NE, ineffective DNA replication or accumulation of cells in cell cycle stages where the chromosomes are unstable. Perhaps the finding that overexpression of *SRP1* suppresses the *cse1* mutation is also indicative of the relationship of the NPC and/or nuclear envelope to the chromosome segregation machinery. Both the *srp1-49* mutant and the *srp1-49 nup2Δ* mutant exhibit a loss of chromatin compaction or organization that may reflect a role for the NPC in higher order chromatin organization. This might present a plausible explanation for the finding that alleles of *SRP1* suppress defects in the RNA polymerase I transcription apparatus (Yano et al., 1992). Specifically, *SRP1* mutants may relax nucleolar chromatin structure in such a way as to ameliorate the partially defective PolI enzyme.

Nuclear segregation is also perturbed in the *srp1-49 nup2Δ* double mutants. Many multinucleate and anucleate cells are observed even at the permissive temperature, and at the non-permissive temperature nuclei become fragmented. Mitotic spindles are formed in these mutant cells, however they are abnormal in a number of ways. The nuclei of cells with short spindles are often found improperly positioned away from the bud neck, and elongated bipolar spindles are occasionally observed within the mother cell. In more severely affected cells, multiple spindle pole bodies are observed, probably as a result of multiple rounds of endomitosis. These

phenotypes probably reflect the need for *SRP1* and *NUP2* function to coordinate spindle elongation with nuclear positioning and proper spindle orientation. Another finding that suggests that Srp1 is involved in regulating the actions of the mitotic spindle is our recent isolation of two alleles of *SRP1* in a screen for genes that are synthetic lethal with *bik1* mutants. Bik1p is a microtubule associated protein in yeast that has previously been shown to be required for nuclear fusion during karyogamy and efficient nuclear segregation during vegetative growth. Perhaps in *bik1* cells, the role of the NPC in spindle regulation is more critical, consistent with the observation of the terminal phenotype of *bik1 srp1* strains where extremely multinucleate cells are commonly observed. We propose these results linking NPC proteins to nuclear segregation indicate that the NE plays a positive role in the timing and/or alignment of the mitotic spindle with respect to the axis of cell division.

The isolation of *srp1-49 nup2Δ* conditional mutant strain enabled us to detect a requirement for NPC components in the maintenance of nuclear envelope structure. When observed by electron microscopy, major defects in the nuclear envelope were apparent. First, many cells had round inclusions of cytoplasm within their nuclei encircled by a NE double membrane including NPCs, and the overall boundary defined by the nuclear envelope has numerous protrusions and irregularities never seen in wild-type cells. Furthermore, sheets of excess double membranes on either side of the nuclear envelope were observed in many cells. These membranes were identified as NE because they are perforated by NPCs. These extra layers of NE are somewhat reminiscent of the NE karmellae induced by the overexpression of the gene for HMG-CoA reductase reported by Wright et al. (1988). However the extra NE stacks observed in *srp1-49 nup2Δ* strains are much more irregular and disordered than HMG-CoA reductase induced karmellae. In contrast to HMG-CoA reductase overexpression, this phenotype is likely to be the result of loosened structural restraints on the NE, rather than the uncoordinated membrane biogenesis proposed for karmellae.

Potential functions of the nucleoporin/Srp1 complexes

The *SRP1* gene is related in sequence to the β-catenins, a family of proteins that are components of desmosomes and adherens plaques which

are required to link the cell surface, via cadherins, to the cytoskeleton (Kemler, 1993; Peifer et al., 1994; Yano et al., 1994). Perhaps the nucleoporin/Srp1 complex is part of an analogous system that links the nuclear envelope to the nucleo- or cytoskeleton. The possible targets of such a linkage could be chromatin, as yet unidentified intermediate filaments within the nucleus (possibly related to lamins?), actin or intermediate filaments in the cytoplasm. It is interesting to note another NPC protein, nup153 from amphibians, has been reported to be associated with filaments that project long distances into the nucleus from the NPC (Cordes et al., 1993).

Attachment of chromatin to the NE might be necessary for proper segregation of the nuclear contents during mitosis. It is well documented in yeast that telomeres are restricted to foci at the nuclear periphery during interphase (Klein et al., 1992), which leads us to speculate that perhaps the site of connection between chromosome ends and the NE are NPCs. Furthermore, connections to the nuclear contents might be required to maintain the regular spherical topology of the NE. The severing of the linkage between the NPC and these elements is a plausible explanation for the accumulation of the extra layers of membrane observed in the double mutant strain. That is, if the binding of the Nup/Srp1 complexes to elements within the nucleus is necessary to restrict the NPCs and NE to encircle the nuclear contents, in their absence the nuclear envelope may be induced to increase and project away from the chromatin. In this way the NPCs might be responsible for defining the difference between the outer nuclear envelope and the endoplasmic reticulum membrane.

A more indirect alternative model for the manner in which these NPC constituents affect nuclear envelope structure might be based on the more firmly established role of the NPC as the transporter of proteins and RNAs across the nuclear envelope. These mutants might selectively affect the transport of macromolecules (such as regulatory or structural proteins) that in turn are necessary for normal nuclear structure. One argument against this model is the existence of several mutants that are globally defective for protein and or RNA transport, such as $nsp1^{ts}$, $nup49^{ts}$ and $npl3^{ts}$, but do not effect nuclear morphology in this manner. Therefore, if these structural phenotypes are due to defect in transport, there must be a difference in transport substrate specificity between mutations in different NPC components.

Acknowledgments

The authors would like to thank Ya-Huei Tu for her excellent technical assistance with the electron microscopy. Peter Murray for kindly preparing the cell sections used for immunoelectron microscopy. We thank Hans Mosch for constructing the *GAL1-10* GFP vector and Steve Kron for assistance in visualizing GFP fluorescence. Susan Wente for providing strains and advice about electron microscopy. Laura Davis for sharing data prior to publication. We thank P.S. McCaw his critical comments on this manuscript, as well as all the members of the Fink lab for constructive suggestions.

Chapter 4

The yeast nuclear import receptor is required for mitosis

4.1 Introduction

The import of proteins into eukaryotic nuclei is comprised of two separable steps: the binding of import substrate to the nuclear envelope, and its subsequent translocation across the nuclear pore complex (NPC) into the nucleoplasm (Newmeyer and Forbes, 1988; Richardson et al., 1988) The recognition of proteins for nuclear localization is mediated by the interaction of short signal sequences (Nuclear Localization Sequence: NLS) within the targeted protein (Garcia-Bustos et al., 1991) with specific receptors. The major NLS binding protein (NBP) in animals, fungi, and plants is an approximately 60-70 kilodalton protein identified by chemical cross linking and blot-overlay assays (Adam et al., 1989; Silver et al., 1989). These NLS binding proteins are distributed between the cytoplasm and the nucleus. The activity of these proteins is necessary for the binding step of the import reaction by specific antibody inhibition of binding in vitro (Stochaj et al., 1991) and by reconstitution of a partially fractionated import reaction based on permeabilized mammalian cells with addition of purified NBP (Adam and Gerace, 1991). Furthermore, these proteins are highly phosphorylated, and their phosphorylation is required for NLS binding in vitro (Stochaj and Silver, 1992).

A protein required for targeting a nuclear import substrate to the nuclear envelope *in vitro* has recently been isolated and cloned (Görlich et al., 1994). This 60kD protein, importin, is absolutely required for the accumulation of import substrate on the nuclear envelope and binds to NLS sequences *in vitro* (Görlich et al., 1995; Moroianu et al., 1995). Importin was shown to be 44% identical to Srp1, a previously identified protein from *Saccharomyces cerevisiae* (Yano et al., 1992). Mutations in *SRP1* have pleiotropic effects, including suppression of conditional mutations in RNA polymerase I (Yano et al., 1992), defects in nucleolar structure (Yano et al., 1994), and synthetic lethality with mutations in the nuclear pore component, *NUP1* (Belanger et al., 1994). Furthermore, Srp1 is physically associated with Nup1 and Nup2, a related NPC constituent (Belanger et al., 1994).

In this work we demonstrate that Srp1/importin is essential for nuclear protein import in yeast both *in vivo* and *in vitro*, consistent with its role as a NLS receptor. Surprisingly, conditional *srp1* mutants arrest in the cell cycle during the G2/M phase. Moreover, degradation of the mitotic cyclin

Clb2 is impaired in *srp1-31*, suggesting that the import of a critical cell cycle regulator necessary for mitotic degradation is especially sensitive to defects in nuclear import.

4.2 Materials and Methods

Yeast strains and genetic techniques

All strains in this study are derivatives of W303-1a. We replaced the *SRP1* chromosomal locus with the *srp1-31* mutant allele (Yano et al., 1994) to avoid copy number effects. Unlike the plasmid-borne version, the integrated *srp1-31* allele has a recessive, extreme temperature sensitive growth defect. Four independent strains of *srp1-31* were constructed by plasmid integration and pop-out (Winston et al., 1983), and each isolate had identical growth properties.

To test genetic interaction with the cyclin dependent kinase, a *srp1-31* strain containing a *SRP1 URA3 CEN* plasmid was crossed with congenic strains bearing either the *cdc28-1N* or *cdc28-4* mutations. Tetrads were dissected and the genotypes of the resultant spores were determined by complementation tests and a PCR based assay for the *SRP1* genotype. The *srp1-31* mutation destroys a XbaI restriction site; this change was detected by amplification of a 630 nucleotide fragment of the *SRP1* gene with oligonucleotide primers of the sequence CTGCAGATGAACTTCGTCGTC and GTCCACGTAGCGGTCCTGATC, followed by restriction digestion and gel electrophoresis.

Microscopy

Immunofluorescence on yeast spheroplasts was performed as previously described (Loeb et al., 1993), except that formaldehyde fixation was performed for 60 minutes at room temperature. MAb 9C4 (Bossie et al., 1992) was detected with CY3 labeled goat anti-mouse IgG (Jackson Immunoresearch). To observe microtubules, we used YOL1/34 (Accurate Chemicals) as the primary antibody and goat anti-rat IgG conjugated with Texas red (Jackson Immunoresearch) as the secondary antibody.

In vitro assay for nuclear import

Components of the *in vitro* assay were prepared as previously described (Schlenstedt et al., 1993). *In vitro* assays were performed with 2 mg/ml final concentration of cytosol at 30°C. The experiment was repeated three times and for each sample >175 cells were scored for binding and import of the substrate.

Determination of Clb2 stability

Steady state levels of ectopically expressed Clb2 were determined by western blot. Derivatives of *SRP1* and *srp1-31* strains containing the *GAL1::CLB2* gene integrated at the *LEU2* locus were constructed by transformation with YIpG2::*CLB2* (Stueland et al., 1993). Protein concentrations in extracts (Yaffe and Schatz, 1984) were determined using the BCA reagent (Pierce), and normalized prior to electrophoresis.

For the pulse-chase experiment, the *GAL1::CLB2-lacZ* fusion was constructed from a polymerase chain reaction (PCR) derived fragment containing residue -6 to the last codon of the *CLB2* gene cloned into the BamHI and EcoRI sites of pKB64 (Kornitzer et al., 1994). This plasmid was introduced in *SRP1* and *srp1-31* strains and pulse chase analysis was performed as described (Kornitzer et al., 1994).

4.3 Results

Srp1 is necessary for nuclear import

To establish a functional role for Srp1, we first examined the nuclear protein import properties of the *srp1-31* mutant using an *in vitro* permeabilized cell assay (Schlenstedt et al., 1993). Because Srp1 protein is present in the permeabilized cell preparation and the cytosolic fraction (Figure 1A), both fractions were prepared from mutant and wild-type cells. Mutant Srp1 was also present in both fractions after temperature shift, indicating that the *srp1-31* defect is not due to decreased expression or instability. Binding of fluorescent substrate to the nuclear envelope and import into the nucleus are both affected in *srp1-31* mutants (Figure 1B). Import competence decreases in *srp1-31* with time at 37°C. After six hours at 37°C, *srp1-31* loses greater than 95% of its ability to import the substrate into nuclei. Mixing mutant cells with wild-type cytosol or vice versa resulted in intermediate levels of import, suggesting that both soluble and nuclear envelope bound Srp1 contributes to import. To confirm the observed *in vitro* import defect, we examined the localization of several nuclear proteins *in vivo* by immunofluorescence. We found localization defects for some, but not all, nuclear proteins tested. The nucleolar protein recognized by the 9C4 antibody (Bossie et al., 1992) accumulates aberrantly in the cytoplasm of *srp1-31* cells at the non-permissive temperature (Figure 1C). In contrast, the Npl3 protein (Bossie et al., 1992) and a histone-βGal fusion (Moreland et al., 1987) are only moderately mislocalized and the Nop1 protein (Aris and Blobel, 1988) is properly localized under these conditions (data not shown). In contrast, the *srp1-31* mutation has no effect on mRNA export, as determined by *in situ* hybridization to poly(A)$^+$ RNA (data not shown).

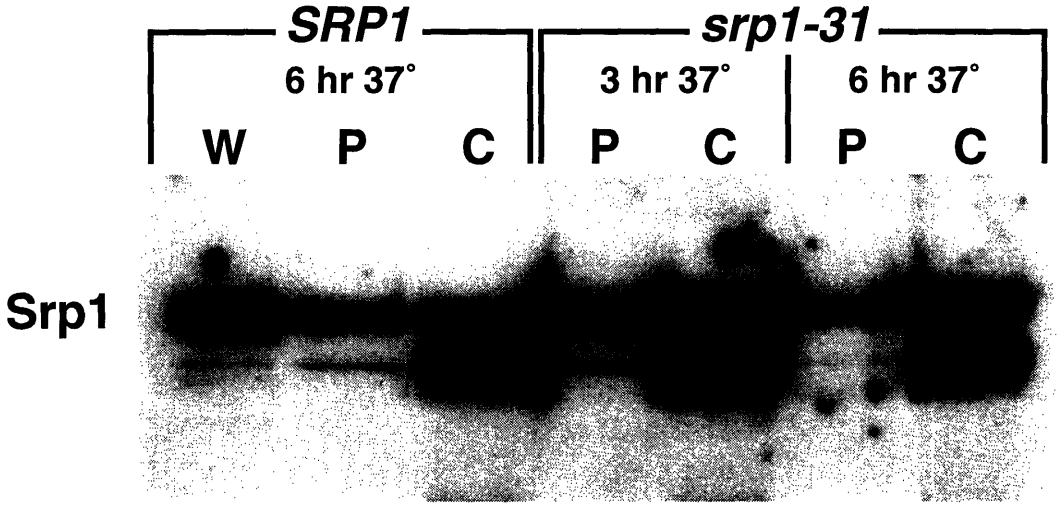
A *srp1* mutant arrests in mitosis

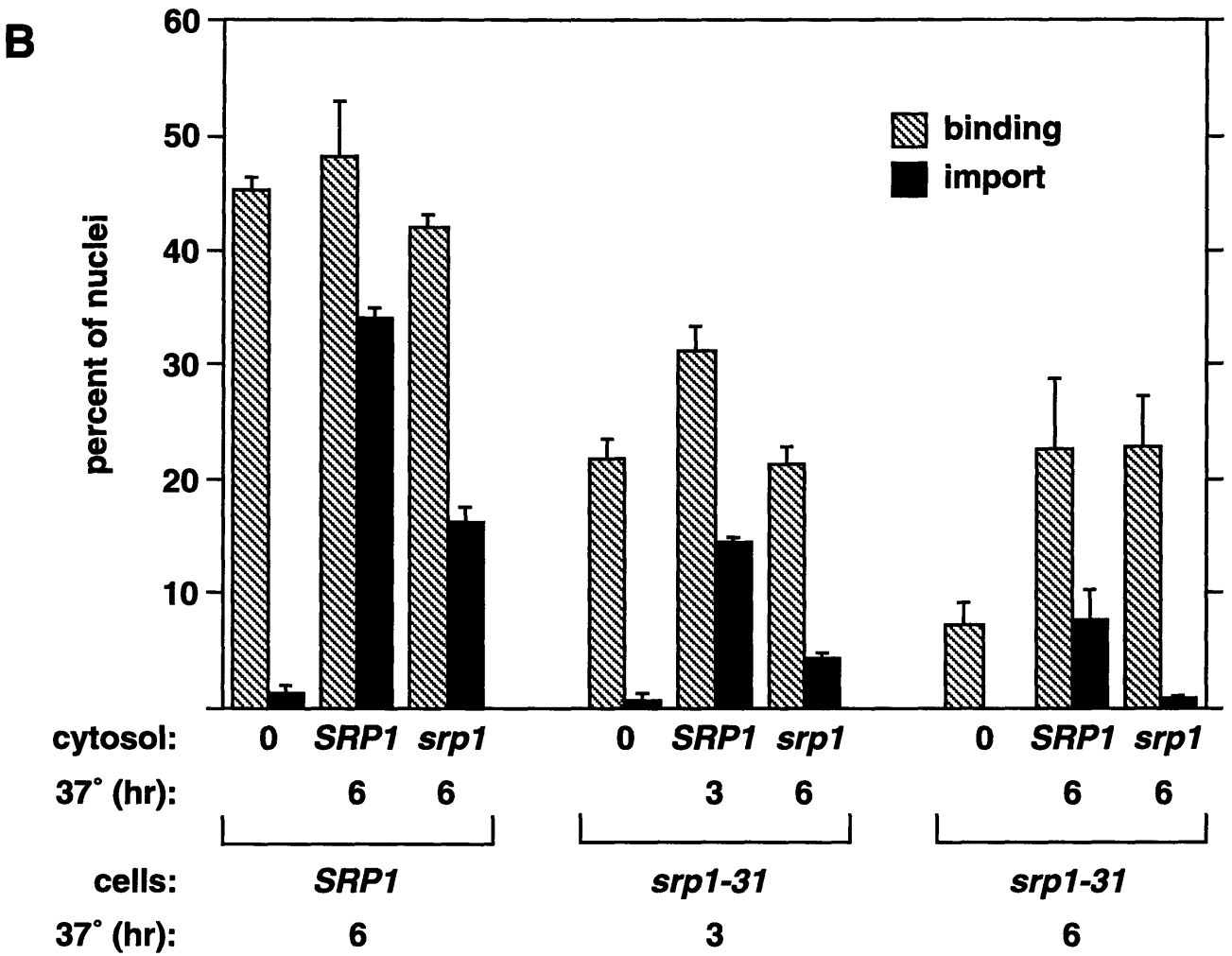
srp1-31 strains show a uniform G2/M arrest phenotype at the non-permissive temperature (Figure 2). After six hours of incubation at 37°C, about 85% of cells have large buds with a single nucleus at the bud neck and a short bipolar spindle consistent with a block just prior or during mitosis (Figure 2A). DNA content analysis of these cells shows that greater than 90 % have replicated DNA (data not shown), consistent with a defect in the cell

Figure 4.1 Srp1 is essential for nuclear import.

A. Srp1 is present in both permeabilized cells and the cytosolic fraction of the *in vitro* nuclear import assay. Permeabilized cells and cytosol were prepared from *SRP1* and *srp1-31* cells grown at 25°C and 37°C for three and six hours. An immunoblot of components used in the *in vitro* assay prepared from *SRP1* and *srp1-31* cells grown at 37° for the indicated times was probed with anti-Srp1 antiserum (Yano et al., 1992). W: whole cells, P: permeabilized cells, C: cytosol. Approximately equal numbers of cells were loaded in each lane. The lower band observed in cytosol preparations is probably a degradation product. B. *srp1-31* blocks the import of nuclear proteins *in vitro*. Cell equivalents of permeabilized cells and cytosol were mixed as shown along with the import substrate, a rhodamine labeled HSA-SV40 NLS conjugate, then import was observed by fluorescence microscopy. In contrast to *in vitro* assays using permeabilized mammalian cells (Moore and Blobel, 1992), some binding of substrate to the yeast nuclear envelope occurs in the absence of added cytosolic proteins (Schlenstedt et al., 1993). C. *In vivo* mislocalization of a nucleolar protein in the *srp1-31* mutant. Immunofluorescence with monoclonal antibody 9C4 (Bossie et al., 1992) was performed on *SRP1* and *srp1-31* cells that had been shifted to 37°C for six hours in YPD. Each frame is an identical exposure.

A




B
60
50
40
30
20
10
percent of nuclei
binding
import
cytosol:
0
SRP1
srp1
0
SRP1
srp1
0
SRP1
srp1
37° (hr):
6
6
3
6
6
6
cells:
SRP1
srp1-31
srp1-31
37° (hr):
6
3
6

C

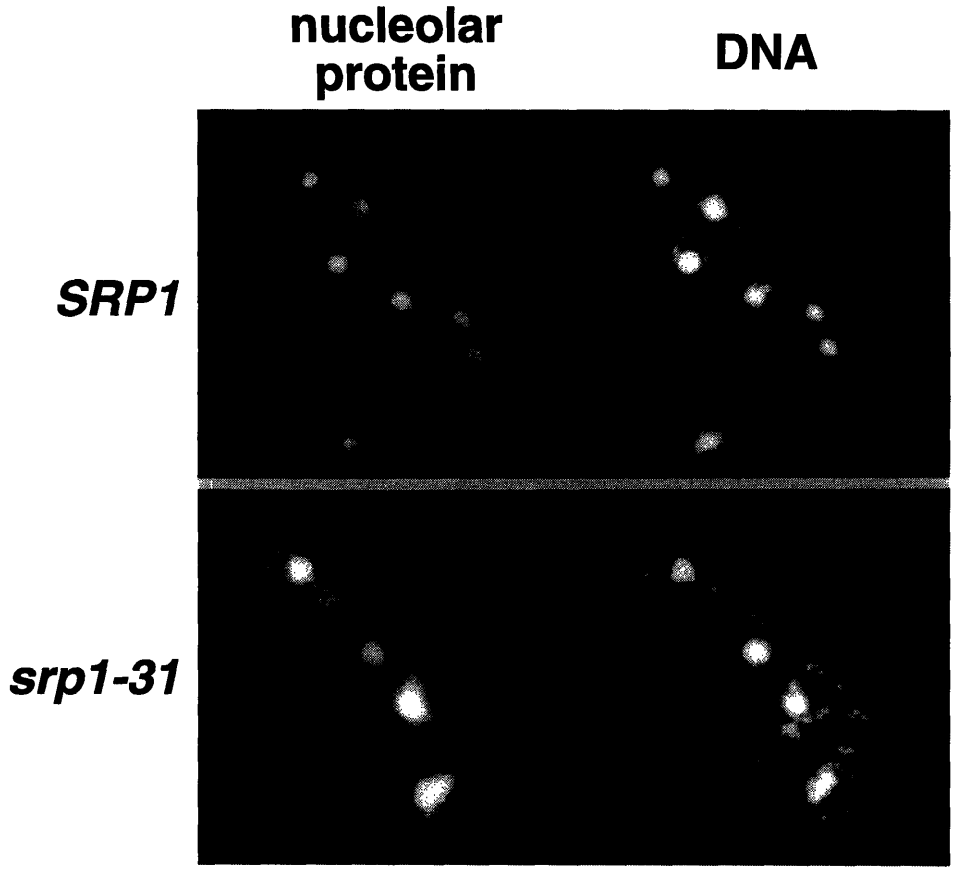
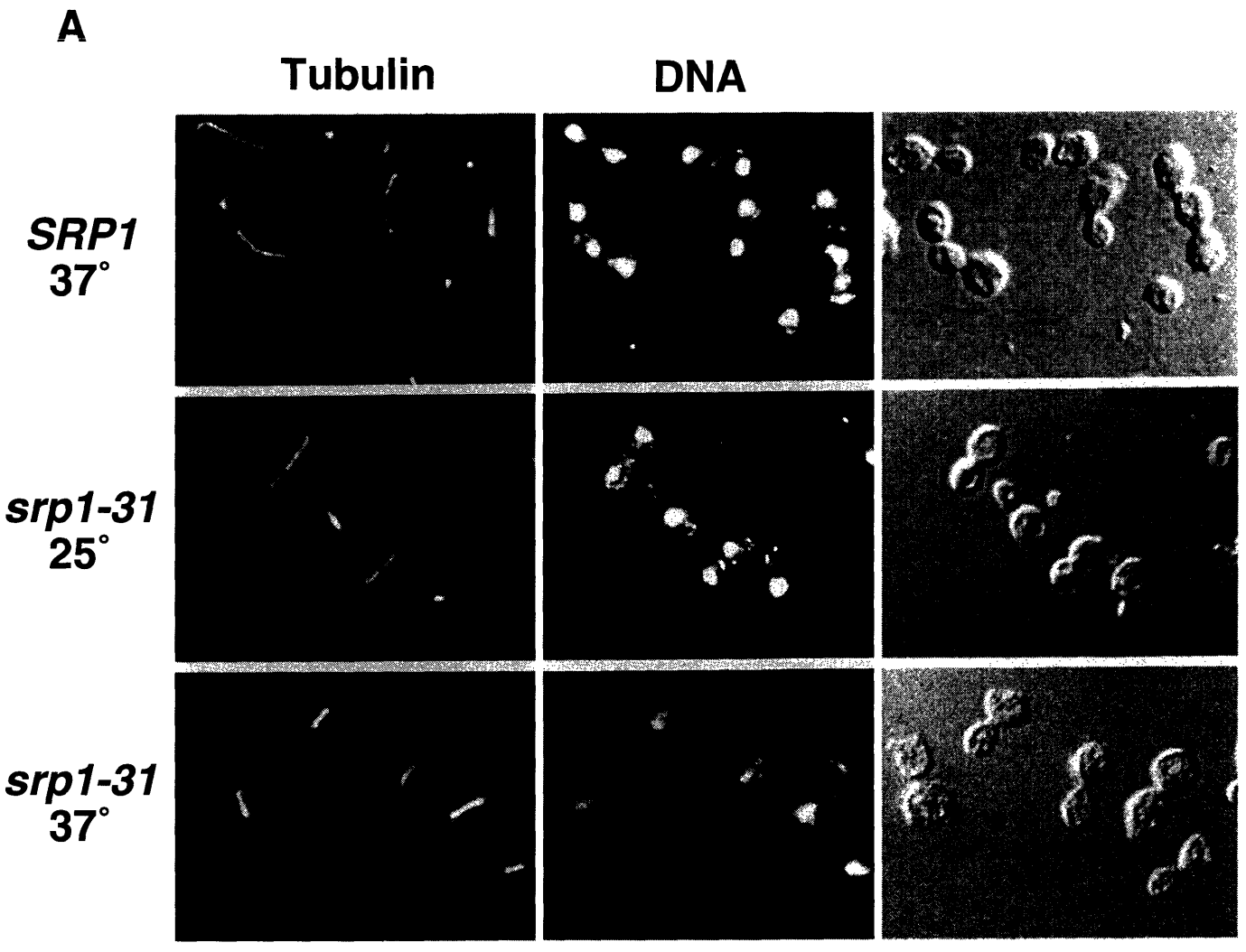
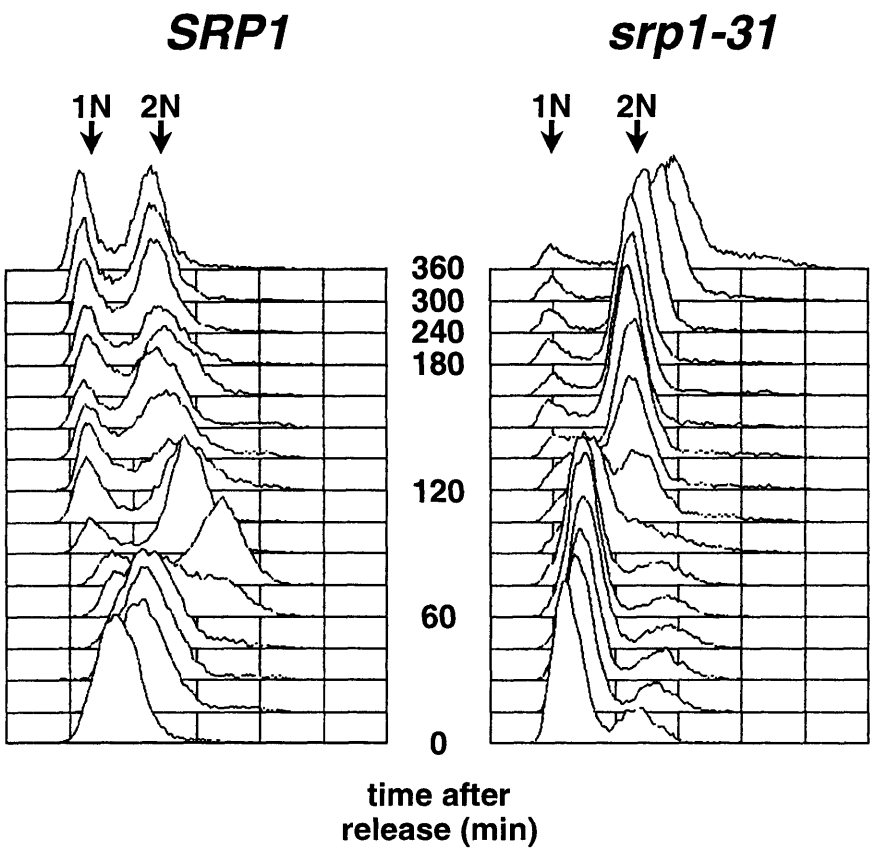


Figure 4.2 A temperature sensitive mutation in *SRP1* leads to arrest at the G2/M phase of the cell cycle.

A. Tubulin localization in *srp1-31* mutants. *SRP1* and *srp1-31* strains were prepared for immunofluorescence six hours after shift to 37°C, then microtubules were visualized with the YOL1/34 antibody. B. DNA replication after synchronization by alpha-factor mating pheromone. *SRP1* and *srp1-31* cultures were synchronized in G1 by treatment with 3μM alpha-factor in YPD medium at 25°C for 2.5 hours. Cultures were then released from pheromone arrest into fresh media at 37°C. Samples were withdrawn at 15 minute intervals, then fixed and analyzed by flow cytometry (Epstein and Cross, 1992). Histograms of fluorescence intensity versus cell number are shown.


A
Tubulin
DNA
SRP1
37°
srp1-31
25°
srp1-31
37°

B


SRP1
srp1-31
1N
2N
1N
2N
360
300
240
180
120
60
0
time after
release (min)

cycle after S phase. Furthermore, when synchronized in G1, then released into the non-permissive temperature, *srp1-31* cells arrest with replicated DNA in the first cycle after the shift (Figure 2B). Initiation of S phase is delayed by 30-45 minutes in *srp1-31* cells. The nuclear envelope, nuclear pores and the mitotic spindles of arrested *srp1-31* cells appear identical by transmission electron microscopy to those found in large budded *SRP1* cells (data not shown), indicating that the cell cycle defect is not a consequence or cause of global defects in nuclear envelope structure. The G2/M arrest phenotype is not unique to the *srp1-31* allele; another temperature sensitive allele, *srp1-49*, also accumulates cells with replicated DNA. However, *srp1-31* has both the most dramatic effect on nuclear import, and the most uniform cell cycle defect of the conditional alleles isolated. In strains containing a deletion of *SRP1* whose survival depends upon expression of a galactose inducible *SRP1* gene, depletion of the protein by glucose repression leads to a similar large budded arrest phenotype. However, Srp1 is an extremely stable protein, so the phenotype is only manifested after long periods of growth on glucose.

Cyclin proteolysis is defective in *srp1-31*

The simplest explanation for the G2/M arrest caused by the *srp1-31* mutation is that an event required for progression through mitosis depends on the import of a cell cycle regulator into the nucleus and is thereby sensitive to a block in Srp1-mediated transport. A good candidate is the wave of protein degradation that is required for mitosis and that persists through G1 (King et al., 1994). Therefore, we compared the levels of Clb2 (a mitotic cyclin degraded during anaphase and throughout G1, whose degradation is required to exit mitosis) (Amon et al., 1994; Surana et al., 1993) in *SRP1* and *srp1-31* cells. Steady state levels of Clb2 were tested in G1 arrested cells because any mutant that arrests at G2/M will have high levels of Clb2, whereas only mutants that specifically affect mitotic cyclin regulation should affect the degradation of Clb2 in G1. Clb2 ectopically expressed from the *GAL1* promoter is present in at least 20-fold higher levels in *srp1-31* mutants arrested in alpha-factor as compared to *SRP1* (Figure 3A). The difference in steady state level of Clb2 protein is not a result of increased expression of

Figure 4.3 Accumulation of Clb2 in alpha-factor arrested *srp1-31* cells ectopically expressing *CLB2* from the *GAL1* promoter.

Cycling and alpha-factor arrested cells containing a *GAL1::CLB2* gene were induced with galactose at 25°C. One hour after addition of galactose, samples were split and half were shifted to 37°C. After two hours further incubation, total protein and RNA were prepared and FACS analysis was performed. Top: An immunoblot of equal amounts of total protein probed with polyclonal rabbit anti-Clb2 (Amon et al., 1992). Bottom: The FACS profile of each sample at the time of harvest. Galactose induction of *GAL1::CLB2* is much more efficient at 25°C, therefore the level of Clb2 present in arrested cells should be compared to that of cycling cells grown under the same temperature regimen.

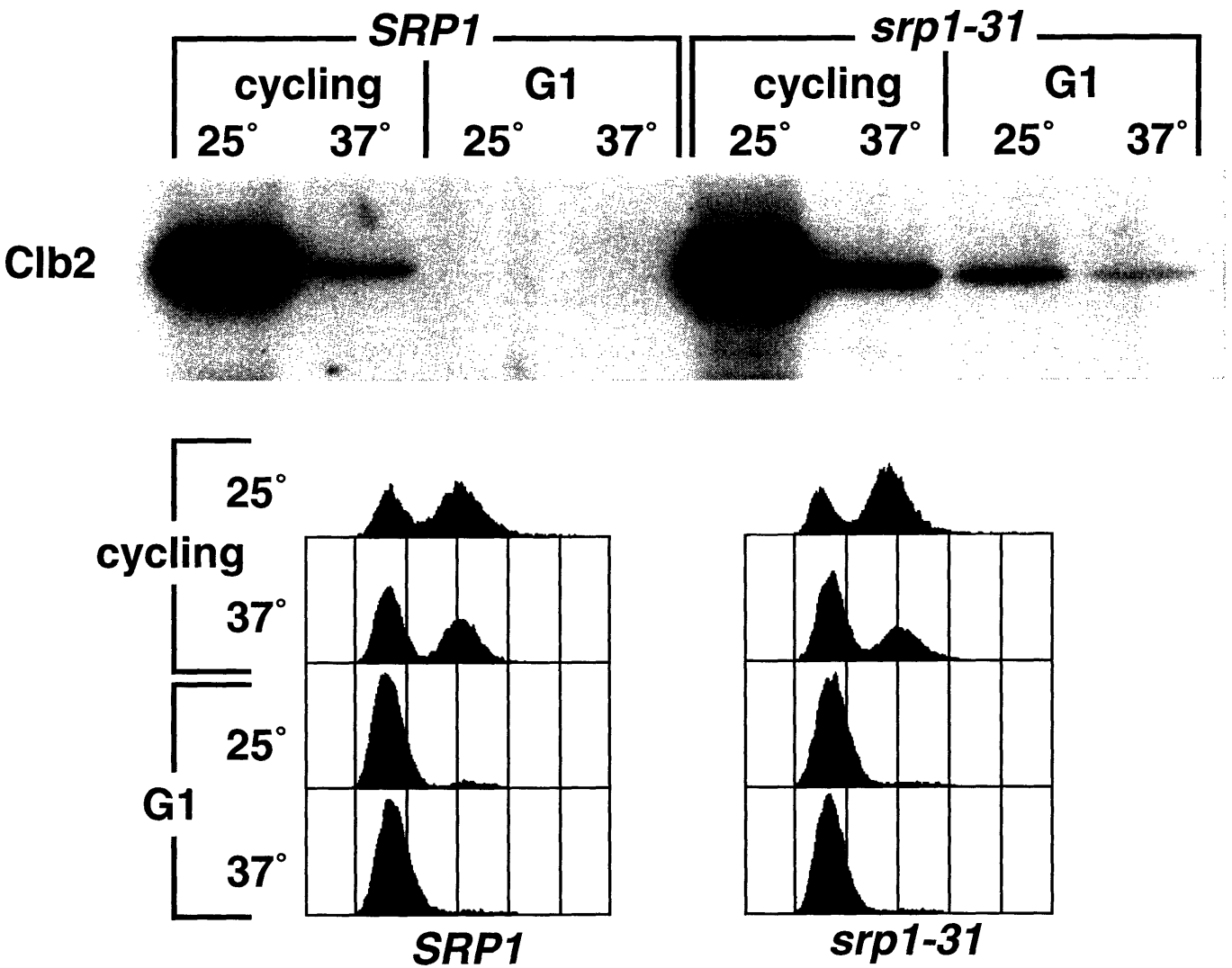
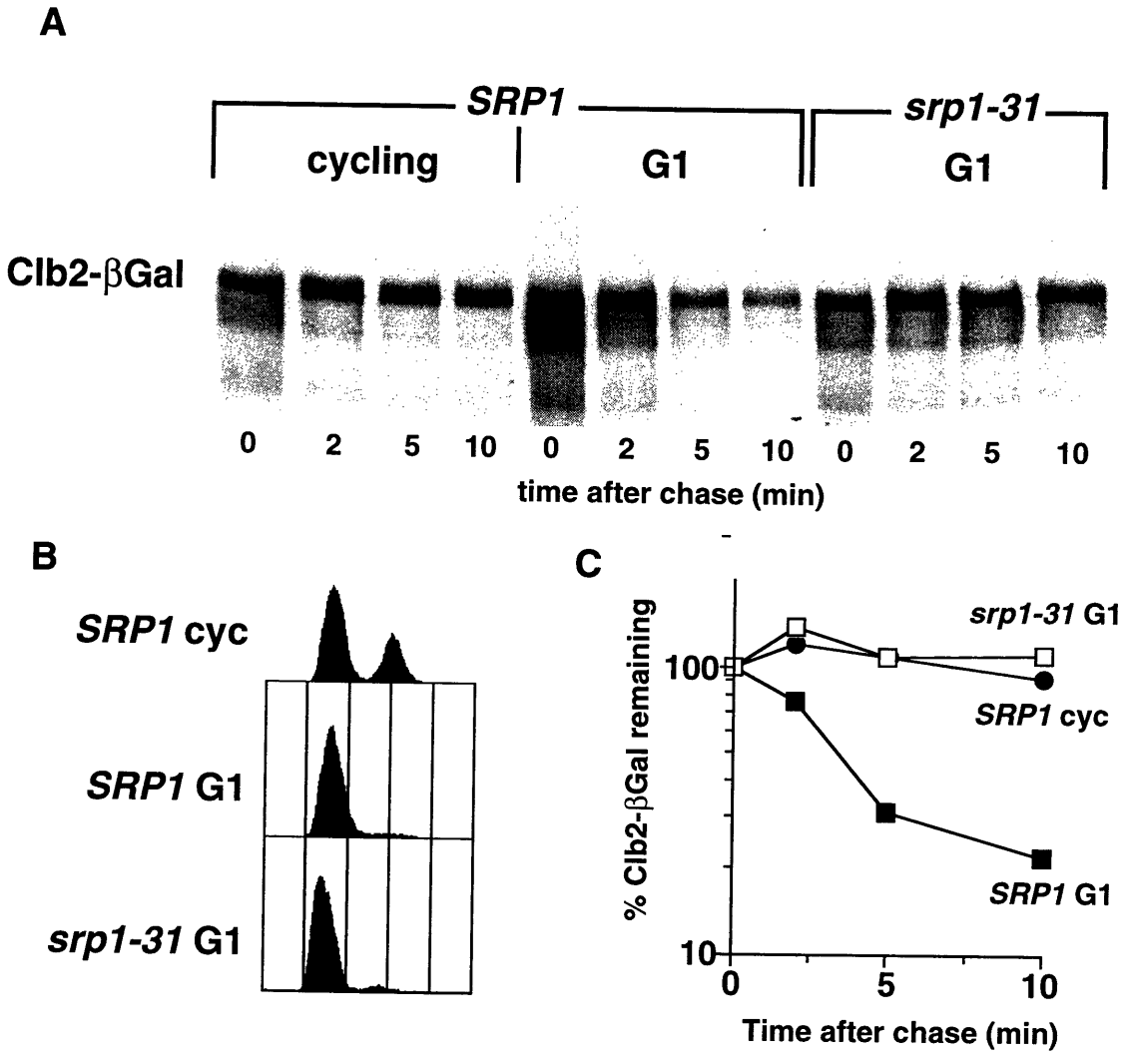

SRP1
srp1-31
cycling
G1
cycling
G1
25°
37°
25°
37°
25°
37°
25°
37°
Clb2
25°
cycling
37°
25°
G1
37°
SRP1
srp1-31

Figure 4.4 Clb2-βGal is stabilized in *srp1-31* mutants during G1.
Cycling and alpha-factor arrested *SRP1* cells and alpha-factor arrested *srp1-31* cells grown at 25°C were induced by the addition of galactose. After 40 minutes, the cultures were shifted to 37°C for 80 minutes, pulse labeled for 2 minutes with ^{35}S methionine, and chased with excess unlabeled methionine for the indicated periods. Clb2-βGal was immunoprecipitated and subjected to gel electrophoresis (Kornitzer et al., 1994). A. Autoradiograph of immunoprecipated samples. B. The FACS profile of each strain at the time of labeling. C. A comparison of the decay rates of Clb2-βGal from each culture determined by quantifying the amount of labeled immunoprecipitate in each lane of the gel in part A. with a Fuji phosphoimager device.


A
SRP1
srp1-31
cycling
G1
G1
Clb2-βGal
0 2 5 10 0 2 5 10 0 2 5 10
time after chase (min)
B
SRP1 cyc
SRP1 G1
srp1-31 G1
C
% Clb2-βGal remaining
100
10
srp1-31 G1
SRP1 cyc
SRP1 G1
0 5 10
Time after chase (min)

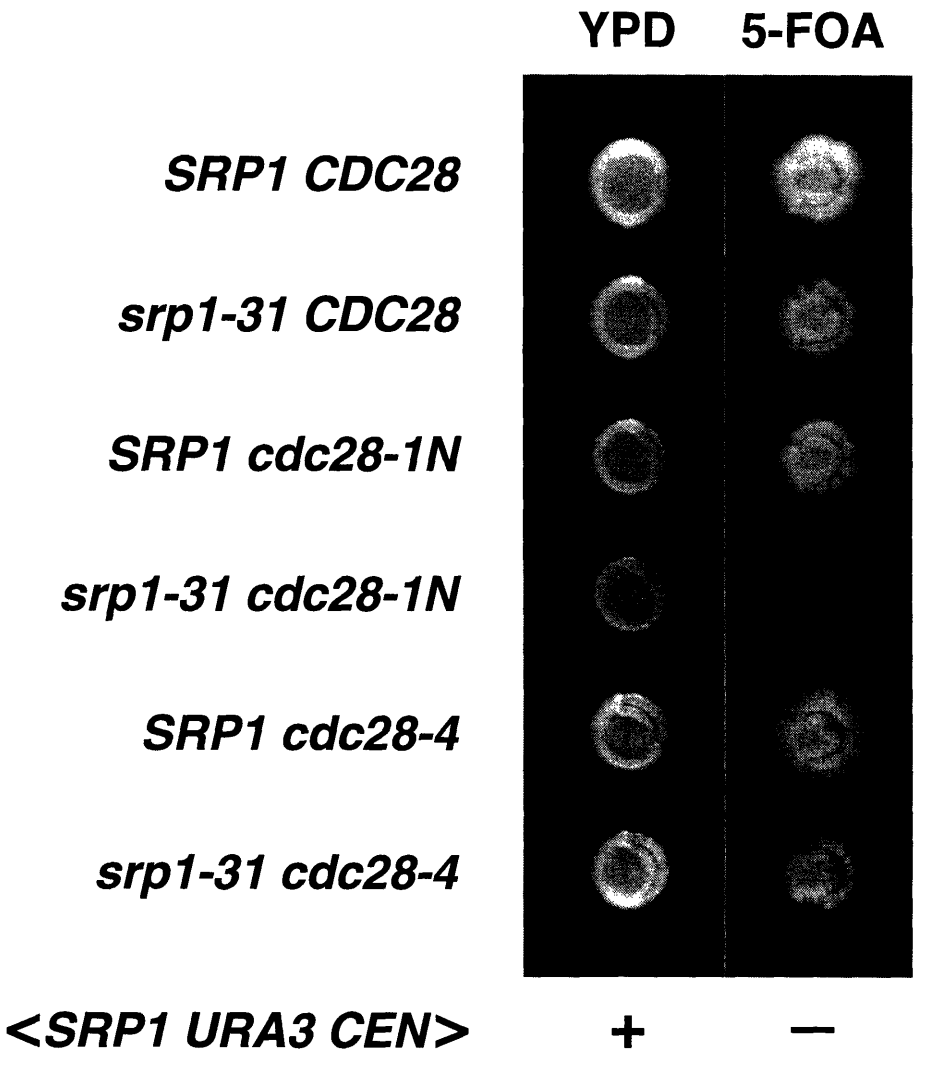
CLB2 RNA (not shown) or incomplete arrest of the *srp1-31* mutant strain (Figure 3B).

The increased levels of Clb2 are due to a defect in cyclin proteolysis: pulse chase analysis revealed a dramatic increase in the half life of a Clb2-βGal fusion in the *srp1-31* mutant strain arrested in G1 at 37°C as compared to *SRP1* (Figure 4). In the *srp1-31* mutant, the stability of Clb2-βGal in G1 is comparable to the apparent half-life of Clb2-βGal in cycling *SRP1* cells. The half-life of another unstable protein, Gcn4-βGal, is not significantly affected in the *srp1-31* mutant (data not shown).

SRP1 interacts genetically with a mitotic arrest allele of _CDC28_

Yeast mutants defective in enzymes involved in protein degradation, such as *UBC9*, *PRG1*, *CIM3* and *CIM5*, have cell cycle arrest phenotypes similar to that of *srp1-31* (Friedman and Snyder, 1994; Ghislain et al., 1993; Seufert et al., 1995). *CIM3* and *CIM5* were isolated as mutants that are lethal in combination with *cdc28-1N*, an allele of the cyclin dependent kinase that causes arrest at G2/M. These putative components of the 26S protease accumulate Clb2 and Clb3 proteins at the non-permissive temperature. Because *srp1-31* also appears to be involved in cyclin degradation, we tested for possible genetic interactions between *SRP1* and *CDC28* (Figure 5). We found that *srp1-31* is synthetically lethal with cdc28-1N, but not with cdc28-4, which arrests in G1. Moreover, *srp1-31* is not lethal in combination with other mutants that arrest in G2/M, such as *tub2-401*. These data suggest a specific interaction between srp1-31 and the G2/M kinase.

Figure 4.5 Synthetic lethality between *srp1-31* and *cdc28-1N*.
Saturated liquid cultures of strains of the indicated genotypes containing a *SRP1 URA3 CEN* plasmid (pDP168) were spotted onto medium permissive for the presence of the plasmid (YPD) or medium that selects against the presence of the plasmid (5-FOA) at 25°C. YPD and 5-FOA plates were photographed after two and three days, respectively. Identical results were obtained with reciprocal crosses with a *CDC28 CEN URA3* plasmid.


YPD
5-FOA
SRP1 CDC28
srp1-31 CDC28
SRP1 cdc28-1N
srp1-31 cdc28-1N
SRP1 cdc28-4
srp1-31 cdc28-4
<SRP1 URA3 CEN>
+
—

4.4 Discussion

The effects of the *srp1-31* mutant on protein uptake *in vitro* and *in vivo* suggest that Srp1 and importin have equivalent roles in nuclear import. This conclusion is supported by their sequence similarity and the ability of Srp1 to bind to nuclear localization sequences (Görlich et al., 1995). Furthermore, Srp1 is likely to be identical to NBP70, a previously isolated yeast NLS binding protein (Stochaj et al., 1991), because Srp1 purified from *E. coli* is recognized by an antibody raised against NBP70 (G.S., unpublished observation). The *srp1-31* mutation (Ser 116 to Phe) is in an invariant residue among all five published importin homologs. Therefore, it may be useful to introduce this mutation into importins from other species as a probe for structure-function analysis of this protein family.

The genetic and physical interactions between Srp1 and XFXFG nucleoporins (Belanger et al., 1994) suggest that the targeting step of nuclear import requires the interaction of Srp1/importin•substrate complexes with these components of the NPC. It is not known whether the interaction between substrate and receptor occurs in the cytoplasm and is followed by docking, or if the recognition step occurs within the NPC. Although we found that both the soluble and insoluble pools of Srp1 participate in import *in vitro*, most Srp1 protein in intact cells is associated with the nuclear envelope and nuclear interior ((Yano et al., 1992) and J.L., unpublished observations).

In addition to its nuclear import defect, the *srp1-31* mutant arrests in the cell cycle with a G2/M terminal phenotype. We found that the *srp1-31* mutant does not properly execute the proteolysis of the B-type cyclin Clb2 that normally occurs during mitosis and persists during G1. Clb2 stability is similarly altered by a mutation in *CSE1*, a gene required for normal mitotic segregation of chromosomes (Irniger et al., 1995; Xiao et al., 1993). Because *SRP1* has been isolated as a multi-copy suppressor of *cse1*, these proteins may function together in the import process.

We suggest that the G2/M arrest observed in the *srp1-31* mutant is due to the inability of the mutant strain to degrade and thereby inactivate key mitotic regulators, including Clb2. The *srp1-31* mutant also specifically interacts with the *cdc28-1N* mutation of the cyclin dependent kinase that is defective in the G2/M stage. Therefore, in the *srp1-31 cdc28-1N* double

mutant at 25°C, the deficient kinase and the stabilized mitotic regulators may synergistically prevent exit from mitosis. Accumulation of Clb2 is unlikely to be the sole cause of the arrest phenotype because Clb2 stabilized by the deletion of its cyclin destruction box leads to a later, anaphase arrest phenotype (Surana et al., 1993). Rather, the G2/M arrest of *srp1-31* is probably a consequence of the inability to destroy multiple proteins whose degradation is coordinately regulated with Clb2, such as other B-type cyclins and the proposed sister chromatid adhesion factor (Holloway et al., 1993) .

Because Srp1 functions as the nuclear import receptor, the mechanism of arrest in *srp1-31* cells probably involves the failure to import a nuclear protein required for mitosis. The similar arrest phenotypes and common defects in cyclin degradation make components or regulators of the mitotic degradation program appealing candidates for the nuclear import substrate(s) whose transport is limiting in *srp1-31*. It is possible that under normal conditions, regulated import of this substrate is a trigger for the execution of mitosis.

Acknowledgments

We are indebted to M. Nomura and M. Tabb for providing *srp1* mutants and antibodies prior to publication and to K. Nasmyth for suggesting we test the stability of mitotic cyclins in *srp1* mutants. We thank D. Görlich for a sample of purified Srp1 protein. Furthermore, we thank Y.-H. Tu for assistance with electron microscopy, and D. Lew for plasmids. We also thank A. Amon, B. Bartel, J. Bender and J. Celenza for critical reading of the manuscript and all the members of the Fink laboratory for helpful suggestions. G.R.F. is an American Cancer Society Professor of Genetics. This work was supported by NIH grant GM-40266 (to G.R.F.) and GM-36373 (to P.A.S.).

Chapter 5

Conclusions and prospects

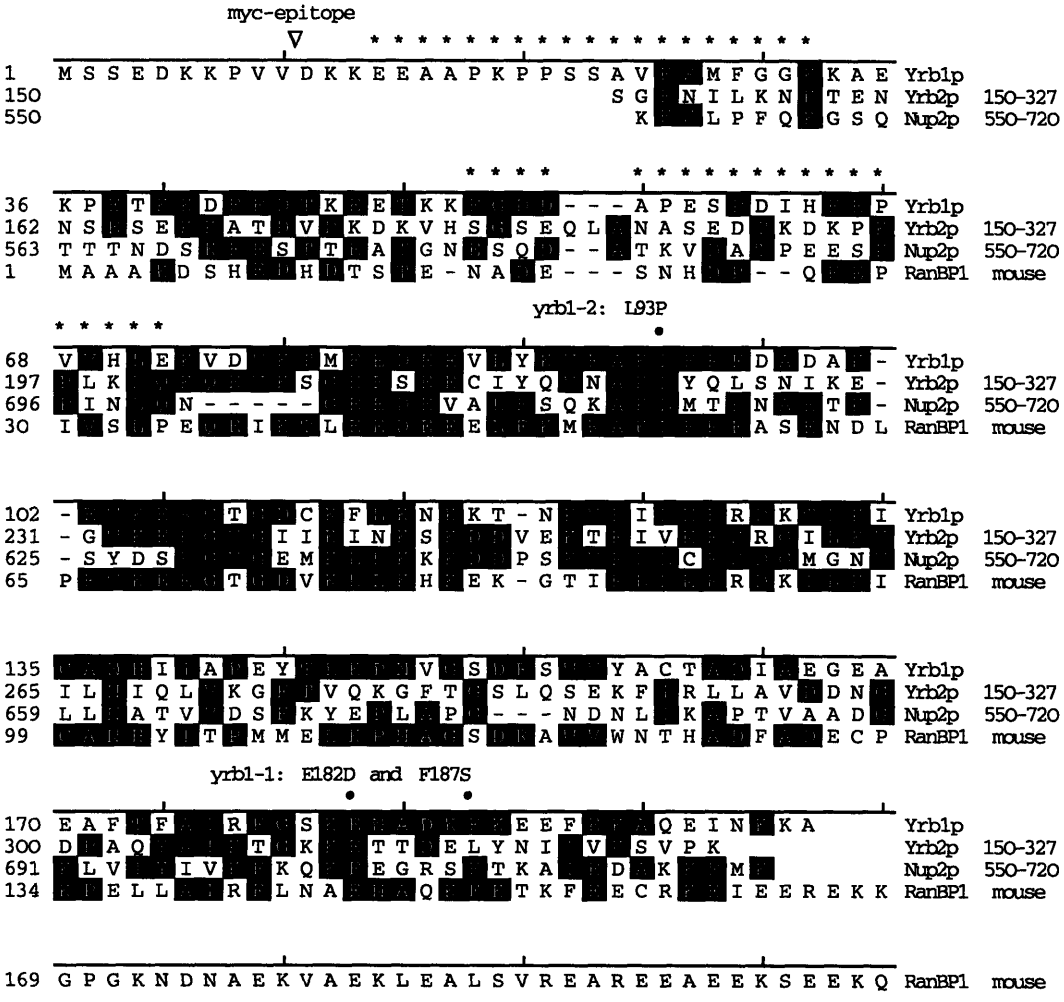
5.1 The mechanism of nuclear import

The molecular mechanism by which proteins are imported into the nucleus is beginning to emerge. Components of the system that targets proteins fated for nuclear import to the nuclear envelope have been isolated from a variety of organisms by a combination of biochemical and genetic techniques. Among the factors that have been shown to play a role in this process are integral components of the nuclear pore belonging to the XFXFG nucleoporin family. In yeast, at least two of these proteins, Nup1 and Nup2, serve directly as the docking site for the nuclear import receptor, Srp1. In mammalian cells, an equivalent interaction exists between the NLS receptor complex and several nucleoporins. A great deal of work remains to be done to understand the nature of these interactions. For example, it is not known which domains of XFXFG nucleoporins mediate the interaction with the receptor. Furthermore, the binding of receptor/substrate complexes directly to nucleoporins raises the possibility that the nucleoporins are themselves the translocation machinery. Perhaps the receptor substrate complex is passed from one nucleoporin to the next from those situated in the peripheral cytoplasmic region of the pore to nucleoporins embedded in the inner nuclear ring. In the future I suspect that workers in this field will try to address these issues in detail by reproducing the steps of the import process *in vitro* with more precision, as well as characterizing further interactions between known pore components and identifying more constituents of the NPC.

The Nup2 protein, discussed in Chapter 2, may play a unique role in the import reaction. The carboxy terminal domain of this protein is homologous to a recently identified family of proteins (RanBPs) that bind to the RanGTPase and stimulate the hydrolysis of the guanine triphosphate nucleotide (Figure 5.1). RanBPs appear to play a direct role in the import reaction because both overexpression and conditional mutants in the essential yeast RanBP Yrb1 result in aberrant nuclear transport (G. Schlenstedt and P. Silver, personal communication). Therefore, I speculate that Nup2 may also bind to the GTPase Gsp1, in some part of the import cycle. If this is true, then perhaps Nup2 also could stimulate the RanGAP, Rna1 *in vitro*. One caveat to this model is the fact that the Nup2 protein is not essential for yeast growth. Therefore, either the binding of Gsp1 to the pore complex is not

Figure 5.1 Sequence homology between the carboxy terminus of Nup2 and Ran binding proteins

Comparison of the protein sequences of the carboxy terminus (aa 550-720) to RanBP1 of mouse, Yrb1 Ran binding protein of yeast, and Yrb2, a related yeast gene. Any residue shared by two proteins is boxed.


myc-epitope
yrb1-2: L93P
yrb1-1: E182D and F187S
Yrb1p
Yrb2p 150-327
Nup2p 550-720
RanBP1 mouse

essential, which is unlikely, or there is a redundant system for this targeting function. If this is true, then either there is an unidentified nucleoporin with a RanBP domain, or perhaps Yrb1 serves as the targeting protein, perhaps via an interaction with Nup1. There is a homologus protein in yeast, Yrb2, that may participate in nuclear import. An important prediction of the redundancy model would be that mutations that inactivate Nup2 should be incompatible with mutations that diminish the function of Yrb1.

The EM data presented in Chapter 3 suggest that the majority of Srp1 is located inside the nucleus. However, it is clear that some Srp1 is in the cytoplasm (Chapter 4), and acts there to target NLS containing substrates to the NPC. Taken together, these facts predict that Srp1/importin should shuttle across the nuclear pore complex, probably in association with its transport cargo. I am currently testing this possibility *in vivo* using a Srp1 protein that has been tagged with a GFP domain. By expressing Srp1-GFP in a haploid cell, allowing fluorescent protein to accumulate in the nucleus, repressing further expression, then mating to a *kar1-1* mutant cell that is incapable of nuclear fusion, I will test whether Srp1 can leave the nucleus then re-enter. If Srp1-GFP is found in both nuclei of these heterokaryons, it would indicate that this protein can shuttle. It is also unknown whether the localization of Srp1 depends on its association with a transport substrate. From the *in vitro* nuclear import experiments described in Chapter 4, it would appear that NE bound or nuclear Srp1 is participating in the import reaction. This might mean that Srp1 might first bind to the NPC, then attract import substrate. Alternatively, the mutant Srp1 associated with the NPC at the beginning of the experiment might simply block the binding sites on nucleoporins from productive interaction with cytosolic, substrate bound Srp1.

How does Srp1/importin recognize the wide variety of NLS sequences that appear to be recognized by this pathway? Srp1 would be an interesting choice for structural studies because of the broad specificity of its binding interactions. It is known that Srp1 is a highly phosphorylated protein, and that this phosphorylation is required for the binding of NLS substrate (Stochaj and Silver, 1992). Is there a cycle of phosphorylation and dephosphorylation occurring as the receptor/substrate complex is translocated into the nucleus? It is likely that there are other NLS receptor proteins in the cell, acting as receptors responsible for other classes of import substrates, such as snRNP

complexes. If such proteins exist, it would be interesting to know how they recognize the NPC: do they interact with the nuclear pore through Nup1 and Nup2, or through other nucleoporins, such as the Nsp1 complex? Preliminary studies with degenerate polymerase chain reaction have not identified any homologs to Srp1, but other receptors do not necessarily have to have similar sequences to serve analogous roles recognizing other substrates.

The mechanism of translocation across the NPC is still quite mysterious. There may be more components of the NPC that interact with either the NLS receptor/substrate complex or the RanGTPase. Perhaps a NPC ATPase awaits detection. Understanding the mechanism of nuclear import will require the identification of these factors. I propose a variation of the genetic screen used to isolate RNA export mutants as a powerful, unbiased approach to isolating more essential factors for nuclear import. In Chapter 3 I demonstrated the feasibility of employing protein fusions to the green fluorescent protein of *Aquorea victoria* as *in vivo* reporters for nuclear import. I expressed a fusion of histone H2B to GFP and found that it was efficiently transported into the nucleus. Furthermore, this nuclear localization can be disturbed by mutants in nuclear transport [Chapter 3 and Schlenstedt et al., (1995)]. Therefore, it would be possible to screen a temperature sensitive bank of yeast mutants expressing this construct or another NLS-GFP for mutants that affect protein import. This assay is much less labor intensive than the *in situ* assay for poly(A)+ localization that has already been successfully utilized to isolate a number of components of the nuclear import pathway. Interesting variations on this idea include a screen based on the localization of Srp1-GFP, which I have recently demonstrated to be primarily nuclear. This screen might reveal mutants in receptor/substrate targeting. Another interesting reporter might be a snRNA binding protein, in order to characterize a different recognition pathway.

5.2 The role of nuclear import in the cell cycle

The arrest in the cell cycle during the G2/M phase of the *srp1-31* mutant coincides with an loss of general protein import. It appears that this cell cycle affect is not specific to this allele of *SRP1*, as depletion of Srp1 from the cell can have the same consequences. Therefore, we have concluded that there is a nuclear import substrate that is particularly sensitive to the loss of

Srp1 activity, and the mislocalization of this protein leads to the inability to exit mitosis. The critical question is the identity of this cell cycle regulator. As described in Chapter 4, the arrest phenotype of *srp1-31* mimics the phenotypes of factors that control the exit from mitosis by modulating the stability of mitotic cyclins. My experiments could not discern the difference between an upstream or downstream participant in this process. Therefore the mislocalized component may be part of the enzymatic machinery that targets cyclins and other mitotic proteins for destruction, such as Ubc9, a ubiqutin conjugating enzyme for these substrates (Seufert, et al., 1995), or a regulatory molecule involved signalling the moment in mitosis when this proteolysis should occur. Even the localization a target of the proteolysis event could be affected, in the sense that mislocalization of a cyclin might protect it from the degradation machinery. Perhaps the import of the critical substrate disturbed by *srp1-31* is regulated during the cell cycle. Other proteins in yeast, such as the S phase regulators of the Cdc46 family, are only transported into the nucleus during a narrow window of the cell cycle. Furthermore, regulated import of a key regulator into the nucleus at the time of mitosis would be a logical substitute for the vertebrate strategy involving NE breakdown at this time of the cell cycle.

How will it be possible to define the critical import substrate affected by *srp1-31*? Because Srp1 is likely to bind to hundreds of nuclear proteins, a biochemical approach is not feasible. Therefore, a genetic strategy is more appropriate. One could imagine that oversupply of the substrate might make up for inefficient transport. However, I have already performed a multi-copy suppressor screen of the temperature sensitive phenotype of *srp1-31*, and I was unable to find any suppressing genes other than *SRP1*. A more promising strategy to study this problem has been a traditional chromosomal extragenic suppressor screen. I have identified greater than ten recessive, non-allelic extragenic suppressors of *srp1-31*. Characterization of these genes may clarify the role of Srp1 in the cell cycle, and identify more players in the nucleocytoplasmic import process.

Bibliography

Adam, E. J., and Adam, S. A. (1994). Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. J Cell Biol *125*, 547-55.

Adam, S. A., and Gerace, L. (1991). Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. Cell *66*, 837-47.

Adam, S. A., Lobl, T. J., Mitchell, M. A., and Gerace, L. (1989). Identification of specific binding proteins for a nuclear location sequence. Nature *337*, 276-9.

Adam, S. A., Marr, R. S., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol *111*, 807-16.

Aebi, U., Jarnik, M., Reichelt, R., and Engel, A. (1990). Structural analysis of the nuclear pore complex by conventional and scanning transmission electraon microscopy. EMSA Bull. *20*, 69-76.

Akey, C. W. (1989). Interactions and structure of the nuclear pore complex revealed by cryo-electron microscopy. J Cell Biol *109*, 955-70.

Akey, C. W. (1990). Visualization of transport-related configurations of the nuclear pore transporter. Biophys. J. *58*, 341-355.

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

Akey, C. W., and Radermacher, M. (1993). Architecture of the Xenopus nuclear pore complex revealed by three-dimensional cryo-electron microscopy. J Cell Biol *122*, 1-19.

Aldrich, T. L., Di Segni, G., McConaughy, B. L., Keen, N. J., Whelen, S., and Hall, B. D. (1993). Structure of the yeast TAP1 protein: dependence of transcription activation on the DNA context of the target gene. Mol Cell Biol *13*, 3434-44.

Allen, J. L., and Douglas, M. (1989). Organization of the nuclear pore complex in *Saccharomyces cerevisiae*. J. Ultrastruct. Mol. Struct. Res. *102*, 95-108.

Amberg, D. C., Fleischmann, M., Stagljar, I., Cole, C. N., and Aebi, M. (1993). Nuclear PRP20 protein is required for mRNA export. Embo J *12*, 233-41.

Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992). Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev *6*, 1173-89.

Amon, A., Irniger, S., and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell *77*, 1037-50.

Amon, A., Surana, U., Muroff, I., and Nasmyth, K. (1992). Regulation of p34CDC28 tyrosine phosphorylation is not required for entry into mitosis in S. cerevisiae. Nature *355*, 368-71.

Aris, J. P., and Blobel, G. (1988). Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. J Cell Biol *107*, 17-31.

Aris, J. P., and Blobel, G. (1989). Yeast nuclear envelope proteins cross react with an antibody against mammalian pore complex proteins. J Cell Biol *108*, 2059-67.

Baeuerle, P. A., and Baltimore, D. (1988). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. Cell *53*, 211-7.

Baeuerle, P. A., and Baltimore, D. (1988). I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science *242*, 540-6.

Baserga, S. J., Gilmore-Hebert, M., and Yang, X. W. (1992). Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. Genes and Dev *6*, 1120-1130.

Bataille, N., Helser, T., and Fried, H. M. (1990). Cytoplasmic transport of ribosomal subunits microinjected into the Xenopus laevis oocyte nucleus: a generalized, facilitated process. J Cell Biol *111*, 1571-82.

Belanger, K. D., Kenna, M. A., Wei, S., and Davis, L. I. (1994). Genetic and physical interactions between Srp1p and nuclear pore complex proteins Nup1p and Nup2p. J Cell Biol *126*, 619-30.

Belhumeur, P., Lee, A., Tam, R., Di Paolo, T., Fortin, N., and Clark, M. W. (1993). GSP1 and GSP2, genetic suppressors of the prp20-1 mutant in Saccharomyces cerevisiae: GTP-binding proteins involved in the maintenance of nuclear organization. Mol Cell Biol *13*, 2152-61.

Bender, A., and Pringle, J. R. (1991). Use of a screen for synthetic lethal and multicopy suppressee mutants to identify two new genes involoved in morphogenesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *11*, 1295-1305.

Benditt, J. O., Meyer, C., Fasold, H., Barnard, F. C., and Riedel, N. (1989). Interaction of a nuclear location signal with isolated nuclear envelopes and identification of signal-binding proteins by photoaffinity labeling. Proc Natl Acad Sci U S A *86*, 9327-31.

Berlin, V., Styles, C. A., and Fink, G. R. (1990). BIK1, a protein required for microtubule function during mating a mitosis in *Saccharomyces cerevisiae*, colocalizes with tubulin. J. Cell. Biol. *111*, 2573-2586.

Bischoff, F. R., Krebber, H., Kempf, T., Hermes, I., and Ponstingl, H. (1995). Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. Proc Natl Acad Sci *92*, 1749-1753.

Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W., and Ponstingl, H. (1995). Co-activation of RanGTPase and inhibition of GTP disscociation by Ran-GTP binding protein RanBP1. EMBO J *14*, 705-715.

Bischoff, F. R., Maier, G., Tilz, G., and Ponstingl, H. (1990). A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by RCC1, a gene implicated in onset of chromosome condensation. Proc Natl Acad Sci U S A *87*, 8617-21.

Bischoff, F. R., and Ponstingl, H. (1991). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature *354*, 80-2.

Bischoff, F. R., and Ponstingl, H. (1991). Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. Proc Natl Acad Sci U S A *88*, 10830-4.

Blobel, G. (1985). Gene gating: a hypothesis. Proc Natl Acad Sci U S A *82*, 8527-9.

Boeke, J. D., Lacroute, F., and Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosophate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. *197*, 345-347.

Bogerd, A. M., Hoffman, J. A., Amberg, D. C., Fink, G. R., and Davis, L. I. (1994). nup1 mutants exhibit pleiotropic defects in nuclear pore complex function. J Cell Biol *127*, 319-32.

Bonner, W. M. (1975). 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*, 431-7.

Borer, R. A., Lehner, C. F., Eppenberger, H. M., and Nigg, E. A. (1989). Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell *56*, 379-90.

Bossie, M. A., De Horatius, C., Barcelo, G., and Silver, P. (1992). A mutant nuclear protein with similarity to RNA binding proteins interferes with nuclear import in yeast. Mol Biol Cell *3*, 875-93.

Burglin, T. R., and De Robertis, E. M. (1987). The nuclear migration signal of Xenopus laevis nucleoplasmin. Embo J *6*, 2617-25.

Buss, F., Kent, H., Stewart, M., Bailer, S. M., and Hanover, J. A. (1994). Role of different domains in the self-association of rat nucleoporin p62. J Cell Sci *107*, 631-8.

Butler, G., and Wolfe, K. H. (1994). Yeast homologue of mammalian Ran binding protein 1. Biochim Biophys Acta *1219*, 711-2.

Byers, B. (1981). Cytology of the yeast life cycle. In The molecular biology of the yeast *Saccharomyces*, life cycle and inheritance, J. N. Strathern, E. W. Jones and J. R. Broach, eds. (Cold Spring Harbor NY: Cold Spring Harbor Laboratory), pp. 59-96.

Byers, B., and Goetsch, L. (1991). Preparation of yeast cells for thin-section electron microscopy. Methods Enzymol *194*, 602-8.

Callan, H. G., Randall, J. R., and Tomlin, S.G. (1949). An electron microscope study of the nuclear membrane. Nature *163*, 280.

Carlson, M., and Botstein, D. (1982). Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell *28*, 145-154.

Carmo-Fonseca, M., Kern, H., and Hurt, E. C. (1991). Human nucleoporin p62 and the essential yeast nuclear pore protein NSP1 show sequence homology and a similar domain organization. Eur J Cell Biol *55*, 17-30.

Carter, K. C., Bowman, D., Carrington, W., Fogarty, K., McNeil, J. A., Fay, F. S., and Lawrence, J. B. (1993). A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. Science *259*, 1330-5.

Chalfie, M., Tu, Y., Euskiren, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802-805.

Clark, K. L., Ohtsubo, M., Nishimoto, T., Goebl, M., and Sprague, G., Jr. (1991). The yeast SRM1 protein and human RCC1 protein share analogous functions. Cell Regul *2*, 781-92.

Clark, K. L., and Sprague, G., Jr. (1989). Yeast pheromone response pathway: characterization of a suppressor that restores mating to receptorless mutants. Mol Cell Biol *9*, 2682-94.

Clary, D. O., Griff, I. C., and Rothman, J. E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell *61*, 709-721.

Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W. (1993). Chemical structure of the hexapeptide chromophore of the Aequorea green-fluorescent protein. Biochemistry *32*, 1212-8.

Copeland, C. S., and Snyder, M. (1993). Nuclear pore complex antigens delineate nuclear envelope dynamics in vegetative and conjugating Saccharomyces cerevisiae. Yeast *9*, 235-49.

Cordes, V., Waizenegger, I., and Krohne, G. (1991). Nuclear pore complex glycoprotein p62 of Xenopus laevis and mouse: cDNA cloning and identification of its glycosylated region. Eur J Cell Biol *55*, 31-47.

Cordes, V. C., and Krohne, G. (1993). Sequential O-glycosylation of nuclear pore complex protein gp62 in vitro. Eur J Cell Biol *60*, 185-95.

Cordes, V. C., Reidenbach, S., Kohler, A., Stuurman, N., van Driel, R., and Franke, W. W. (1993). Intranuclear filaments containing a nuclear pore complex protein. J Cell Biol *123*, 1333-44.

Cortes, P., Ye, Z. S., and Baltimore, D. (1994). RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. Proc Natl Acad Sci U S A *91*, 7633-7.

Coutavas, E., Ren, M., Oppenheim, J. D., D'Eustachio, P., and Rush, M. G. (1993). Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. Nature *366*, 585-7.

Cuomo, C. A., Kirch, S. A., Gyuris, J., Brent, R., and Oettinger, M. A. (1994). Rch1, a protein that specifically interacts with the RAG-1 recombination-activating protein. Proc Natl Acad Sci U S A *91*, 6156-60.

D'Onofrio, M., Starr, C. M., Park, M. K., Holt, G. D., Haltiwanger, R. S., Hart, G. W., and Hanover, J. A. (1988). Partial cDNA sequence encoding a nuclear pore protein modified by O-linked N-acetylglucosamine. Proc Natl Acad Sci U S A *85*, 9595-9.

Dabauvalle, M.-C., Schulz, B., Scheer, U., and Peters, R. (1988). Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. Exp. Cell Res. *174*, 291-296.

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

Dasso, M., Nishitani, H., Kornbluth, S., Nishimoto, T., and Newport, J. W. (1992). RCC1, a regulator of mitosis, is essential for DNA replication. Mol Cell Biol *12*, 3337-45.

Dasso, M., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1994). A mutant form of the Ran/TC4 protein disrupts nuclear function in Xenopus laevis egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation. Embo J *13*, 5732-44.

Davis, L. I. (1992). Control of nucleocytoplasmic transport. Curr. Opin. Cell Biol. *4*, 424-429.

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

Davis, L. I., and Blobel, G. (1987). Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. Proc Natl Acad Sci U S A *84*, 7552-6.

Davis, L. I., and Fink, G. R. (1990). The *NUP1* gene encodes an essential component of the yeast nuclear pore complex. Cell *61*, 965-978.

De Gregori, J., Russ, A., von Melchner, H., Rayburn, H., Priyaranjan, P., Jenkins, N. A., Copeland, N. G., and Ruley, H. E. (1994). A murine homolog of the yeast RNA1 gene is required for postimplantation development. Genes Dev *8*, 265-76.

De Robertis, E. M., Lienhard, S., and Parisot, R. F. (1982). Intracellular transport of microinjected 5S and small nuclear RNAs. Nature *295*, 572-576.

De Robertis, E. M., Longthorne, R. F., and Gurdon, J. B. (1978). Intracellular migration of nuclear proteins in Xenopus oocytes. Nature *272*, 254-6.

Demeter, J., Morphew, M., and Sazer, S. (1995). A mutation in the RCC1-related protein pim1 results in nuclear envelope fragmentation in fission yeast. Proc Natl Acad Sci USA *92*, 1436-1440.

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

Di Segni, G., McConaughy, B. L., Shapiro, R. A., Aldrich, T. L., and Hall, B. D. (1993). TAP1, a yeast gene that activates the expression of a tRNA gene with a defective internal promoter. Mol Cell Biol *13*, 3424-33.

Dingwall, C., and Laskey, R. (1992). The nuclear membrane. Science *258*, 942-7.

Dingwall, C., Robbins, J., Dilworth, S. M., Roberts, B., and Richardson, W. D. (1988). The nucleoplasmin nuclear location sequence is larger and more complex than that of SV-40 large T antigen. J Cell Biol *107*, 841-9.

Dingwall, C., Sharnick, S. V., and Laskey, R. A. (1982). A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell *30*, 449-58.

Doye, V., Wepf, R., and Hurt, E. C. (1994). A novel nuclear pore prtein Nup133p with distinct roles in poly(A)$^+$ RNA transport and nuclear pore distribution. EMBO J *13*, 6062-6075.

Dujon, B. (1993). Mapping and sequencing the nuclear genome of the yeast Saccharomyces cerevisiae: strategies and results of the European enterprise. Cold Spring Harb Symp Quant Biol *58*, 357-66.

Duverger, E., Carpentier, V., Roche, A. C., and Monsigny, M. (1993). Sugar-dependent nuclear import of glycoconjugates from the cytosol. Exp Cell Res *207*, 197-201.

Dworetzky, S. I., and Feldherr, C. M. (1988). Translocation of RNA-coated gold particles through the nuclear pores of oocytes. J Cell Biol *106*, 575-84.

Dworetzky, S. I., Lanford, R. E., and Feldherr, C. M. (1988). The effects of variations in the number and sequence of targeting signals on nuclear uptake. J Cell Biol *107*, 1279-87.

Eisenberg, D., Weiss, R. M., and Terwilliger, T. C. (1984). The hydrophobic moment detects periodicity in protein hydrophobicity. Proc. Natl. Acad. Sci. *81*, 140-144.

Elliott, D. J., Stutz, F., Lescure, A., and Rosbash, M. (1994). mRNA nuclear export. Curr Opin Genet Dev *4*, 305-9.

Enoch, T., Peter, M., Nurse, P., and Nigg, E. A. (1991). p34cdc2 acts as a lamin kinase in fission yeast. Journal of Cell Biology *112*, 797-807.

Epstein, C. B., and Cross, F. R. (1992). CLB5: a novel B cyclin from budding yeast with a role in S phase. Genes Dev *6*, 1695-706.

Fabre, E., Boelens, W. C., Wimmer, C., Mattaj, I. W., and Hurt, E. C. (1994). Nup145p is required for nuclear export of mRNA and binds homopolymeric RNA in vitro via a novel conserved motif. Cell *78*, 275-89.

Featherstone, C., Darby, M. K., and Gerace, L. (1988). A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. J Cell Biol *107*, 1289-97.

Feldherr, C. M., and Akin, D. (1990). EM visualization of nucleocytoplasmic transport processes. Electron Microsc Rev *3*, 73-86.

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

Feldherr, C. M., Kallenbach, E., and Schultz, N. (1984). Movement of a karyophilic protein through the nuclear pores of oocytes. J Cell Biol *99*, 2216-22.

Finlay, D. R., and Forbes, D. J. (1990). Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. Cell *60*, 17-29.

Finlay, D. R., Meier, E., Bradley, P., Horecka, J., and Forbes, D. J. (1991). A complex of nuclear pore proteins required for pore function. J Cell Biol *114*, 169-83.

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

Fischer, U., Darzynkiewicz, E., Tahara, S. M., Dathan, N. A., Luhrmann, R., and Mattaj, I. W. (1991). Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. J Cell Biol *113*, 705-14.

Flach, J., Bossie, M., Vogel, J., Corbett, A., Jinks, T., Willins, D. A., and Silver, P. A. (1994). A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. Mol Cell Biol *14*, 8399-407.

Fleischmann, M., Clark, M. W., Forrester, W., Wickens, M., Nishimoto, T., and Aebi, M. (1991). Analysis of yeast prp20 mutations and functional complementation by the human homologue RCC1, a protein involved in the control of chromosome condensation. Mol Gen Genet *227*, 417-23.

Forbes, D. J., Kirschner, M. W., and Newport, J. W. (1983). Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. Cell *34*, 13-23.

Forrester, W., Stutz, F., Rosbash, M., and Wickens, M. (1992). Defects in mRNA 3'-end formation, transcription initiation, and mRNA transport associated with the yeast mutation prp20: possible coupling of mRNA processing and chromatin structure. Genes Dev *6*, 1914-26.

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

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

Friedman, H., and Snyder, M. (1994). Mutations in PRG1, a yeast proteasome-related gene, cause defects in nuclear division and are suppressed by deletion of a mitotic cyclin gene. Proc Natl Acad Sci U S A *91*, 2031-5.

Fuchs, R. (1991). MacPattern: Protein pattern searching on the Apple Macintosh. Comput. Applic. Biosci. *7*, 105-106.

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

Gerace, L., Blum, A., and Blobel, G. (1978). Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. Interphase and mitotic distribution. J Cell Biol *79*, 546-66.

Gerace, L., Ottaviano, Y., and Kondor, K. C. (1982). Identification of a major polypeptide of the nuclear pore complex. J Cell Biol *95*, 826-37.

Gerace, L., Ottaviano, Y., and Kondor-Koch, C. (1982). Identification of a major polypeptide of the nuclear pore complex. J Cell Biol *95*, 826-37.

Gerring, S. L., Spencer, F., and Hieter, P. (1990). The CHL 1 (CTF 1) gene product of Saccharomyces cerevisiae is important for chromosome transmission and normal cell cycle progression in G2/M. Embo J *9*, 4347-58.

Ghislain, M., Udvardy, A., and Mann, C. (1993). S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature *366*, 358-62.

Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature *344*, 678-682.

Goldberg, M. W., and Allen, T. D. (1992). High resolution scanning electron microscopy of the nuclear envelope: demonstration of a new, regular, fibrous lattice attached to the baskets of the nucleoplasmic face of the nuclear pores. J Cell Biol *119*, 1429-40.

Goldberg, M. W., and Allen, T. D. (1993). The nuclear pore complex: three-dimensional surface structure revealed by field emission, in-lens scanning electron microscopy, with underlying structure uncovered by proteolysis. J Cell Sci *106*, 261-74.

Goldfarb, D. S. (1992). Are the cytosolic components of the nuclear, ER, and mitochondrial import apparatus functionally related? Cell *70*, 185-8.

Goldfarb, D. S. (1991). Shuttling proteins go both ways. Curr Biol *1*, 212-214.

Goldfarb, D. S., Gariepy, J., Schoolnik, G., and Kornberg, R. D. (1986). Synthetic peptides as nuclear localization signals. Nature *322*, 641-4.

Görlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R. A., Hartmann, E., and Prehn, S. (1995). Two different subunits of importin cooperate to recognized nuclear localization signals and bind them to the nuclear envelope. Curr Biol *5*, 383-392.

Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. Cell *79*, 767-778.

Grandi, P., Doye, V., and Hurt, E. C. (1993). Purification of NSP1 reveals complex formation with 'GLFG' nucleoporins and a novel nuclear pore protein NIC96. Embo J *12*, 3061-71.

Grandi, P., Schlaich, N., Tekotte, H., and Hurt, E. C. (1995). Functional interaction of Nic96p with a core nucleoporin complex consisting of Nsp1p, Nup49p and a novel protein Nup57p. EMBO J *14*, 76-87.

Greber, U. F., and Gerace, L. (1992). Nuclear protein import is inhibited by an antibody to a lumenal epitope of a nuclear pore complex glycoprotein. J Cell Biol *116*, 15-30.

Greber, U. F., Senior, A., and Gerace, L. (1990). A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large lumenal domain and a small cytoplasmic tail. Embo J *9*, 1495-502.

Gu, Z., Moerschell, R. P., Sherman, F., and Goldfarb, D. S. (1992). NIP1, a gene required for nuclear transport in yeast. Proc Natl Acad Sci U S A *89*, 10355-9.

Gumbiner, B. M., and McCrea, P. D. (1993). Catenins as mediators of the cytoplasmic functions of cadherins. J Cell Sci Suppl *17*, 155-8.

Guthrie, C., and Fink, G. R. (1991). Guide to yeast genetics and molecular biology. In Methods in Enzymology, J. N. Abelson and M. I. Simon, eds. (San Diego: Academic Press, Inc.).

Hall, M. N., Hereford, L., and Herskowitz, I. (1984). Targeting of E. coli beta-galactosidase to the nucleus in yeast. Cell *36*, 1057-65.

Hallberg, E., Wozniak, R. W., and Blobel, G. (1993). An integral membrane protein of the pore membrane domain of the nuclear envelope contains a nucleoporin-like region. J Cell Biol *122*, 513-21.

Hamm, J., and Mattaj, I. W. (1990). Monomethylated cap structures facilitate RNA export from the nucleus. Cell *63*, 109-18.

Harel, A., Zlotkin, E., Nainudel-Epszteyn, S., Feinstein, N., Fisher, P. A., and Gruenbaum, Y. (1989). Persistence of major nuclear envelope antigens in an envelope-like structure during mitosis in Drosophila melanogaster embryos. J Cell Sci *94*, 463-70.

Hauber, J., Stucka, J., Krieg, R., and Feldman, H. (1988). Analysis of yeast chromosomal regions carrying members of the glutamate tRNA gene family: various transposable elements are associated with them. Nucl. Acids Res. *16:*, 10623-10634.

Heath, I. B. (1980). Variant mitoses in lower eukaryotes: Indicators of the evolution of mitosis. Int Rev Cytol *64*, 1-80.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene *28*, 351-359.

Hennessy, K. M., Clark, C. D., and Botstein, D. (1990). Subcellular localization of yeast CDC46 varies with the cell cycle. Genes Dev *4*, 2252-63.

Hennessy, K. M., Lee, A., Chen, E., and Botstein, D. (1991). A group of interacting yeast DNA replication genes. Genes Dev *5*, 958-69.

Hinshaw, J. E., Carragher, B. O., and Milligan, R. A. (1992). Architecture and design of the nuclear pore complex. Cell *69*, 1133-41.

Holloway, S. L., Glotzer, M., King, R. W., and Murray, A. W. (1993). Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. Cell *73*, 1393-402.

Holm, C., Goto, T., Wang, J. C., and Botstein, D. (1985). DNA topoisomerase II is required at the time of mitosis in yeast. Cell *41*, 553-63.

Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., and Hart, G. W. (1987). Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. J Cell Biol *104*, 1157-64.

Hopper, A. K., Traglia, H. M., and Dunst, R. W. (1990). The yeast *RNA1* gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. J. Cell Biol. *111*, 309-321.

Huffaker, T. C., Hoyt, M. A., and Botstein, D. (1987). Genetic analysis of the yeast cytoskeleton. Ann. Rev. Genet. *21*, 259-284.

Hurt, E. C. (1988). A novel nucleoskeletal-like protein located at the nuclear periphery is required for the life cycle of *Saccharomyces cerevisiae*. EMBO J. *7*, 4323-4334.

Hurt, E. C. (1990). Targeting of a cytosolic protein to the nuclear periphery. J. Cell Biol. *111*, 2829-2837.

Hutchinson, H. T., Hartwell, L. H., and McLaughlin, C. S. (1969). Temperature sensitive yeast mutant defective in ribonucleic acid production. J. Bacteriol. *99*, 807-814.

Imamoto, N., Matsuoka, Y., Kurihara, T., Kohno, K., Miyagi, M., Sakiyama, F., Okada, Y., Tsunasawa, S., and Yoneda, Y. (1992). Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. J Cell Biol *119*, 1047-61.

Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell *in press*.

Ito, H., Fukada, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells with alkalai cation. J. Bacteriol. *153*, 163-168.

Izaurralde, E., and Mattaj, I. W. (1992). Transport of RNA between nucleus and cytoplasm. Semin Cell Biol *3*, 279-88.

Izaurralde, E., Stepinski, J., Darzynkiewicz, E., and Mattaj, I. W. (1992). A cap binding protein that may mediate nuclear export of RNA polymerase II-transcribed RNAs. J Cell Biol *118*, 1287-95.

Jarmolowski, A., Boelens, W. C., Izaurralde, E., and Mattaj, I. W. (1994). Nuclear export of different classes of RNA is mediated by specific factors. J Cell Biol *124*, 627-35.

Jarnik, M., and Aebi, U. (1991). Toward a more complete 3-D structure of the nuclear pore complex. J Struct Biol *107*, 291-308.

Jongens, T. A., Ackerman, L. D., Swedlow, J. R., Jan, L.Y., and Jan, Y.N. (1994). *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. Genes and Development *8*, 2123-2136.

Jongens, T. A., Hay, B., Jan, L. Y., and Jan, Y. N. (1992). The germ cell-less gene product: a posteriorly localized component necessary for germ cell development in Drosophila. Cell *70*, 569-84.

Kadowaki, T., Chen, S., Hitomi, M., Jacobs, E., Kumagai, C., Liang, S., Schneiter, R., Singleton, D., Wisniewska, J., and Tartakoff, A. M. (1994). Isolation and characterization of Saccharomyces cerevisiae mRNA transport-defective (mtr) mutants. J Cell Biol *126*, 649-59.

Kadowaki, T., Goldfarb, D., Spitz, L. M., Tartakoff, A. M., and Ohno, M. (1993). Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. Embo J *12*, 2929-37.

Kadowaki, T., Zhao, Y., and Tartakoff, A. M. (1992). A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm. Proc Natl Acad Sci U S A *89*, 2312-6.

Kaiser, C. A., and Schekman, R. (1990). Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. Cell *61*, 723-733.

Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984). Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature *311*, 33-8.

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

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet *9*, 317-21.

King, R. W., Jackson, P. K., and Kirschner, M. W. (1994). Mitosis in transition. Cell *79*, 563-571.

Kita, K., Omata, S., and Horigome, T. (1993). Purification and characterization of a nuclear pore glycoprotein complex containing p62. J Biochem *113*, 377-82.

Klein, F., Laroche, T., Cardenas, M. E., Hofmann, J. F., Schweizer, D., and Gasser, S. M. (1992). Localization of RAP1 and topoisomerase II in nuclei and meiotic chromosomes of yeast. J Cell Biol *117*, 935-48.

Kolodziej, P. A., and Young, R. A. (1991). Epitope tagging and protein surveillance. Methods Enzymol *194*, 508-19.

Kornbluth, S., Dasso, M., and Newport, J. (1994). Evidence for a dual role for TC4 protein in regulating nuclear structure and cell cycle progression. J. Cell Biol. *125*, 705-719.

Kornitzer, D., Raboy, B., Kulka, R. G., and Fink, G. R. (1994). Regulated degradation of the transcription factor Gcn4. EMBO J. *13*, 6021-6030.

Kraemer, D., Wozniak, R. W., Blobel, G., and Radu, A. (1994). The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. Proc Natl Acad Sci U S A *91*, 1519-23.

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site specific mutagenesis without phenotypic selection. In Methods in Enzymology, R. Wu and G. L., eds. (Orlando Florida: Academic Press), pp. 367-382.

Lanford, R. E., and Butel, J. S. (1984). Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell *37*, 801-13.

Laskey, R. A., and Dingwall, C. (1993). Nuclear shuttling: the default pathway for nuclear proteins? Cell *74*, 385-6.

Lawrence, J. B., Singer, R. H., and Marselle, L. M. (1989). Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. Cell *57*, 493-502.

Lee, A., Tam, R., Belhumeur, P., Di Paolo, T., and Clark, M. W. (1993). Prp20, the Saccharomyces cerevisiae homolog of the regulator of chromosome condensation, RCC1, interacts with double-stranded DNA through a multi-component complex containing GTP-binding proteins. J Cell Sci *106*, 287-98.

Lee, W.-C., Xue, Z., and Melese, T. (1991). The *NSR1* gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. J. Cell Biol. *113*, 1-12.

Lee, W. C., and Melese, T. (1989). Identification and characterization of a nuclear localization sequence-binding protein in yeast. Proc Natl Acad Sci U S A *86*, 8808-12.

Lee, W. C., Zabetakis, D., and Melese, T. (1992). NSR1 is required for pre-rRNA processing and for the proper maintenance of steady-state levels of ribosomal subunits. Mol Cell Biol *12*, 3865-71.

Li, R., Shi, Y., and Thomas, J. O. (1992). Intracellular distribution of a nuclear localization signal binding protein. Exp Cell Res *202*, 355-65.

Liu, Z., and Gilbert, W. (1994). The yeast KEM1 gene encodes a nuclease specific for G4 tetraplex DNA: implication of in vivo functions for this novel DNA structure. Cell *77*, 1083-92.

Loeb, J. D., Davis, L. I., and Fink, G. R. (1993). NUP2, a novel yeast nucleoporin, has functional overlap with other proteins of the nuclear pore complex. Mol Biol Cell *4*, 209-22.

Macaulay, C., Meier, E., and Forbes, D. J. (1995). Differential mitotic phosphorylation of proteins of the nuclear pore complex. J Biol Chem *270*, 254-262.

Mandell, R. B., and Feldherr, C. M. (1990). Identification of two HSP70-related Xenopus oocyte proteins that are capable of recycling across the nuclear envelope. J Cell Biol *111*, 1775-83.

Matsumoto, T., and Beach, D. (1991). Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. Cell *66*, 347-60.

Maul, G. G. (1977). Nuclear pore complexes. Elimination and reconstruction during mitosis. J Cell Biol *74*, 492-500.

Maul, G. G., Deaven, L. L., Freed, J. J., Campbell, G. L., and Becak, W. (1980). Investigation of the determinants of nuclear pore number. Cytogenet Cell Genet *26*, 175-90.

McMorrow, I., Bastos, R., Horton, H., and Burke, B. (1994). Sequence analysis of a cDNA encoding a human nuclear pore complex protein, hnup153. Biochim Biophys Acta *1217*, 219-23.

Mehlin, H., Daneholt, B., and Skoglund, U. (1992). Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. Cell *69*, 605-13.

Meier, U. T., and Blobel, G. (1992). Nopp140 shuttles on tracks between nucleolus and cytoplasm. Cell *70*, 127-38.

Meier, U. T., and Blobel, G. (1990). A nuclear localization signal binding protein in the nucleolus. J Cell Biol *111*, 2235-45.

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J Cell Biol *123*, 1649-59.

Michaud, N., and Goldfarb, D. (1992). Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. J Cell Biol *116*, 851-61.

Michaud, N., and Goldfarb, D. S. (1993). Most nuclear proteins are imported by a single pathway. Exp Cell Res *208*, 128-36.

Michaud, N., and Goldfarb, D. S. (1991). Multiple pathways in nuclear transport: the import of U2 snRNP occurs by a novel kinetic pathway. J. Cell Biol. *112*, 215-223.

Miller, M. W., and Hanover, J. A. (1994). Functional nuclear pores reconstituted with beta 1-4 galactose-modified O-linked N-acetylglucosamine glycoproteins. J Biol Chem *269*, 9289-97.

Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5. Cell *66*, 743-58.

Moore, M. S., and Blobel, G. (1994). A G protein involved in nucleocytoplasmic transport: the role of Ran. Trends Biochem Sci *19*, 211-6.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-3.

Moore, M. S., and Blobel, G. (1994). Purification of a Ran-interacting protein that is required for protein import into the nucleus. Proc Natl Acad Sci U S A *91*, 10212-6.

Moore, M. S., and Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. Cell *69*, 939-50.

Moreland, R. B., Langevin, G. L., Singer, R. H., Garcea, R. L., and Hereford, L. M. (1987). Amino acid sequences that determine the nuclear localization of yeast histone 2B. Mol Cell Biol *7*, 4048-57.

Moroianu, J., Blobel, G., and Radu, A. (1995). Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. Proc Natl Acad Sci USA *92*, 2008-2011.

Mutvei, A., Dihlmann, S., Herth, W., and Hurt, E. C. (1992). NSP1 depletion in yeast affects nuclear pore formation and nuclear accumulation. Eur J Cell Biol *59*, 280-95.

Nasmyth, K., Adolf, G., Lydall, D., and Seddon, A. (1990). The identification of a second cell cycle control on the HO promoter in yeast: cell cycle regulation of SW15 nuclear entry. Cell *62*, 631-47.

Nehrbass, U., Fabre, E., Dihlmann, S., Herth, W., and Hurt, E. C. (1993). Analysis of nucleo-cytoplasmic transport in a thermosensitive mutant of nuclear pore protein NSP1. Eur J Cell Biol *62*, 1-12.

Nehrbass, U., Kern, H., Mutvei, A., Horstmann, H., Marshallsay, B., and Hurt, E. (1990). NSP1: a yeast nuclear envelope protein localized at the nuclear pores exerts its essential function by its carboxy terminal domain. Cell *61*, 979-989.

Nelson, M., and Silver, P. (1989). Context affects nuclear protein localization in Saccharomyces cerevisiae. Mol Cell Biol *9*, 384-9.

Newmeyer, D. D. (1993). The nuclear pore complex and nucleocytoplasmic transport. Curr Opin Cell Biol *5*, 395-407.

Newmeyer, D. D., and Forbes, D. J. (1988). Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. Cell *52*, 641-53.

Newmeyer, D. D., Lucocq, J. M., Burglin, T. R., and De Robertis, E. M. (1986). Assembly in vitro of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. Embo J *5*, 501-10.

Newport, J. W., and Forbes, D. J. (1987). The nucleus: structure, function and dynamics. Ann. Rev. Biochem. *56*, 535-565.

Nieuwint, R. T. M., Molenaar, C. M. T., J.H., v. B., van Raamsdonk-Duin, M. M. C., Mager, W. H., and Planta, R. J. (1985). The gene for yeast ribosomal protein S31 contains an intron in the leader sequence. Curr. Genet. *10*, 1-5.

Nigg, E. A. (1992). Assembly and cell cycle dynamics of the nuclear lamina. Semin Cell Biol *3*, 245-53.

Nigg, E. A., Baeuerle, P. A., and Luhrmann, R. (1991). Nuclear import-export: in search of signals and mechanisms. Cell *66*, 15-22.

Nigg, E. A., Hilz, H., Eppenberger, H. M., and Dutly, F. (1985). Rapid and reversible translocation of the catalytic subunit of cAMP-dependent protein kinase type II from the Golgi complex to the nucleus. Embo J *4*, 2801-6.

Nishimoto, T., Eilen, E., and Basilico, C. (1978). Premature chromosome condensation in a ts DNA- mutant of BHK cells. Cell *15*, 475-483.

Nishitani, H., Ohtsubo, M., Yamashita, K., Iida, H., Pines, J., Yasudo, H., Shibata, Y., Hunter, T., and Nishimoto, T. (1991). Loss of RCC1, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. Embo J *10*, 1555-64.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 205-215.

O'Neill, R. E., and Palese, P. (1995). NPI-1, the human homolog of *SRP1*, interacts with Influenza virus nucleoprotein. Virology *206*, 116-125.

Ohashi, A., Gibson, J., Gregor, I., and Schatz, G. (1982). Import of proteins into mitochondria. J. Biol. Chem. *257*, 13042-13047.

Ohtsubo, M., Okazaki, H., and Nishimoto, T. (1989). The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. J Cell Biol *109*, 1389-97.

Ohtsubo, M., Yoshida, T., Seino, H., Nishitani, H., Clark, K. L., Sprague, G., Jr., Frasch, M., and Nishimoto, T. (1991). Mutation of the hamster cell cycle gene RCC1 is complemented by the homologous genes of Drosophila and S.cerevisiae. Embo J *10*, 1265-73.

Paine, P. L., Moore, L. C., and Horowitz, S. B. (1975). Nuclear envelope permeability. Nature *254*, 109-14.

Pandey, S., Karande, A. A., Mishra, K., and Parnaik, V. K. (1994). Inhibition of nuclear protein import by a monoclonal antibody against a novel class of nuclear pore proteins. Exp Cell Res *212*, 243-54.

Pandey, S., and Parnaik, V. K. (1991). Identification and characterization of nuclear location signal-binding proteins in nuclear envelopes. Biochim Biophys Acta *1063*, 81-9.

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

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

Pante, N., Bastos, R., McMorrow, I., Burke, B., and Aebi, U. (1994). Interactions and three-dimensional localization of a group of nuclear pore complex proteins. J Cell Biol *126*, 603-17.

Park, M. K., D'Onofrio, M., Willingham, M. C., and Hanover, J. A. (1987). A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked N-acetylglucosamine is part of the immunodeterminant. Proc Natl Acad Sci U S A *84*, 6462-6.

Peifer, M., Berg, S., and Reynolds, A. B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. Cell *76*, 789-791.

Pellman, D., Bagget, M., Tu, Y.-H., and Fink, G. R. (1995). Yeast microtubule associated proteins required for anaphase spindle elongation. J. Cell Biol. *in press*.

Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo J *6*, 3333-40.

Pinol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature *355*, 730-2.

Powers, M. A., Macaulay, C., Masiarz, F. R., and Forbes, D. J. (1995). Reconstituted nuclei depleted of a vertebrate GLFG nuclear pore protein, p97, import but are defective in nuclear growth and replication. J Cell Biol *128*, 721-736.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992). Primary structure of the Aequorea victoria green-fluorescent protein. Gene *111*, 229-33.

Privalsky, M. L., Sharif, M., and Yamamoto, K. R. (1990). The viral erbA oncogene protein, a constitutive repressor in animal cells, is a hormone-regulated activator in yeast. Cell *63*, 1277-86.

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

Reichelt, R., Holzenburg, A., Buhle, E. L., Jarnik, M., Engel, A., and Aebi, U. (1990). Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. J. Cell Biol. *110*, 883-894.

Ren, M., Coutavas, E., D'Eustachio, P., and Rush, M. G. (1994). Effects of mutant Ran/TC4 proteins on cell cycle progression. Mol Cell Biol *14*, 4216-24.

Ren, M., Drivas, G., D'Eustachio, P., and Rush, M. G. (1993). Ran/TC4: a small nuclear GTP-binding protein that regulates DNA synthesis. J Cell Biol *120*, 313-23.

Ren, M., Villamarin, A., Shih, A., Coutavas, E., Moore, M. S., LoCurcio, M., Clarke, V., Oppenheim, J. D., D'Eustachio, P., and Rush, M. G. (1995). Separate

domains of the Ran GTPase interact with different factors to regulate nuclear protein import and RNA processing. Mol Cell Biol *15*, 2117-2124.

Richardson, H. E., Wittenberg, C., Cross, F., and Reed, S. I. (1989). An essential G1 function for cyclin-like proteins in yeast. Cell *59*, 1127-1133.

Richardson, W. D., Mills, A. D., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1988). Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell *52*, 655-64.

Richardson, W. D., Mills, A. D., Diworth, S. M., Laskey, R. A., and Dingwall, C. (1988). Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell *52*, 655-664.

Ris, H. (1989). Three-dimensional imaging of cell ultrastructure with high resolution low voltage SEM. Inst. Phys. Conf. Ser. *98*, 657-662.

Ris, H. (1991). The three-dimensional structure of the nuclear pore complex as seen by high voltage electron micrscopy and high resolution low voltage scanning electron microscopy. EMSA Bull. *21*, 54-56.

Rosbash, M., and Singer, R. H. (1993). RNA travel: tracks from DNA to cytoplasm. Cell *75*, 399-401.

Roth, S., Hiromi, Y., Godt, D., and Nusslein-Volhard, C. (1991). cactus, a maternal gene required for proper formation of the dorsoventral morphogen gradient in Drosophila embryos. Development *112*, 371-88.

Roth, S., Stein, D., and Nusslein-Volhard, C. (1989). A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell *59*, 1189-202.

Rout, M. P., and Blobel, G. (1993). Isolation of the yeast nuclear pore complex. J Cell Biol *123*, 771-83.

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

Rushlow, C. A., Han, K., Manley, J. L., and Levine, M. (1989). The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. Cell *59*, 1165-77.

Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J., and Silver, P. (1989). A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an Escherichia coli heat shock protein. J Cell Biol *109*, 2665-75.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sanchez, E. R., Toft, D. O., Schlesinger, M. J., and Pratt, W. B. (1985). The 90kD non-steroid-binding phosphoprotein that binds to the un-transformed glucocorticoid receptor in molybdate-stabilized L-cell cytosol is the murine 90kD heat shock protein. J. Biol. Chem *260*, 12398-12401.

Sazer, S., and Nurse, P. (1994). A fission yeast RCC1-related protein is required for the mitosis to interphase transition. Embo J *13*, 606-15.

Schena, M., and Yamamoto, K. R. (1988). Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. Science *241*, 965-7.

Schlenstedt, G., Hurt, E., Doye, V., and Silver, P. A. (1993). Reconstitution of nuclear protein transport with semi-intact yeast cells. J Cell Biol *123*, 785-98.

Schlenstedt, G., Saavedra, C., Loeb, J. D. J., Cole, C. N., and Silver, P. (1995). The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)+ RNA in the cytoplasm. Proc. Natl. Acad. Sci. USA *92*, 225-229.

Schmidt-Zachmann, M. S., Dargemont, C., Kuhn, L. C., and Nigg, E. A. (1993). Nuclear export of proteins: the role of nuclear retention. Cell *74*, 493-504.

Seufert, W., Futcher, B., and Jentsch, S. (1995). Role of ubiquitin-conjugation enzyme in degradation of S- and M-phase cyclins. Nature *373*, 78-81.

Sharp, P. A. (1994). Split genes and RNA splicing. Cell *77*, 805-15.

Shen, W. C., Selvakumar, D., Stanford, D. R., and Hopper, A. K. (1993). The Saccharomyces cerevisiae LOS1 gene involved in pre-tRNA splicing encodes a nuclear protein that behaves as a component of the nuclear matrix. J Biol Chem *268*, 19436-44.

Sherman, F., Hicks, J. B., and Fink, G. R. (1986). Methods in yeast genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Shi, Y., and Thomas, J. O. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol Cell Biol *12*, 2186-92.

Sikorski, R. S., and Heiter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. Genetics *122*, 19-27.

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

Silver, P. A., Sadler, I., and Osborne, M. (1989). Yeast proteins that recognize nuclear localization sequences. J. Cell Biol. *109*, 983-989.

Snow, C. M., Senior, A., and Gerace, L. (1987). Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J Cell Biol *104*, 1143-56.

Soderqvist, H., and Hallberg, E. (1994). The large C-terminal region of the integral pore membrane protein, POM121, is facing the nuclear pore complex. Eur J Cell Biol *64*, 186-91.

Stafstrom, J. P., and Staehelin, L. A. (1984). Dynamics of the nuclear envelope and of nuclear pore complexes during mitosis in the Drosophila embryo. Eur J Cell Biol *34*, 179-89.

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

Sterne-Marr, R., Blevitt, J. M., and Gerace, L. (1992). O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for nuclear protein import. J Cell Biol *116*, 271-80.

Steward, R. (1989). Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell *59*, 1179-88.

Stewart, M. (1992). Nuclear pore structure and function. Semin Cell Biol *3*, 267-77.

Stochaj, U., Osborne, M., Kurihara, T., and Silver, P. A. (1991). A yeast protein that binds nuclear localization signals: purification, localization, and antibody inhibition of binding activity. J. Cell Biol. *113*, 1243-1254.

Stochaj, U., and Silver, P. A. (1992). A conserved phosphoprotein that specifically binds nuclear localization sequences is involved in nuclear import. J Cell Biol *117*, 473-82.

Stueland, C. S., Lew, D. J., Cismowski, M. J., and Reed, S. I. (1993). Full activation of p34CDC28 histone H1 kinase activity is unable to promote entry into mitosis in checkpoint-arrested cells of the yeast Saccharomyces cerevisiae. Mol Cell Biol *13*, 3744-55.

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

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. Embo J *12*, 1969-78.

Tachibana, T., Imamoto, N., Seino, H., Nishimoto, T., and Yoneda, Y. (1994). Loss of RCC1 leads to suppression of nuclear protein import in living cells. J Biol Chem *269*, 24542-5.

Tartakoff, A. M., and Schneiter, R. (1995). The nuclear GTPase cycle: promoting peripheralization? Trends Cell Biol *5*, 5-8.

Thomas, B., and Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. Cell *56*, 619-630.

Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987). Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependant protein kinase. Cell *50*, 277-287.

Traglia, H. M., Atkinson, N. S., and Hopper, A. K. (1989). Structural and functional analyses of Saccharomyces cerevisiae wild-type and mutant RNA1 genes. Mol Cell Biol *9*, 2989-99.

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

Wagner, P., and Hall, M. N. (1993). Nuclear protein transport is functionally conserved between yeast and higher eukaryotes. Febs Lett *321*, 261-6.

Wallis, J. W., Hereford, L., and Grunstein, M. (1980). Histone H2B genes of yeast encode two different proteins. Cell *22*, 799-805.

Wente, S. R., and Blobel, G. (1994). NUP145 encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure. J Cell Biol *125*, 955-69.

Wente, S. R., and Blobel, G. (1993). A temperature-sensitive NUP116 null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleocytoplasmic traffic. J Cell Biol *123*, 275-84.

Wente, S. R., Rout, M. P., and Blobel, G. (1992). A new family of yeast nuclear pore complex proteins. J Cell Biol *119*, 705-23.

Whytock, S., and Stewart, M. (1988). Preparation of shadowed nuclear envelopes from Xenopus oocyte germinal vesicles for electron microscopy. J Microsc *151*, 115-26.

Wilken, N., Kossner, U., Senecal, J. L., Scheer, U., and Dabauvalle, M. C. (1993). Nup180, a novel nuclear pore complex protein localizing to the cytoplasmic ring and associated fibrils. J Cell Biol *123*, 1345-54.

Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984). The structure of an antigenic determinant in a protein. Cell *37*, 767-778.

Wimmer, C., Doye, V., Grandi, P., Nehrbass, U., and Hurt, E. C. (1992). A new subclass of nucleoporins that functionally interact with nuclear pore protein NSP1. Embo J *11*, 5051-61.

Winston, F., Chumley, F., and Fink, G. R. (1983). Eviction and transplacement of mutant genes in yeast. Methods Enzymol *101*, 211-28.

Wozniak, R. W., Bartnik, E., and Blobel, G. (1989). Primary structure analysis of an integral membrane glycoprotein of the nuclear pore. J Cell Biol *108*, 2083-92.

Wozniak, R. W., and Blobel, G. (1992). The single transmembrane segment of gp210 is sufficient for sorting to the pore membrane domain of the nuclear envelope. J Cell Biol *119*, 1441-9.

Wozniak, R. W., Blobel, G., and Rout, M. P. (1994). POM152 is an integral protein of the pore membrane domain of the yeast nuclear envelope. J Cell Biol *125*, 31-42.

Wright, R., Basson, M., D'Ari, L., and Rine, J. (1988). Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. J Cell Biol *107*, 101-14.

Xiao, Z., McGrew, J. T., Schroeder, A. J., and Fitzgerald-Hayes, M. (1993). CSE1 and CSE2, two new genes required for accurate mitotic chromosome segregation in Saccharomyces cerevisiae. Mol Cell Biol *13*, 4691-702.

Xing, Y., Johnson, C. V., Dobner, P. R., and Lawrence, J. B. (1993). Higher level organization of individual gene transcription and RNA splicing [see comments]. Science *259*, 1326-30.

Yaffe, M. P., and Schatz, G. (1984). Two nuclear mutations that block mitochondrial protein import in yeast. Proc Natl Acad Sci U S A *81*, 4819-23.

Yamada, M., and Kasamatsu, H. (1993). Role of nuclear pore complex in simian virus 40 nuclear targeting. J Virol *67*, 119-30.

Yan, H., Merchant, A. M., and Tye, B. K. (1993). Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. Genes Dev *7*, 2149-60.

Yang, J., and De Franco, D. B. (1994). Differential roles of heat shock protein 70 in the in vitro nuclear import of glucocorticoid receptor and simian virus 40 large tumor antigen. Mol Cell Biol *14*, 5088-98.

Yano, R., Oakes, M., Yamaghishi, M., Dodd, J. A., and Nomura, M. (1992). Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations, in Saccharomyces cerevisiae. Mol Cell Biol *12*, 5640-51.

Yano, R., Oakes, M. L., Tabb, M. M., and Nomura, M. (1994). Yeast Srp1p has homology to armadillo/plakoglobin/beta-catenin and participates in apparently multiple nuclear functions including the maintenance of the nucleolar structure. Proc Natl Acad Sci U S A *91*, 6880-4.

Yoneda, Y., Imamoto-Sonobe, N., Yamaizumi, M., and Uchida, T. (1987). Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. Exp Cell Res *173*, 586-95.

Zasloff, M. (1983). tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. Proc Natl Acad Sci U S A *80*, 6436-40.