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Brr3 FUNCTIONS IN YEAST mRNA EXPORT AND MAY COUPLE EXPORT WITH mRNA PROCESSING

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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San Francisco

[redacted]

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I have saved this part of my thesis, the most important, for last, and I can already feel my heart beating faster as I write. I exit this stage of my life with very mixed emotions. On the one hand, I'm ecstatic that my thesis is done and I'm finally moving on. On the other hand, it's with a heavy heart that I leave the comfort of UCSF and the Guthrie lab. Where else would I have had the opportunity to meet so many amazingly smart and wonderfully wacky people? Plus, I've been able to forge close, lasting friendships with quite a few of them. It is these friends that pulled me through the hardships of graduate school, were with me to celebrate the good times, and never let me forget the importance of enjoying life. I am immensely grateful to all of them.

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Brr3 FUNCTIONS IN YEAST mRNA EXPORT AND MAY COUPLE EXPORT WITH mRNA PROCESSING

Tanya L. Awabdy

ABSTRACT

Nuclear processing of mRNAs and mRNA export to the cytoplasm are integral steps in gene expression. In the nucleus, newly transcribed mRNAs undergo multiple processing steps, acquiring a 5' cap and 3' poly(A) tail. Additionally, intron-containing messages are spliced. After processing, the matured mRNAs are exported through the nuclear pore complex to the cytoplasm. Many components required for each pre-mRNA processing step have been identified. In contrast, much less is known about mRNA export; mRNA-binding proteins and nucleoporins have been isolated as potential export factors. Furthermore, virtually nothing is known about factors that may serve to couple mRNA processing and export, acting to direct mature mRNAs from the sites of processing to the export pathway.

To investigate the coupling of mRNA processing and export in yeast, I chose to study the *brr3-1* mutant, which displays strong defects in both pre-mRNA splicing and mRNA export, and contains elongated poly(A) tails. Originally, no significant homologies to any known proteins were found in the *BRR3* gene sequence. However, *BRR3* has been isolated in two other mRNA export screens, as *GLE1* and *RSS1*, and a leucine-rich nuclear export signal was found in the Gle1 protein sequence. Further supporting a role for Brr3 in mRNA export, I have shown that the *brr3-1* mutant is synthetically lethal with

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several nucleoporin mutants implicated in mRNA export. Moreover, Brr3 shares some characteristics of nucleoporins, such as localization to the nuclear rim and detergent extraction. Also, Brr3 interacts stably and quantitatively with the nucleoporin Rip1.

By comparison, the onset of the *brr3-1* general splicing defect is first seen after an export block is detected, and *brr3-1* extracts are only slightly defective for splicing. Thus, Brr3 is unlikely to be an essential splicing factor, although a role in the coupling of splicing and export cannot be ruled out. In contrast, a dramatic block in poly(A) tail shortening is seen when assaying 3'-end formation in *brr3-1* extracts. Therefore, Brr3 could be a coupling factor linking mRNA export with poly(A) tail shortening, possibly at the nuclear pores.

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INTRODUCTION

Nuclear transport: the challenge

In eukaryotic cells, the events crucial to gene expression are spatially separated into two compartments. RNA synthesis and processing occur in the nucleus, while proteins are synthesized on the other side of the nuclear envelope, in the cytoplasm. This poses an immense challenge to the cell: the need to traffic thousands of RNAs and proteins across the nuclear envelope every minute. The main constituents exiting the nucleus are mRNAs, tRNAs, and rRNAs, while all nuclear proteins travel in the opposite direction (Nigg, 1997). Some RNAs, such as the small nuclear RNAs (snRNAs) important for pre-mRNA splicing, are exported to the cytoplasm for processing and assembly with proteins and then re-enter the nucleus (Izaurralde, 1995). Additionally, certain proteins shuttle between the nucleus and cytoplasm (Pinol-Roma, 1992).

The complexity of transport substrates, and the sheer volume of molecules entering and exiting the nucleus predict a transport mechanism comprised of an extensive network of carefully orchestrated export and import machineries. Nonetheless, when I joined the Guthrie lab in 1991, next to nothing was known about the cellular factors that function in nuclear transport. I distinctly remember my first encounter with transport as a biological problem, in one of Christine's BioReg lectures: she had outlined the steps of gene expression, and in stark contrast to the considerable knowledge of transcription, pre-mRNA processing, and translation, a huge question mark sat at the mRNA export step. It was this impressive lack of knowledge that first drew me to the problem of mRNA export. Here was this critical step in gene expression that appeared largely ignored. To me, the huge question mark represented an irresistible challenge that, once undertaken, could lead to many opportunities for discovery.

At that time, the studies of nuclear transport were confined to two experimental approaches. The first approach consisted of microinjecting radiolabeled or fluorescently-labeled transport substrates into the nucleus or cytoplasm of metazoan cells. The final destination of the substrates was determined by swift fractionation of the cell nucleus and cytoplasm or fluorescence microscopy. These experiments defined the general characteristics of RNA export and protein import: saturable, ATP-dependent, and temperature-dependent, implying an active, carrier-mediated mechanism (Zasloff, 1983; Newmeyer, 1988; Guddat, 1990; Hamm, 1990). The second experimental approach was ultrastructural; gold particles coated with protein or RNA were microinjected into *Xenopus* oocytes and examined by electron microscopy (EM). These experiments demonstrated that transport of proteins and RNA proceeds bidirectionally through nuclear pores embedded in the nuclear envelope (Dworetzky, 1988). EM studies of partially purified nuclear pores also revealed the beauty of these enormous structures, with their eight-fold symmetry (Allen, 1989).

The experiments in metazoan cells were important for understanding nuclear transport as a phenomenon, but many questions remained unanswered, especially regarding mRNA export. What are the signals essential for targeting mRNAs to the export pathway? What are the receptors that recognize these signals and transport the mRNA to the nuclear pores? What is the physical mechanism of mRNA movement to the nuclear pores? How are mRNAs translocated through the pores? Are nuclear pore proteins directly involved in mRNA export, and if so, which ones? What makes export unidirectional? It was clear that identification of the cellular proteins underlying the mRNA export pathway, be it an mRNA-binding protein, an export receptor, or a nuclear pore protein, was crucial to answering these questions. I realized one way to isolate such factors is to develop an *in vitro* mRNA export assay, and biochemically purify the required components. This seemed a daunting task, even for an ambitious first year student with many years of graduate school ahead. A more attractive approach was to study mRNA export in a simple, genetically tractable system such as yeast.

The main obstacle to studying mRNA export in yeast at the time was the lack of an assay to directly examine mRNA export in yeast cells. However, there were unpublished reports of a successful strategy employing dT50 oligonucleotide probes for *in situ* hybridization to the poly(A) tails of mRNAs in yeast; the bulk poly(A) RNA are then visualized by fluorescence microscopy, using fluorescently-conjugated antibodies that recognize the dT50 probe (Amberg, 1992; Kadowaki, 1994). Anne de Bruyn Kops, a post-doc who had just joined the Guthrie lab, set forth to figure out the conditions to replicate the *in situ* assay. She planned to use the assay to screen a bank of yeast mutants for those that failed to export bulk poly(A) RNA. I decided to work on developing a complementary strategy to examine mRNA transport in yeast cells, biochemical fractionation of the nuclear and cytoplasmic compartments. This powerful technique would enable us to analyze the localization of specific mRNAs and other RNAs in the mutants, crucial in determining which mutants inhibit mRNA export specifically or block RNA export in general. A rapid fractionation protocol would also allow us to analyze RNA export kinetically.

Fractionating yeast cells proved to be an extremely difficult endeavor. I soon realized it was important to keep the nuclei as intact as possible to prevent leakage of nuclear RNAs; also, separating the nuclei from ribosomes was equally critical, since cytoplasmic mRNAs bound to ribosomes needed to be kept apart from nuclear mRNAs to accurately monitor mRNA localization. However, trying to quickly separate the small yeast nuclei from the dense population of ribosomes, without extensively damaging the nuclear envelope, turned out to be the largest obstacle. After a year and a half and many fruitless attempts, I decided to move on to another project. In retrospect, although it was worth my time and effort, it was probably an overly ambitious task. Even today, five years later, no one has succeeded in cleanly and rapidly separating intact nuclei from the cytoplasm in yeast.

The subsequent projects I undertook involved various strategies to identify novel RNA export factors. To investigate snRNA transport in yeast, I started to develop an *in situ* hybridization assay to detect specific snRNAs in yeast cells, as a means to screen for mutants that mislocalize snRNAs. Preliminary results were promising, but my attention was overtaken by a second project which eventually led me to explore mRNA stability (see Appendix I). Fortunately, Maki Inada, currently a student in the Guthrie lab, has continued the snRNA project, and is making significant progress.

The *brr3-1* project

At this point I had been in the lab for almost four years, and was eager to work on something concrete, a specific factor about which I could ask defined questions. My interest in mRNA export had not waned. I had witnessed an explosion in understanding of the molecular events underlying protein import; this was primarily due to the development of an *in vitro* protein import assay, which resulted in the isolation of multiple soluble import factors (Gorlich, 1997). In contrast, the field of mRNA export had a long way to go. By now, the dT50 *in situ* hybridization assay was standard, and a few yeast mutants that accumulate poly(A) RNA in the nucleus had been identified. Of these mutants, the best understood ones were a handful of nucleoporins, proteins that localize to the nuclear pore complex (Hurt, 1988; Davis, 1990; Wente, 1992). The essential mRNA export signals, and which proteins acted as mRNA export receptors, were still undetermined. Furthermore, being in a lab that predominantly studied pre-mRNA splicing heightened my awareness of the complexity of pre-mRNA processing, and underscored yet another poorly understood process: the coupling of mRNA processing events with nuclear export.

In the nucleus, newly transcribed mRNAs are capped at the 5' end, and the mature 3' ends are formed by cleavage and polyadenylation. Additionally, the messages containing introns are spliced. Once these processing events occur, the matured mRNAs are exported to the cytoplasm. It is critical for mRNAs to be fully processed before export to the cytoplasm; the cap and poly(A) tail of an mRNA are required for its translation and stability (Decker, 1994; Tarun, 1995), and the translation of mRNAs still containing an intron could lead to the production of truncated and aberrant proteins, likely to be detrimental to the cell. Impressive progress had been made in elucidating the chemistry of each of the pre-mRNA processing reactions, and identifying many components of the macromolecular complexes driving each processing event (Moore, 1993a; Guthrie, 1996; Keller, 1997). However, hardly any of this work addressed the temporal and spatial relationship of the processing events, and how the processing machineries communicated with each other, if at all.

To me, a more intriguing question was how mRNAs are directed from the sites of processing to the export pathway. Once again I felt the pull of an important, but barely explored biological problem. Many more questions came to mind: what monitors when an mRNA has completed processing and is ready to be exported? Is there active retention of pre-mRNAs by the processing machineries followed by passive release of the matured mRNA to the export machinery? Or, is there an active hand-off mechanism between the processing and export machineries? If so, which export factors interact with processing factors to mediate this hand-off? Alternatively, are there factors which have overlapping functions in mRNA processing and export? What interested me the most was trying to identify potential factors that interact with both the processing and export machineries to direct mRNAs from one to the other. I figured a good way to start was to study yeast mutants which display strong defects in both pre-mRNA processing and mRNA export. The *brr3-1* mutant fit this criteria.

The *brr3-1* mutant was initially isolated from a bank of cold-sensitive yeast strains in a screen for pre-mRNA splicing mutants (Noble, 1996). Suzanne Noble shifted cultures of each strain to the non-permissive temperature of 16°C for 10 hours, painstakingly prepared RNA from each, and assayed the RNA for accumulation of splicing intermediates and/or the decrease of mature mRNA of three intron-containing messages. The mutants she isolated fell into nine complementation groups; five of these did not correspond to genes encoding known splicing factors and were named *BRR* (Bad Response to Refrigeration). Among the *brr* mutants, *brr3-1* displays a relatively strong block to the first step of splicing when shifted to 16°C.

Soon thereafter, Anne completed her screen of the same cold-sensitive bank for poly(A) RNA export mutants (A. de Bruyn Kops and C. Guthrie, manuscript in preparation). She shifted cultures of each strain to the cold for 15 minutes and 3 hours, then assayed the cells for nuclear accumulation of poly(A) RNA by dT50 *in situ* hybridization. She found that mutants in six complementation groups exhibit this phenotype, and two of them overlap with the mutants from the splicing screen, *brr3-1* and *brr4-1*. *brr4-1* shows weak splicing and poly(A) export defects. However, the onset of the *brr3-1* export defect is very rapid, with mutant cells displaying the phenotype as early as 15 minutes after the cold shift. Importantly, both the export and splicing defects of *brr3-1* co-segregate with the cold-sensitivity, indicating that all three phenotypes are linked to the same mutation. Anne also determined that bulk poly(A) tail length is increased in *brr3-1* cells, a phenotype that had been detected in other mutants defective for poly(A) RNA export (Forrester, 1992).

It was at this point that I picked up the *brr3-1* project, the main subject of my thesis. I was keen to test whether the Brr3 protein functions to couple splicing and export, by interacting with both the splicing and export machineries to direct fully spliced mRNAs from the spliceosome to the export pathway. However, before delving into examining the coupling model, more preliminary work needed to be done, such as sequencing the

BRR3 gene and determining the cellular localization of the Brr3 protein. Also, although the coupling hypothesis was tantalizing, realistically three testable models could explain the primary function of Brr3. First, it could be a splicing factor, with the export defect of the *brr3-1* mutant being an indirect effect of the splicing defect. Second, it could function directly in mRNA export, with the *brr3-1* splicing defect being an indirect effect of the export defect. Third, it could function in both splicing and export directly, as a coupling factor.

I reasoned that if Brr3 is primarily a splicing factor, the onset of the *brr3-1* splicing defect should be similar to that of the export defect, coming on very quickly after a cold shift. Also, one way to determine if the *brr3-1* mutant directly affects splicing is to test splicing efficiency in *brr3-1* extracts. Another prediction of this model is that Brr3 should be nuclear. A final prediction is, given the strong export phenotype of *brr3-1*, the Brr3 protein is likely to be a late-acting splicing factor, required to release fully spliced mRNAs from the spliceosome. In this case, it possibly acts in conjunction with Prp22, an established splicing factor that functions at the mRNA release step. Thus, genetic interactions between *prp22* mutants and *brr3-1* should be tested.

Potentially more difficult to ascertain is a primary role for Brr3 in mRNA export. When I took on the *brr3-1* project, the best defined class of export factors were nucleoporins. If Brr3 is a nucleoporin, an obvious clue could come from the Brr3 sequence, if it contained the signature repeats of nucleoporins. If not, a connection to the NPC could be determined by testing for genetic interactions between *brr3-1* and various nucleoporin mutants, especially those implicated in poly(A) RNA export. Another indication of

Brr3 acting at the NPC is localization of the protein at the nuclear periphery. Beyond the nucleoporin prediction, I could take a process-of-elimination approach to determine if the *brr3-1* mutant specifically affects mRNA export, such as ruling out a general transport defect by demonstrating that protein import is normal in *brr3-1* cells.

The final model, that Brr3 is a splicing-export coupling factor, is the most intriguing, but the most daunting to prove. In the simplest case, Brr3 would be required for splicing *in vitro*, while having an apparent connection with the export pathway, such as interactions with nucleoporins. However, a coupling factor could direct mRNAs from the spliceosome to the export pathway without being required for splicing. One prediction of this scenario is that Brr3 interacts with a splicing factor to coordinate the release of the mRNA from the spliceosome; again, Prp22 comes to mind. In general, a seemingly important feature of a splicing-export coupling factor is the ability to shuttle between the nucleus and the NPC, or between the nucleus and cytoplasm.

Before proceeding to the results from my analysis of the *brr3-1* mutant, I think it worthwhile to give a general review of the current state of the nuclear transport field. This will provide a frame of reference for my results, as well as introducing the known players in nuclear transport, many of which were part of my analysis of the Brr3 protein. Furthermore, I will elaborate on topics I have briefly mentioned thus far: what it means to be a nucleoporin, possible mRNA export signals, the different types of mRNA export factors that are turning up, and a discussion of the idea of coupling mRNA processing and export events. The following is an overview of the nuclear pore complex and nucleoporins, the factors involved in nuclear protein import, the current understanding of RNA export (focusing on the export of mRNAs), and a brief review of the processing steps that precede mRNA export.

The nuclear pore complex

Central to the nuclear transport pathway is the nuclear pore complex (NPC), an enormous structure with a mass of over 100 megadaltons that connects the inner and outer membranes of the nuclear envelope (Hinshaw, 1992). The ultrastructure of the NPC seems to be conserved between yeast and higher eukaryotes, notably the eightfold rotational symmetry of the NPC subunits (Allen, 1989). Besides the symmetric framework of the NPC, electron microscopy studies reveal the presence of a central plug, a basket-like structure extending from the nuclear face, and fibrils extending from the cytoplasmic face of the NPC (Aebi, 1990). Hundreds to thousands of NPCs are estimated to perforate a nuclear envelope, depending on the organism (Allen, 1989).

The NPC provides an aqueous channel for the passage of molecules between the nucleus and cytoplasm. Ions and molecules smaller than 40 kD can passively diffuse through the 10 nm channel of the NPC, but the pore can expand up to 30 nm to allow transport of larger molecules (Pante, 1994). Electron microscopy studies show that RNA-coated gold particles up to 23 nm in diameter can be translocated across the NPC of *Xenopus* oocytes. Furthermore, the pores are bidirectional; protein-coated gold particles enter the nucleus through the same pores from which RNA-coated particles exit (Dworetzky, 1988).

The NPC is predicted to contain 50 to 100 distinct nucleoporins (Rout, 1993). Initially, the working definition of a nucleoporin was a protein that specifically localizes to the NPC, as determined by immuno-EM (Davis, 1990; Nehrbass, 1990). A less definitive assay for NPC localization is indirect immunofluorescence, which results in a punctate signal at the nuclear periphery. Nucleoporins were first identified through binding to monoclonal antibodies raised against rat and yeast nuclear envelope fractions. These antibodies localize to the NPC by both indirect immunofluorescence and electron microscopy (Davis, 1986; Snow, 1987; Hurt, 1988). The antibodies were used to identify and clone several yeast nucleoporin genes, namely *NSP1, NUP1, NUP2, NUP49, NUP100,* and *NUP116* (Davis, 1990; Nehrbass, 1990; Wente, 1992), as well as some of their mammalian homologs (Starr, 1990; Radu, 1995). The proteins encoded by each of these genes localize to the NPC by fluorescence microscopy and EM, and thus fit the definition of a nucleoporin.

The utilization of these nucleoporins as starting points in genetic screens and biochemical assays, in addition to the development of a method to purify yeast nucleoporins, has led to a rapid acceleration in the number of yeast nucleoporins identified (Rout, 1993; Doye, 1997). To date, as many as 30 have been isolated and characterized. They can be grouped into four classes, based on primary sequence structure (Table I). Three of the four classes contain a highly repetitive tetrapeptide or dipeptide motif, either FXFG, GLFG, or the more degenerate XXFG sequence. These motifs are unique to nucleoporins

Table I. Four classes of yeast nucleoporins

	Import defect?	Export defect?	NPC/NE defect?	Disruption phenotype
1. FXFG				
Nup1	yes	yes	yes	ts
Nsp1*	yes	no	yes	lethal
Nup2	nd	nd	nd	viable
Nup36	nd	nd	nd	nd
2. GLFG				
Nup49*	yes	yes	no	lethal
Nup100	nd	nd	nd	viable
Nup116	nd	yes	yes	ts
Nup145/Rat10	yes	yes	yes	lethal
Nup57*	nd	nd	nd	lethal
3. XXFG				
Nup159/Rat7	no	yes	yes	lethal
Rip1	nd	no	nd	viable
4. No repeats				
Nic96*	yes	no	yes	lethal
Pom152	nd	nd	nd	viable
Nup133/Rat3	nd	yes	yes	ts
Nup82*	yes	yes	no	lethal
Nup170	nd	nd	no	viable
Nup157	nd	nd	no	viable
Nup120/Rat2	no	yes	yes	ts
Nup84	no	yes	yes	ts
Nup85/Rat9	no	yes	yes	ts
Seh1	nd	nd	no	cs
Nup188	no	yes	yes	viable
Gle2	no	yes	yes	ts
Nup192	nd	nd	nd	nd

Data in the table taken from Doye and Hurt, 1997. NPC/NE defect = aberrant nuclear pore/nuclear envelope morphology. nd = not determined, ts = temperature-sensitive, cs = cold-sensitive. *contains putative coiled-coil domain but are not a requirement, as evidenced by the growing number of nucleoporins in the fourth class, which lack any repeats. Other notable sequence features include predicted coiled-coil α-helical domains in five nucleoporins, of the repeat and non-repeat classes (marked by an asterisk in Table I). Four of these coiled-coil nucleoporins, Nsp1, Nup49, Nup57, and Nic96 co-immunoprecipitate as a complex from yeast nuclear extracts (Grandi, 1995). The fifth coiled-coil nucleoporin, Nup82, is present in a separate complex with Nsp1 (Grandi, 1995b). Deletion of the putative coiled-coil region in Nic96 or Nup82 abolishes their ability to co-immunoprecipitate the other nucleoporins in their respective complexes; hence, the coiled-coil regions likely mediate the physical interactions between these nucleoporins. One non-repeat nucleoporin, Pom152, contains a transmembrane domain, and is located in the pore membrane domain of the yeast nuclear envelope (Wozniak, 1994). Finally, three GLFG nucleoporins, Nup100, Nup116, and Nup145 contain a novel "nucleoporin RNA-binding motif" (NRM), which has been shown to bind homopolymeric RNAs *in vitro* (Fabre, 1994).

Although the number of nucleoporins identified in recent years has increased at an impressive rate, it is important to note that the definition of a nucleoporin seems to have relaxed. Fewer and fewer reports include immuno-EM localization of the putative nucleoporin at the NPC, and instead rely on the less stringent immunofluorescence assay to demonstrate localization at the nuclear rim. This is problematic, since it is conceivable that transport factors, which localize to the nuclear rim/NPC under steady-state conditions, only transiently associate with nuclear pore components in the process of escorting their substrates from one side of the NPC to the other. For instance, this appears to be the case for Srp1, a soluble protein import factor that is clearly not a nucleoporin (Yano, 1992; Belanger, 1994). Thus, without immuno-EM data, it is increasingly important to utilize additional criteria to distinguish between true nucleoporins, the proteins that permanently reside at the NPC, and transport factors which transiently associate with the NPC. One way to determine if a protein is a nucleoporin is by demonstrating a stable biochemical interaction with nucleoporins that have been localized to the NPC by EM. Also, a true nucleoporin should be present in biochemically purified NPCs; however, the published yeast NPC purification method (Rout, 1993) is difficult and laborious, which makes it less feasible as a standard assay. The nucleoporins listed in Table I fit at least one of these two criteria. The most effective way to rule out if a protein is a nucleoporin is by showing that it can be localized to the nucleus and/or cytoplasm in addition to the NPC, providing evidence that it shuttles between the nucleus and cytoplasm.

Most of the known yeast nucleoporins have been extensively characterized, both biochemically and genetically, resulting in a complex matrix of interactions. A recent compilation of this large body of data can be found in a review by Doye and Hurt (1997). Nonetheless, the primary function of these proteins is still unclear. In principle, nucleoporins could be divided into two functional groups. First are the ones that comprise the structural framework of the NPC, necessary to maintain integrity of the channel. These nucleoporins are assumed not to function directly in nuclear transport. The second group consists of the peripheral NPC nucleoporins, which presumably reside at the areas of the NPC exposed to the central channel and function directly in the translocation of transport substrates. To do so, these nucleoporins likely mediate the gating of the nuclear pore, allowing it to expand for the passage of proteins and RNA complexes. Also, peripheral nucleoporins possibly localize to the cytoplasmic fibrils or nuclear basket that extend from the core NPC structure, and make the initial contacts with the transport substrates.

Thus far, the nucleoporins thought to play a structural role in the NPC are Pom152, Nup188, Nup170, Nup157, Nic96, and Nup192, based on their abundance and central location at the NPC (Doye, 1997). These six nucleoporins are the most abundant proteins of the NPC, making up over 25% of the total mass of purified yeast NPCs (Aitchison, 1995). Pom152 is an obvious candidate for a structural NPC protein, since it is the only known integral membrane nucleoporin (Wozniak, 1994). Furthermore, Nup188 has also been localized to the central region of the NPC (Nehrbass, 1996). Additionally, Pom152, Nup188, and Nic96 interact biochemically and genetically (Aitchison, 1995; Nehrbass, 1996; Zabel, 1996). In addition, each of these three nucleoporins interact genetically with Nup157 and Nup170 (Aitchison, 1995). Furthermore, cells harboring a *nup188* mutation or depleted for Nic96 display nuclear envelope abnormalities (Nehrbass, 1996; Zabel, 1996). However, definitively assigning these nucleoporins to the structural NPC function would be premature. For example, a novel, cold-sensitive allele of *NUP188* displays a strong poly(A) RNA export defect without any obvious NPC structural abnormalities, suggesting a more direct role in nuclear export (A. de Bruyn Kops, K. Stade, and C. Guthrie, manuscript in preparation).

Equally difficult to ascertain is which nucleoporins function directly in nuclear transport. Some clues have come from analyzing the transport phenotypes of yeast nucleoporin mutants; some are defective in protein import and/or RNA export (see Table I). However, most of these mutants also display aberrant nuclear envelope and NPC morpholgy, which could cause a non-specific block to transport (Wente, 1993; Siniossoglou, 1996). More convincing evidence that nucleoporins play a direct role in nuclear transport is provided by the demonstration of a physical interaction between soluble transport factors and nucleoporins (discussed in the following sections on protein import and RNA export). Ideally, the way to prove that a nucleoporin functions directly in transport is by using an *in vitro* transport system where a nucleoporin can be depleted and shown to disrupt a specific transport activity. Unfortunately, such a system has yet to be developed.

Nuclear protein import: factors and mechanisms

The active import of proteins destined for the nucleus is an energy-dependent, receptor-mediated process (Gorlich, 1997). Often, these proteins contain nuclear localization signals (NLSs) that direct their import to the nucleus. The first type of NLS discovered is the one that is best characterized, consisting of one or two short stretches of basic amino acids (Dingwall, 1991). This kind of NLS has been identified in proteins of various organisms, including large T antigen of the SV40 virus, *Xenopus* nucleoplasmin, and yeast histone H2B. In each case, mutating or deleting critical residues of the NLS results in a block to nuclear import, and the NLS peptide is able to drive a non-nuclear reporter protein into the nucleus (Dingwall, 1991).

By visualizing fluorescently labeled NLS-containing proteins in *Xenopus* oocytes, it became clear that nuclear import proceeds in two distinct steps: NLS-dependent docking to the nuclear periphery, followed by energy-dependent translocation into the nucleus (Newmeyer, 1988; Richardson, 1988). A critical turning point in the import field was the development of an *in vitro* system that efficiently and reliably mimicked these two steps (Adam, 1990). The salient feature of this assay is the semi-permeabilization of HeLa or rat liver cells, so that the plasma membrane is perforated while the nuclear envelope remains intact. It was shown that, in these cells, the nuclear import of an exogenously-added protein required the presence of a functional NLS, and the addition of ATP and cytosol. Furthermore, fractionation pared down the cytosolic requirement to two fractions; the first fraction allowed the docking step to occur, and the second fraction, when added to the first, enabled translocation of the import substrate into the nucleus (Moore, 1992).

Expeditious work from many groups led to the purification of the soluble cytosolic factors sufficient to support nuclear protein import *in vitro*: importins α and β, Ran, and p10. Importins α and β (also called karyopherin α/β), as a heterodimer, recognize the NLS of the import substrate and mediate its docking at the NPC (Gorlich, 1995). Specifically, importin α directly binds the NLS while binding importin β (Weis, 1995). The importin β binding domain (IBB domain) is at the N-terminal region of importin α, distinct from its NLS-binding site (Gorlich, 1996; Weis, 1996). Although importin β is not directly associated with the NLS, it does enhance importin α affinity to an NLS in solution binding experiments (Rexach, 1995). Additionally, importin β is responsible for docking the complex to the NPC by interacting with specific nucleoporins. Solution binding and blot overlay experiments have identified interactions between importin β and the repeat regions of yeast nucleoporins Nup1 and Nup159 (Kraemer, 1995; Rexach, 1995). Furthermore, importin β binds to mammalian nucleoporins located at both the cytoplasmic and nuclear faces of the NPC, suggesting that it directs the import complex all the way through the NPC (Radu, 1995b). Supporting this model, an EM snapshot taken at the end of an *in vitro* protein import reaction revealed importin β at the nuclear side of the NPC (Gorlich, 1995b). Interestingly, the same set of experiments also demonstrated that importin β remains at the NPC, while importin α enters the nucleoplasm with the import substrate.

The other two soluble import factors, Ran and p10 (also called NTF2), are required for the second import step, translocation across the NPC into the nucleus (Moore, 1993b; Nehrbass, 1996b). Ran is a member of the *ras* family of small GTPases, and hence alternates between GDP- and GTP-bound states. Thus far, the only two proteins known to govern the switch between Ran-GDP and Ran-GTP are RanGAP1, a potent stimulator of the weak GTPase activity of Ran, and RCC1, which promotes the exchange of GDP for GTP (Bischoff, 1991; Bischoff, 1994). Strikingly, at steady state RanGAP1 and RCC1 are spatially separated in the cell, with RanGAP1 predominantly cytoplasmic and RCC1 mainly nuclear (Ohtsubo, 1989; Hopper, 1990). This predicts that the cytoplasmic form of Ran is mostly GDP-bound, whereas nuclear Ran is in the GTP-bound state, implying distinct roles for the two forms of Ran in protein import.

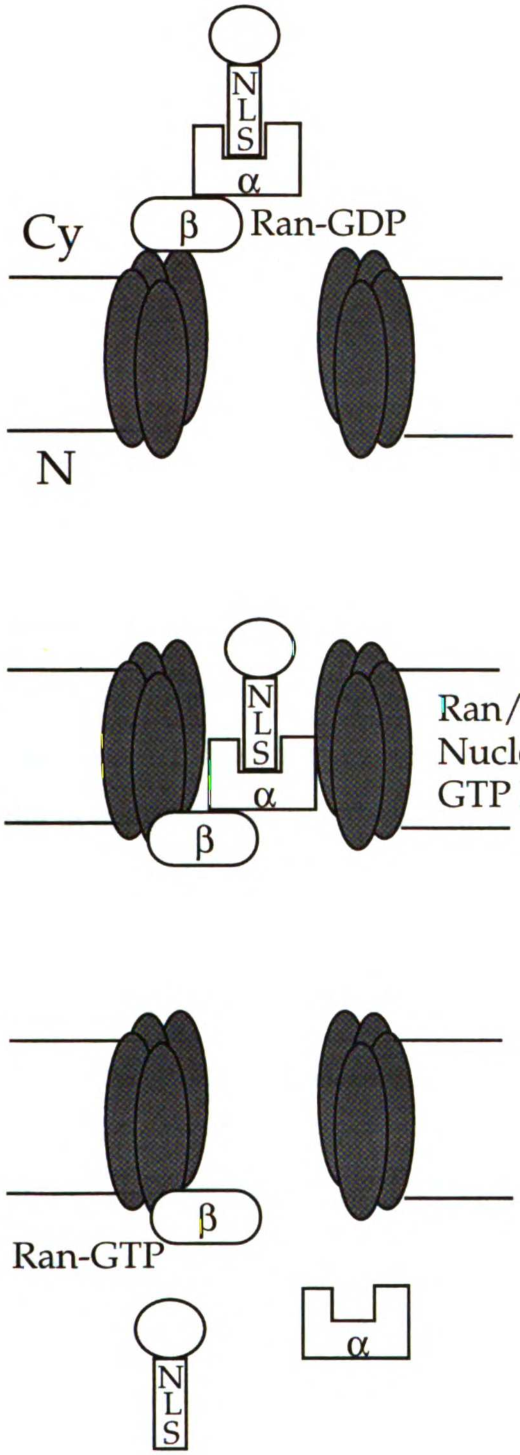
To determine where each form of Ran functions in the protein import pathway, Ran pre-loaded with either GTP or GDP was tested for the ability to import an NLS-protein *in vitro* (Gorlich, 1996b; Weis, 1996b). Ran-GDP allowed import, in the presence of the importins, p10, and free GTP. In contrast, neither docking at the nuclear envelope nor translocation was observed when Ran-GTP was used. Moreover, when incubated in solution, Ran-GTP completely dissociates a complex of importin α, importin β, and a nucleoporin repeat region (Rexach, 1995). Thus, it appears that Ran-GDP supports translocation across the NPC, while Ran-GTP promotes the disassembly of the importin complex from the NPC. This fits nicely with the compartmentalization of the two forms of Ran; Ran-GDP in the cytoplasm initiates translocation of the import complex, while Ran-GTP in the nucleus dissociates the importin heterodimer and its attachment to the NPC, leading to release of the import substrate into the nucleus.

It is unknown if the spatial separation of the two forms of Ran is enough to account for the translocation mechanism across the NPC. The addition of non-hydrolyzable forms of GTP block import *in vitro* (Melchior, 1993; Moore, 1993b); however, it is unclear whether this is solely a result of a block in the docking step (due to depletion of Ran-GDP), or whether it reflects an additional requirement for GTP hydrolysis during translocation. The idea that multiple rounds of nucleotide exchange and GTP hydrolysis occur at the NPC is not inconceivable, especially since import complexes must travel a 100 nm distance from one side of the NPC to the other (Pante, 1996; Gorlich, 1997; Nigg, 1997). Currently, one of the major challenges of the protein import field is to elucidate the molecular mechanism underlying this movement across the NPC.

Also unclear is the nature of the association between the importins and Ran during translocation across the NPC. One proposed function for p10, the second soluble factor required for translocation *in vitro*, is to promote the association of Ran-GDP with the importins and a subset of nucleoporins. This is based on *in vitro* binding studies, which demonstrated p10 interacting with FXFG and GLFG repeat nucleoporins, importin β, and Ran-GDP (but not Ran-GTP; Nehrbass, 1996b). These results imply that Ran-GDP and the importins function as a complex in the early stages of translocation. Contrary to this model, more convincing studies suggest that importin association with Ran is only necessary for the dissociation step on the nuclear side of the NPC (Gorlich, 1996b). Importin β binds directly to Ran *in vitro*, but has a much greater affinity for Ran-GTP than Ran-GDP. Moreover, a mutant form of importin β that is unable to bind Ran allows translocation of an import substrate across the NPC; however, the substrate is trapped at the nuclear face of the NPC, prior to release into the nucleus. If Ran is not directly associated with the import substrate during translocation through the NPC, one general model to explain its requirement is in switching the NPC to an open conformation, thereby allowing passage of the import substrate.

To summarize the *in vitro* data thus far, the mechanism of protein import can be divided into three steps (Figure 1). First, the importin α/β heterodimer binds the NLS-protein substrate; importin α binds the NLS directly, while importin β strenghtens this interaction and mediates docking of the complex at the NPC in the presence of Ran-GDP. Second, the importin-substrate complex is translocated through the NPC by an unknown mechanism that requires p10 and possibly multiple rounds of nucleotide

Figure 1: The current model of nuclear protein import. (Step 1) Importins α and β bind the NLS-containing substrate. Importin β mediates docking of the complex at the NPC, in the presence of Ran-GDP. (Step 2) Translocation of the importin-substrate complex through the NPC requires p10, and possibly multiple rounds of nucleotide exchange and GTP hydrolysis by Ran. (Step 3) At the nuclear face of the NPC, Ran-GTP binds importin β, causing dissociation of the complex and the release of importin α and the NLS-substrate into the nucleus.


N
L
S
α
Cy
β
Ran-GDP
N
N
L
S
α
β
Ran/p10
Nucleotide exchange,
GTP hydrolysis?
β
Ran-GTP
N
L
S
α

1. Substrate binding and docking at the NPC

2. Translocation

3. Substrate release

exchange and GTP hydrolysis by Ran. Finally, once at the nuclear face of the NPC, Ran-GTP binds importin β, causing complete dissociation of the complex. Importin β remains at the NPC, while importin α and the NLS-protein are released into the nucleoplasm.

Clearly, *in vitro* experiments have provided much insight into the factors involved in nuclear protein import. The *in vivo* relevance of these results is confirmed by genetic studies in yeast, since the yeast homologs of the essential import factors have been identified. The yeast importin α gene, *SRP1*, also called *KAP60* (for karyopherin of 60 kD) was originally isolated as a high copy suppressor of RNA polymerase I mutants, and was subsequently localized to the nuclear periphery by immunofluorescence (Yano, 1992). *srp1/kap60* mutants are defective for nuclear protein import; in yeast cells, this is assayed by the cytoplasmic accumulation of nuclear proteins or reporter proteins fused to an NLS, visualized by fluorescence microscopy (Yano, 1994). Additional *srp1/kap60* mutants have been isolated on the basis of genetic interactions with a mutant of *NUP1*, encoding a nucleoporin of the FXFG class (Belanger, 1994). Mutants in the yeast importin β gene, *RSL1/KAP95*, also display a protein import defect, and the wildtype Rsl1/Kap95 protein localizes to the nuclear periphery, in a pattern that significantly overlaps with both Srp1/Kap60 and Nup159 (Koepp, 1996b). The yeast homolog of p10, Ntf2, is concentrated at the nuclear envelope as well, and mutating highly conserved amino acids results in a temperature-sensitive phenotype and mislocalization of nuclear proteins to the cytoplasm (Corbett, 1996).

Studies of the Ran-GDP/GTP cycle in yeast suggest a broader role for Ran in nuclear transport. For example, a mutation in the yeast Ran gene *GSP1* which stabilizes the GTP-bound form leads to a concomitant block in nuclear protein import and poly(A) RNA export (Schlenstedt, 1995). A mutant of the yeast RanGAP1 gene, *rna1-1*, displays defects in both protein import and poly(A) RNA export (Amberg, 1992; Corbett, 1995). The yeast RCC1 homologue, Prp20, blocks poly(A) RNA export when mutated (Forrester, 1992). Furthermore, the *rna1* and *prp20* mutants are defective in mRNA transcription initiation and 3'-end formation, and the *rna1* mutant has additional defects in pre-tRNA splicing and pre-rRNA processing (Hopper, 1978; Forrester, 1992). Whether these pleiotropic effects are due to an alteration in Ran per se, as opposed to another GTPase affected by Rna1 and Prp20, is uncertain; however, Ran seems to be the predominant nuclear GTPase, since it is very abundant and highly conserved (Bischoff, 1991b). It is also possible that the defects in RNA processing are an indirect effect of impairing nuclear transport. Nonetheless, it appears that Ran is involved in the general transport of substrates to and from the nucleus, and possibly functions in other nuclear processes as well.

Although significant attention has been given to the import of proteins harboring the pioneer "classic" NLS, more recent work has identified different types of NLSs that utilize alternative receptor-mediated import pathways (listed in Table II). A novel NLS was discovered in one member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins, hnRNP A1. hnRNP proteins bind to RNA polymerase II transcripts, and influence the processing and export of these transcripts (Dreyfuss, 1993). A subset of these proteins, including hnRNP A1, rapidly shuttle between the nucleus and cytoplasm (Pinol-Roma, 1992). The NLS of hnRNP A1, termed M9, is distinct from the classic NLS in both sequence and function; it is a glycine-rich sequence spanning 38 amino acids, and, interestingly, signals both the import and export of hnRNP A1 (Michael, 1995). *In vitro* competition experiments determined that hnRNP A1 enters the nucleus independent of the importin α/importin β pathway (Pollard, 1996). Subsequently, a novel protein, named transportin, was identified as the import receptor for hnRNP A1, based on specific binding to the M9 sequence and its requirement for the import of an M9-tagged reporter protein in permeabilized HeLa cells. Notably, sequence analysis of transportin revealed homology to a region of importin β. The yeast homolog of transportin, Kap104, binds to repeat-containing nucleoporins by blot overlay, and co-purifies with two yeast hnRNP proteins, Nab2 and Nab4 (Aitchison, 1996). Supporting the role for transportin in this specialized import pathway, *kap104* mutants mislocalize Nab2 to the cytoplasm but do not affect the nuclear localization of a GFP reporter fused to the SV40 classic-type NLS.

Homology searches of the yeast genome database resulted in the identification of two additional members of the importin β family, Kap123 and Pse1/Kap121 (Rout, 1997). Studies of Kap123 identified a third import pathway, specifically for the import of ribosomal proteins into the nucleus. Ribosomal proteins must enter the nucleus en route to the nucleolus, where they assemble with rRNAs into ribosomal subunits. Hence, NLSs have been identified in three yeast ribosomal proteins; the ribosomal NLSs do not follow a consensus pattern but are distinct from the classical type or the M9-type of NLS (Schaap, 1991). Kap123 was shown to bind to ribosomal proteins by blot overlay, and it also co-purifies with ribosomal proteins from yeast cytosolic extracts (Rout, 1997). Moreover, *KAP123*-deleted cells (which are viable) display cytoplasmic accumulation of a reporter protein fused with the NLS of ribosomal protein

Table II. Classes of import substrates, their signals and receptors

Substrate	Import signal	Import receptor
various nuclear proteins	classic NLS (basic)	Importin α/β (Kap60/95 in yeast)
hnRNP A1	M9	Transportin (Kap104 in yeast)
ribosomal proteins	ribosomal NLS	Kap123/121 (yeast)
snRNPs	trimethyl-G cap, signal on associated proteins?	?

L25, while Nab2 and an SV40 NLS-GFP fusion are localized to the nucleus. Induced expression of *PSE1/KAP121* in the *KAP123* deletion background enables the L25 NLS reporter protein to enter the nucleus, suggesting an overlapping function between the two genes.

The existence of distinct receptors dedicated to the nuclear import of hnRNP proteins and ribosomal proteins is not surprising, considering these two classes of proteins are highly abundant in the cell. Another type of import substrate of significant abundance is the small nuclear ribonucleoprotein particles (snRNPs), which, once the snRNAs are processed and assembled with proteins in the cytoplasm, enter the nucleus for participation in pre-mRNA splicing. Competition experiments in *Xenopus* oocytes have shown that some of the factors required for snRNP import are distinct from those needed for import of proteins containing the classic NLS (Fischer, 1991; Michaud, 1992). The snRNP-specific import factors have yet to be identified, and the exact snRNP NLS is unknown, although it is likely to consist of both protein and RNA elements (Fischer, 1993). One feature common to snRNP and protein import is the requirement for Ran; like protein import, the import of snRNPs added to permeabilized HeLa cells is blocked by non-hydrolyzable GTP analogs and in the presence of Ran mutants (Palacios, 1996).

Nuclear export of RNAs

The initial experiments delineating the properties of nuclear RNA export involved microinjection of *Xenopus* oocyte nuclei with various RNA molecules. In this system, RNA export has been shown to be protein-mediated, saturable, temperature and ATP-dependent, which strongly predicts an active, receptor-mediated mechanism (Izaurralde, 1995). These properties have been demonstrated for the export of many classes of RNA molecules, including tRNA, 5S rRNA, snRNAs, and mRNA (Zasloff, 1983; Guddat, 1990; Hamm, 1990; Dargemont, 1992). Moreover, competition experiments showed that tRNA, snRNA, and mRNA molecules can specifically inhibit the export of the same class of RNA, but not the export of the other classes of RNA (Jarmolowski, 1994). Thus, RNA export is very likely to be mediated, in part, by distinct factors that are specific for each class of RNA. This implies the existence of multiple RNA export pathways involving substrate-specific receptors, reminiscent of the various protein import pathways.

In the case of 5S rRNA, potential export factors were discovered by utilizing export-defective 5S rRNA mutants. Proteins which fail to bind the mutant RNAs, but could bind wildtype 5S rRNA, were identified. Two such proteins are L5, a ribosomal protein, and TFIIIA, necessary for the transcription of 5S rRNA (Guddat, 1990). Although binding of these proteins to RNA correlates with RNA export ability, one caveat of this approach is that loss of export could be due to nuclear retention of the mutant RNAs by a novel interaction. Thus, it remains to be seen whether these proteins are truly required for RNA export.

In contrast to 5S rRNA export, snRNA export has been characterized more extensively. The spliceosomal snRNAs transcribed by RNA polymerase II (U1, U2, U4, and U5) are capped at the 5' end with 7-methyl guanosine and exported to the cytoplasm, where they are further processed and assembled with proteins into mature snRNPs (Izaurralde, 1995). One signal determined to be important for the export of these snRNAs is the 5' cap structure (Hamm, 1990). Furthermore, competition experiments involving U1 snRNAs carrying different cap structures demonstrated that snRNA export requires a saturable cap-binding factor (Jarmolowski, 1994). This cap-binding complex (CBC) has been purified from HeLa and *Xenopus* cells, and consists of two proteins, CBP20 and CBP80 (Izaurralde, 1992; Kataoka, 1995). Supporting a role for CBC in snRNA export, antibodies against CBP20 which block CBC binding to the cap also specifically inhibit snRNA export (Izaurralde, 1995b). CBC is thought to shuttle between the nucleus and cytoplasm, but the mechanism by which CBC mediates snRNA export has yet to be clarified (Visa, 1996b). Notably, the yeast homologs of CBP20 and CBP80 are not essential for cell viability (Colot, 1996; Gorlich, 1996c).

Recently, an interesting link has been established between snRNA export and protein import, involving CBC and the α/β importins (Gorlich, 1996c). Both yeast and HeLa importin α were found in a complex with CBC and capped RNA in the nucleus, via an interaction between importin α and the classic type-NLS of CBP80. Strikingly, the RNA was released from the complex when importin β was added, but not when Ran-GTP was present. Since Ran-GTP is mainly nuclear, this implies that the importin α/CBC/RNA complex is stable in the nucleus. However, once exported to the cytoplasm, importin β can release the RNA from the complex, and CBC is free to be re-imported to the nucleus by the importin pathway. Thus, the unidirectional transport of RNA is ensured by the same mechanism that mediates recycling of CBC back to the nucleus. This elegant model is supported by the observation that snRNA export in *Xenopus* oocytes is inhibited under conditions that block nuclear entry of importin α (Gorlich, 1996c).

Of all the studies of RNA export, the most rapid advances have been made in the area of mRNA export, due to the added advantage of utilizing yeast genetics and biochemistry. This is mainly a consequence of the ability to visualize bulk poly(A) RNA in yeast cells. The standard method entails *in situ* hybridization of a digoxygenin-labeled oligo dT50 probe to poly(A) RNA; the location of the RNA is then visualized by fluorescence microscopy, detecting anti-digoxygenin antibodies with a fluorescently-conjugated secondary antibody (Amberg, 1992). In wildtype cells, the fluorescent signal is seen throughout the cell, or on occasion exclusively in the cytoplasm. In cells unable to export poly(A) RNA, the signal is confined to the nucleus. Banks of conditionally lethal yeast mutants have been screened for this phenotype, in pursuit of factors involved in mRNA export (Amberg, 1992; Kadowaki, 1994; A. de Bruyn Kops, unpublished data). In addition, this assay has been used to ascertain whether factors implicated in nuclear transport, such as nucleoporins, also affect mRNA export (Izaurralde, 1995). The remainder of this section will focus on mRNA export, starting with a discussion of the known export signals, and proceeding with a review of the factors currently thought to play a role in the mRNA export pathway.

In considering what signals could determine the active export of mRNA, two general classes can be envisioned. First are specialized structural features of the mRNA, and second are signals within the proteins that interact with mRNA. The structural features of mRNAs, transcribed by RNA polymerase II, include a 7-methyl guanosine cap at the 5' end and a poly(A) tail at the 3' end (in most cases). *Xenopus* microinjection experiments showed that mRNA export is much less dependent on the 5' cap than snRNA export; although the 5' cap, as well as the poly(A) tail, stimulate mRNA export rates, they are not absolutely required for export per se (Jarmolowski, 1994). Although other *cis*-acting signals may be required, the failure to identify one thus far has led increasingly to the idea that export signals reside in the proteins that associate with mRNAs.

Major support for this idea came from the identification of a nuclear export signal (NES) in PKI, a mammalian protein kinase inhibitor. This NES, consisting of a short stretch of regularly spaced leucine residues, is necessary and sufficient to mediate the rapid nuclear export of reporter proteins microinjected into cultured fibroblasts (Wen, 1995). Supporting the model that NES-containing proteins also direct the export of RNA, HIV Rev protein, which binds and mediates the export of unspliced viral pre-mRNAs, possesses a functional NES essential for Rev activity (Fischer, 1995). Additionally, TFIIIA, the protein implicated in 5S rRNA export, harbors a sequence very similar to the PKI/Rev NES (Fridell, 1996), although whether it is a functional export signal has yet to be determined.

Two other types of NESs have since been discovered in mammalian hnRNP proteins, which bind directly to cellular mRNAs. Best characterized is the M9 sequence of hnRNP A1, a protein that has been shown to associate with newly transcribed mRNAs, accompany the RNA through the nuclear pores to the cytoplasm, and shuttle back to the nucleus (Pinol-Roma, 1992; Visa, 1996). The M9 sequence is unlike the Rev NES; as previously mentioned, it is a glycine-rich sequence, and appears to function as both the nuclear export and import signal for hnRNP A1 (Michael, 1995). In addition, an NES comprised of a 24 amino acid sequence unrelated to M9 or to the leucine-rich motif has been identified as the export/import signal for hnRNP K (Michael, 1997).

The presence of NESs in mRNA-binding proteins underscores the importance of *trans*-acting factors in the export of mRNAs. The numerous genetic and biochemical approaches employed to identify yeast transport factors has lengthened the list of proteins implicated in mRNA export (Izaurralde, 1995). The lack of *in vitro* RNA export assays has made it difficult to assign precise roles for these factors, however, those that are best understood thus far can be placed into three categories. First are members of the hnRNP family, second are importin β homologs that act as receptors for export substrates, and third are the nucleoporins likely to be directly involved in the export pathway.

The hnRNP family of proteins was originally identified in HeLa extracts by virtue of uv-crosslinking to poly(A) RNA *in vivo* (Dreyfuss, 1993). hnRNP proteins are now known to be highly conserved and abundant RNA-binding proteins. They harbor at least one of three RNA-binding motifs, the most common being the RNP domain, also called the RNA recognition motif (RRM); less common are the KH motif and the RGG box (Burd, 1994). While a subset of hnRNP proteins shuttle between the nucleus and cytoplasm, such as hnRNP A1, others remain nuclear. Interestingly, for one of the non-shuttling hnRNP proteins, hnRNP C, nuclear retention appears to be an active process; it contains a nuclear retention sequence (NRS) that can override the hnRNP A1 NES, since fusion of the NRS to A1 blocks its export to the cytoplasm (Nakielny, 1996).

mRNA molecules are continuously packaged with hnRNP proteins, from the point of transcription in the nucleus, through the NPC, and in the cytoplasm (Pinol-Roma, 1992; Visa, 1996). This association of hnRNP proteins with mRNA is dynamic, since the strictly nuclear hnRNP proteins dissociate from the messenger RNP (mRNP) complex before or at the NPC, while others, such as hnRNP A1, accompany the mRNA all the way to the cytoplasm (Daneholt, 1997). Although the exact functions of hnRNP proteins are unknown, they are believed to influence the processing and export of mRNAs. For example, mammalian nuclear extracts depleted for hnRNP C are unable to form spliceosomes, and hnRNP A1 influences the regulation of 5' splice site choice *in vitro* (Dreyfuss, 1993). In addition to harboring an NES, a role in mRNA export for hnRNP A1 was demonstrated by competition experiments in which saturating amounts of A1 protein microinjected into *Xenopus* oocyte nuclei inhibited the nuclear export of mRNA (Pante, 1997).

More evidence for the involvement of hnRNP proteins in mRNA export has been provided by experiments in yeast. A handful of essential yeast proteins that display some of the same characteristics as metazoan hnRNP proteins have been identified, including Npl3, Nab2, and Nab4/Hrp1 (Bossie, 1992; Anderson, 1993; Henry, 1996). Mutant alleles of *NPL3* and *NAB2* accumulate poly(A) RNA in the nucleus (Wilson, 1994; Lee, 1996). Nab4/Hrp1 is closely connected with these proteins, since it interacts with Nab2 by two-hybrid analysis (M. Swanson, personal communication), and a mutant allele of *NAB4/HRP1* was isolated as a suppressor of *npl3-1* (Henry, 1996).

Significantly, there is evidence that, like hnRNP A1, all three of these proteins shuttle between the nucleus and cytoplasm. Specifically, yeast heterokaryon experiments demonstrated that Npl3 shuttles (Flach, 1994). In addition, employing yeast strains defective in nuclear protein import, Npl3, Nab2, and Nab4 accumulated in the cytoplasm upon induction of the import block (Aitchison, 1996; Lee, 1996). In striking contrast to hnRNP A1, the yeast hnRNP proteins do not contain a sequence similar to the M9 NES/NLS. Furthermore, no compelling M9 homologies have been found in the yeast protein database (C. Siebel, personal communication). This suggests a diversion from the mammalian mechanism, in that the yeast proteins may utilize a novel export/import signal, or perhaps associate with proteins that contain these signals.

The second class of factors involved in mRNA export are members of the importin β family of transport receptors. It was unclear whether this family of receptors functions in nuclear export until very recently, with the identification of Xpo1, also called Crm1, as the newest member (Stade, 1997). Unlike the rest of the importin β family, Xpo1 seems to be required for nuclear export, not import. Utilizing an engineered protein export substrate, consisting of the PKI NES and a classic NLS fused to GFP, a temperature-sensitive *xpo1* yeast mutant was shown to display a strong and rapid block in the export of the reporter when shifted to the non-permissive temperature, with only a slight protein import defect at later times. Characteristic of the importin family, the yeast Xpo1 protein specifically interacts with both its putative substrate, the PKI NES, and Ran in a two-hybrid assay, and was shown to shuttle between the nucleus and cytoplasm. Substantiating these results, the human homolog of Xpo1 binds cooperatively to Rev/PKI NES peptides and Ran-GTP *in vitro* (Fornerod, 1997b). The requirement for Ran-GTP to form this complex is consistent with its assembly occurring only in the nucleus, which would ensure the unidirectional transport of the complex. Supporting this model and a general role for Ran-GTP in export, the conversion of nuclear Ran-GTP into Ran-GDP in *Xenopus* oocytes or mammalian cells leads to inhibition of NES-protein export as well as inhibition of the export of several mRNAs (Izaurralde, 1997; Richards, 1997). Human Xpo1 also binds to nucleoporins, another feature common to the importin β family (Fornerod, 1997).

Xpo1 binding to the Rev-like NES suggests a possible involvement in RNA export. Indeed, the *xpo1* mutant also accumulates poly(A) RNA in the nucleus with the same kinetics as the protein export block (Stade, 1997). However, inconsistent with a role in mRNA export, human Xpo1 affects snRNA and Rev protein export, but not mRNA export in *Xenopus* oocytes (Fornerod, 1997b). At this point, the reason for this discrepancy is unknown; it is possible that yeast and metazoans employ distinct mechanisms for mRNA export.

Two other members of the importin β family, Pse1/Kap121 and Kap 123 have been implicated in mRNA export (Seedorf, 1997). A yeast strain deleted for *KAP123* and harboring a temperature-sensitive allele of *PSE1/KAP121* rapidly accumulates poly(A) RNA in the nucleus. Since these factors are also involved in ribosomal protein import, they may have dual transport functions. One interesting possibility is that they couple mRNA export with ribosomal protein import, much like the coupling of snRNA export with classic NLS-protein import via importin α.

A third category of proteins involved in the mRNA export pathway are nucleoporins. The extensive influence of nucleoporins on mRNA export is evident from the results of a screen for poly(A) RNA export mutants among a bank of temperature-sensitive yeast strains: approximately half of the export-defective strains isolated (called *rat*, for ribonucleic acid trafficking) contain mutations in nucleoporin genes (listed in Table I, also see Doye, 1997). Overall, of the 30 or so yeast nucleoporins isolated thus far, mutations in 12 of them result in a poly(A) RNA export defect (Table I). Since most of these mutations also cause aberrant nuclear envelope and NPC morphology, and/or defective protein import, a direct effect on mRNA export is difficult to assess (Wente, 1993; Siniossoglou, 1996). Nonetheless, there is evidence that nucleoporins could function directly in RNA export. For example, certain yeast nucleoporin mutants, such as *nup159-ts*, block poly(A) export abruptly after a shift to the non-permissive temperature (Gorsch, 1995). Furthermore, microinjecting antibodies specific to vertebrate Nup98, of the GLFG class, into *Xenopus* oocyte nuclei blocks mRNA, rRNA, and snRNA export without affecting protein import (Powers, 1997).

Another line of evidence pointing to nucleoporin involvement in RNA export is the observed associations between RNA export factors and nucleoporins. As previously mentioned, human Xpo1 directly binds to FG-repeat nucleoporins (Fornerod, 1997). Additionally, in two-hybrid assays, Rev interacts with numerous FG-repeat nucleoporins located at both the nuclear and cytoplasmic faces of the NPC, including mammalian Nup98 and yeast Nup159; these interactions are dependent on the Rev NES (Stutz, 1995; Fritz, 1996). The Rev data support the idea of NES-mediated translocation through the NPC, possibly via Xpo1. An interesting extension of this model postulates that translocation occurs by sequential interactions with nucleoporins (Fritz, 1996).

The various export substrates, their associated factors, signals, and putative receptors are summarized in Table III. Although the identification of export factors is steadily increasing, clearly many questions remain unanswered. In the case of mRNA export in yeast, the nature of the export signal, whether it resides in hnRNP proteins or associated proteins, has yet to be discovered. Also, a specific role for Xpo1 or Kap123/121 in yeast mRNA export is still unclear. In general, the role of Ran-GTP in export and the mechanism of translocation across the NPC is unknown. Even the known export factors are likely to have as yet unidentified partners, working together as complexes in the various export pathways. Thus, despite the recent progress in the RNA export field, these issues are among many that still need to be resolved.

Nuclear mRNA processing events: coupled to the export pathway?

Before mRNAs are exported to the cytoplasm, they must undergo specific processing events in the nucleus. As mRNA molecules are synthesized by RNA polymerase II, they are packaged with hnRNP proteins. The mRNA precursors are capped at the 5' end, and the 3' ends of virtually all of them are formed by endonucleolytic cleavage and polyadenylation. Furthermore, the pre-mRNAs containing introns are spliced. Although these processing events consist of relatively simple enzymatic reactions, surprisingly large and dynamic macromolecular complexes are required to carry them out. The

Table III. Export substrates and export factors

Substrate	Binding proteins	Export signal	Putative receptor
5S rRNA	TFIIIA L5	Leu-rich? ?	? ?
snRNAs	CBC/ Importin α	?	?
PKI		Leu-rich	Xpo1
viral pre-mRNA	Rev	Leu-rich	Xpo1
cellular mRNA: mammals	hnRNP A1 hnRNP K	M9 KNS	? ?
cellular mRNA: yeast	Nab4/Hrp1, Nab2, Npl3	?	? (Xpo1, Kap123/121)

components of these complexes, especially those involved in 3' end formation and splicing, have been the focus of intense research for the past 10 to 20 years (Keller, 1995; Guthrie, 1996). Most of this work has treated these processing machineries as separate entities, however it is becoming increasingly apparent that they are tightly coupled (Steinmetz, 1997). Coupling in this case refers to coordination between the different processing machineries, most likely to ensure complete and accurate processing of each mRNA; this coordination probably occurs via physical interactions between components of the different processing reactions. The exact mechanism of this coupling, and how the processed mRNAs are transferred to the export pathway, are imperative questions just starting to be examined.

The 3' ends of all eukaryotic mRNAs (except those encoding metazoan histones) are processed by endonucleolytic cleavage and polyadenylation of the 5' cleavage product. In mammalian mRNAs, consensus sequences direct where this occurs; the highly conserved hexanucleotide AAUAAA is found just upstream of the cleavage site, and a GU-rich stretch resides downstream of the cleavage site (Proudfoot, 1991). In contrast, the yeast poly(A) signals are much more degenerate, and hence less defined. Also, while the poly(A) tails synthesized on mammalian mRNAs are approximately 250 nucleotides long, yeast poly(A) tails consist of only 70 to 90 nucleotides (Wickens, 1990). Despite hese differences, the factors that bind to the RNA elements, and other omponents necessary for 3' end formation are generally conserved between east and mammals (Manley, 1996).

dentification of the factors involved in 3' end formation has been greatly cilitated by the development of *in vitro* systems that efficiently reproduce site-specific cleavage and polyadenylation (Moore, 1985; Butler, 1990). These assays utilize either yeast or HeLa cell-free extracts which accurately cleave and polyadenylate an exogenous mRNA transcript. From these extracts, specific fractions necessary for cleavage, polyadenylation, or both, have been isolated. Much progress has been made in the identification of the polypeptides that comprise each of these fractions, which currently number over a dozen each for the mammalian and yeast systems. A comprehensive list of these factors, and the fractions from which they were purified, can be found in a review by Keller and Minvielle-Sebastia (1997). Remarkably, the endonuclease responsible for the cleavage reaction is still undetermined. The enzyme that catalyzes the polyadenylation reaction is poly(A) polymerase (PAP); however, multiple accessory factors are needed to make PAP substrate-specific and processive (Lingner, 1991; Wahle, 1991; Wahle, 1991b). In general, the accessory factors of the 3' processing machinery have a wide range of functions, including RNA recognition and binding, formation of protein-protein interactions to promote complex assembly, cleavage specificity, and oly(A) tail length control (Keller, 1997).

addition to 3' end formation, the pre-mRNAs containing introns are
ocessed by the splicing machinery. At the most basic level, the excision of
ons from pre-mRNAs occurs by two successive transesterification
tions (Moore, 1993b). Despite this simplicity, pre-mRNA splicing in
ryotes requires an elaborate assembly called the spliceosome, consisting
e snRNAs and an estimated 100 proteins (Moore, 1993a). As is the case
e 3' processing machinery, the mechanism of splicing and the factors
ved are turning out to be conserved between yeast and higher
yotes. This was initially unexpected, since, in contrast to mammalian

pre-mRNAs, less than 5% of yeast pre-mRNAs posses introns. However, those that do are highly abundant, encoding ribosomal proteins and structural proteins, such as actin and tubulin.

Central to the two splicing reactions are the spliceosomal snRNAs, U1, U2, U4, U5, and U6 (Madhani, 1994). Containing sequences complementary to those in pre-mRNAs, all snRNAs except U4 are able to directly base-pair with the exonic and intronic pre-mRNA sequences essential for splicing. Furthermore, base-pairing interactions among the snRNAs aid in the correct positioning of the snRNAs at the pre-mRNA. For splicing to occur, these RNA-RNA interactions must be disrupted to form new, mutually exclusive structures. For example, U6 enters the spliceosome tightly basepaired with U4. Prior to the first catalytic step, the U4/U6 structure is disrupted, and U6 forms new interactions with U2 and the 5' splice site of the intron, resulting in the placement of U6 near the reactive nucleotides (Madhani, 1992).

In order to achieve efficient and accurate splicing, the multiple RNA-RNA rearrangements must occur with precision in the correct order. This requires he action of the 50 to 100 spliceosomal proteins. Numerous genetic screens, s well as homology to mammalian splicing factors, have identified many of hese proteins in yeast (Rymond, 1992); most have been verified as splicing ctors based on their requirement for splicing exogenous pre-mRNAs *in tro.* Some of these proteins are core constituents of snRNP particles, others e required for proper spliceosome assembly, and yet others act transiently at e first or second steps of splicing. Late-acting splicing proteins are involved release of the spliced messages from the spliceosome and recycling of NPs for further rounds of splicing (it is this group that is likely to be most important in the transfer of fully processed mRNAs to the export pathway). The splicing proteins contain various sequence motifs, but one group is especially notable. This group of six proteins, functioning at different stages of the splicing cycle, belong to the DEAD-box family of RNA-dependent ATPases (Strauss, 1991; Schmid, 1992; Noble, 1996). The founding member of this family, eIF4a, has also been shown to unwind RNA duplexes in an ATP-dependent manner. Thus, these splicing proteins are prime candidates for mediating the remodeling of RNA-RNA structures in the spliceosome.

The coupling of mRNA processing events is proving to be essential in the efficient maturation of mRNAs. This is best exemplified by the functional and physical interactions observed between the C-terminal domain (CTD) of RNA polymerase II and factors required for pre-mRNA splicing and 3' processing. Strikingly, in mammalian cells where the only functional RNA polymerase II is CTD-truncated, both splicing and 3' processing of mRNAs are inhibited (McCracken, 1997). SR-type splicing factors, which function in spliceosome assembly, co-immunoprecipitate with the CTD in mammalian uclear extracts (Mortillaro, 1996). Furthermore, proteins in two fractions ecesssary for 3' processing co-purify with the CTD in mammalian nuclear xtracts (McCracken, 1997). Direct binding was demonstrated between the TD and two cleavage stimulatory factors (CstF), p77 and p64. A recent report rovides strong evidence for the CTD targeting 5' capping enzymes to mRNA anscripts as well (McCracken, 1997b); CTD-truncation decreases the fraction capped mRNAs, and capping enzymes from human, mouse, and yeast ysically associate with the CTD.

Much less is known about how mRNAs are directed from the sites of processing to the export pathway. Two general models can be envisioned: first, that mRNA processing and export are coupled by active coordination between processing and export factors, or second, that release of mature mRNAs from the site of processing is followed by a passive transfer to the export machinery, without any physical interactions between processing and export factors.

The main prediction of the first model is a direct interaction between components of the processing and export machineries. This interaction could occur at an early stage of mRNA processing, like the association of capping, 3' processing, and splicing factors with RNA polymerase II during transcription. Early coupling of mRNA processing and export could be mediated by factors that associate with the pre-mRNA during or soon after transcription, and affect both mRNA processing and export; hnRNP proteins are poised for such a role. In support of this model, Nab4/Hrp1 has recently been identified as a protein in a fraction of yeast cell extracts required for 3' cleavage and polyadenylation, designated CF1B (Kessler, 1997). Also, Npl3 exhibits homology to the metazoan SR-family of splicing factors, and *npl3* mutant extracts splice certain pre-mRNAs inefficiently (C. Siebel, unpublished data). lternatively, coupling of mRNA processing and export could occur after rocessing is completed, with a processing factor actively handing-off the RNA to an export factor. In this case, the processing factors involved are kely to be those that act late in the processing reactions, such as a lyadenylation factor or a late-acting splicing factor. For most transcripts, it not known which proceeds first, splicing or polyadenylation, or whether y occur concomitantly. Also, it is important to note that the majority of mRNAs in yeast do not contain introns, and thus do not associate with splicing factors. Therefore, it is worth examining polyadenylation factors for effects on mRNA export, such as testing if mutants in yeast polyadenylation factors are also defective in poly(A) mRNA export, and determining if polyadenylation factors associate with a putative mRNA export receptor like Xpo1.

Regarding the second model, that mRNAs are passively transfered to the export pathway after release from the processing machinery, there is evidence to suggest that intron-containing mRNAs are sequestered from the export machinery until fully spliced mRNAs are released from the spliceosome. Deletion of the 5' splice site of an intron-containing reporter mRNA in yeast, presumably unable to assemble with splicing factors, results in the export of the pre-mRNA to the cytoplasm (Legrain, 1989). Also, a mutation in *PRP22*, encoding a member of the DEAD-box protein family involved in the release of mRNAs from the spliceosome, causes a block to poly(A) RNA export in yeast (A. de Bruyn Kops, unpublished data). However, a retention model does not preclude an active hand-off mechanism between splicing and export factors. On the contrary, Prp22 could function in the transfer of the spliced mRNA to the export pathway; it would be interesting to determine if Prp22 directly interacts with an mRNA export factor, potentially Brr3. Nonetheless, to date there is no evidence to distinguish between a passive or active hand-off mechanism.

Having introduced the *brr3-1* project and given a review of the nuclear transport literature, a hopefully informative preamble to my results, I now turn to the main chapters of my thesis. As evident in Chapters 1 and 2, my experimental path took some unexpected twists and turns. Chapter 1 primarily describes the Brr3 sequence, secondary structure predictions, and homologies. Devoid of any blazingly obvious motifs, such as an FXFG repeat or an RNP domain, a small ray of hope came in the form of a short leucine-rich region similar to the Rev/PKI NES, first identified by Murphy and Wente (Murphy, 1996). Chapter 2 includes data that further substantiates a primary role for Brr3 in mRNA export. The *brr3-1* mutant is synthetically lethal in combination with a number of nucleoporin mutants implicated in mRNA export. Moreover, Brr3 shares some features of nucleoporins, such as localization to the nuclear rim and detergent extraction. The *brr3-1* splicing defect is not as robust as originally anticipated, both *in vivo* and *in vitro*. Quick to overshadow this phenotype is the one involving poly(A) tail length; the longer poly(A) tails discovered in *brr3-1* cells is likely due to a block in poly(A) tail shortening, seen when assaying 3'-end formation *in vitro*. Therefore, Brr3 may be a coupling factor, linking mRNA export with poly(A) tail shortening, possibly at the NPC. All in all, many questions still remain concerning the primary function of Brr3, such as whether it acts anywhere in the mRNA export pathway besides the NPC, whether it is involved in the export of other RNAs, and the precise nature of its interaction with the mRNA processing machineries. Hopefully, others will use my work as a guide to the ultimate answers.

CHAPTER 1

Brr3 sequence analysis

Abstract

The *brr3-1* mutant is defective in both poly(A) RNA export and pre-mRNA splicing, suggesting that the Brr3 protein could play a primary role in export, splicing, or both processes. To determine if the *BRR3* gene is homologous to any known export and splicing factors, it was cloned by complementation of the *brr3-1* cold-sensitive growth defect and sequenced. One striking feature of the secondary structure predicted for Brr3 is a long α-helical rod in the center of the protein. It was also determined that the Brr3 sequence is not homologous to any known protein, and does not contain any motifs common to splicing and export factors, except for a Rev/PKI-like nuclear export signal. Identification of the *brr3-1* mutation, an arginine to lysine change at the C-terminal end, points to a possible RNA-binding function for that region.

Introduction

The *brr3-1* mutant was isolated from a bank of cold-sensitive yeast strains in wo independent screens. It was first identified as a mutant defective in pre-nRNA splicing (Noble, 1996), and additionally isolated in a screen for nutants that accumulate poly(A) RNA in the nucleus (A. de Bruyn Kops and . Guthrie, manuscript in preparation). The mutant displays a rapid onset of e export defect and a strong first step splicing block at the non-permissive mperature of 16°C. The original mutant strain has been outcrossed, and the licing and export phenotypes co-segregate with the cold-sensitivity.

Furthermore, a clone from a yeast genomic library that complements the cold-sensitive growth defect has been isolated (Noble, 1996).

Based on the strong export and splicing defects, it was initially thought that the *brr3-1* mutant may directly affect mRNA export, pre-mRNA splicing, or possibly both. The yeast splicing pathway has been carefully dissected and a large number of splicing proteins are known (Moore, 1993a). Of these, twelve mutants in *PRP* (pre-mRNA processing) genes have been tested for a poly(A) RNA export defect; only two demonstrate nuclear accumulation of RNA, *prp22* and *prp27*, both of which act late in the splicing pathway (A. de Bruyn Kops, unpublished data). This points to a possible link between the splicing machinery and export pathway, the coupling of which has not been studied. The mRNA export field is relatively new, but an increasing number of yeast mutants with a poly(A) RNA export defect have been identified, including numerous nucleoporins, hnRNP-like proteins, and factors involved in the Ran GTPase cycle (Izaurralde, 1995).

first step in determining the primary role of Brr3 includes sequence alysis and homology searches, to assess any relation to previously entified splicing or export factors. An obvious motif to expect is one or ore of the known RNA-binding domains present in splicing factors as well hnRNP proteins, which function in both splicing and export (Burd, 1994). er sequences of interest are the eIF4A-like RNA helicase motifs, shared by bset of splicing factors including Prp22 (Company, 1991). Features which ld firmly place Brr3 in the export pathway are the GLFG or FXFG repeats cleoporins (Doye, 1997), or homologies to factors involved in the Ran

GTPase cycle, such as GTPase activating proteins or guanine-nucleotide exchange factors (Koepp, 1996).

This chapter describes the subcloning and sequencing of the *brr3-1* complementing clone, results of inactivating the gene, and secondary structure predictions of the encoded protein. Unfortunately none of the expected motifs were found in the Brr3 protein sequence; however, it contains a nuclear export signal, strongly implicating a direct role for Brr3 in mRNA export (Murphy, 1996). Finally, sequencing of the *brr3-1* mutant allele revealed a single conservative mutation, from arginine to lysine, which raises the interesting possibility of a novel RNA-binding sequence in Brr3.

Results/Discussion

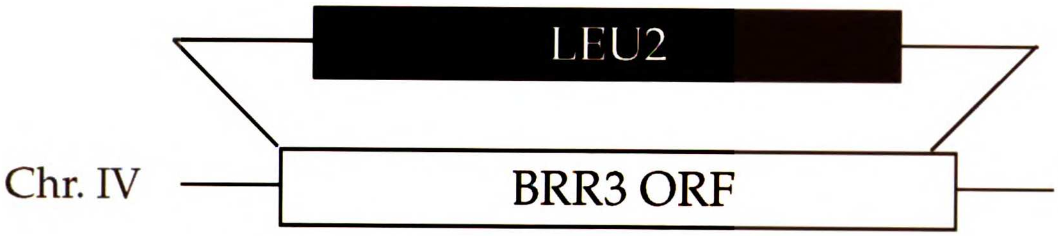
The BRR3 sequence encodes an essential gene

Subcloning of the original *brr3-1* complementing clone narrowed down the complementing region to 2.6 kb. This DNA fragment, on a centromeric plasmid, was capable of restoring wildtype growth to the *brr3-1* mutant strain t 16°C (data not shown). Both strands of the DNA was fully sequenced, evealing an open reading frame (ORF) of approximately 1.6 kb. Subsequent lease of the complete *S. cerevisiae* genome sequence confirmed the accuracy the *brr3-1* complementing sequence, and identified the ORF as a locus of romosome IV (Jacq, 1997).

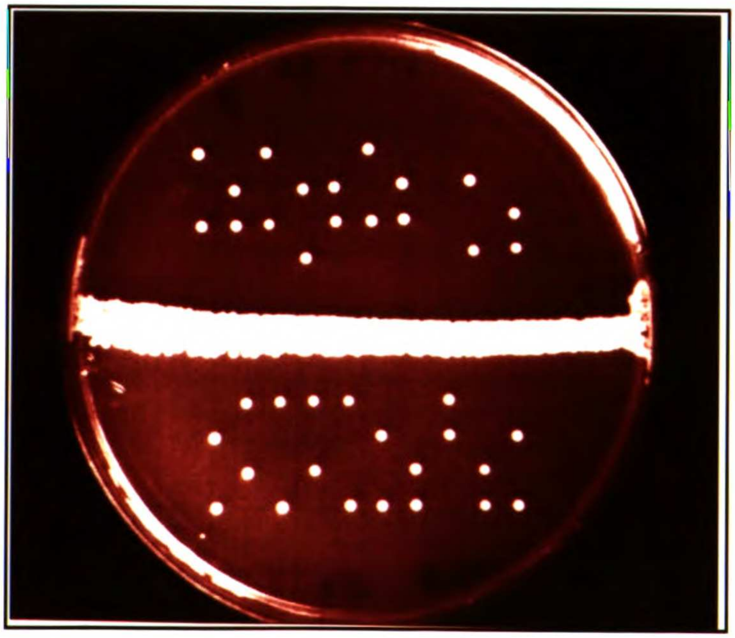
To determine whether the *brr3-1* complementing ORF was required for cell viability, a chromosomal copy of the gene was inactivated by targeted replacement with the *LEU2* gene (Figure 1A). All but the last 150 nucleotides of the ORF were substituted at one of the two loci in a wildtype diploid cell, as confirmed by Southern analysis (data not shown). The diploid was sporulated, and tetrads were dissected. Only two of the four spores in each tetrad grew on rich media at 30°C (Figure 1B). These spores were unable to grow on media lacking leucine, and thus did not contain the inactivated copy of the complementing ORF. This confirmed that the gene is essential for cell viability. Importantly, a cross between the deletion strain and the *brr3-1* mutant strain demonstrated co-segregation of the *LEU2* gene with the cold-sensitive *brr3-1* mutant allele. Of the 54 viable spores from 18 dissected tetrads, 33 were cold-sensitive; all of the cold-sensitive spores were also Leu$^-$. Thus, the *brr3*::*LEU2* deletion is closely linked to the *brr3-1* cold-sensitive mutation, and the complementing ORF is very likely the wildtype *BRR3* gene.

Figure 1: The *BRR3* gene is required for cell viability. One copy of the *BRR3* gene, located on chromosome IV, was inactivated in a wildtype diploid strain by replacement with the *LEU2* gene. The resulting heterozygous diploids were sporulated, and tetrads were dissected and germinated on YEPD plates at 30°C. *(A)* Schematic of the *BRR3* gene replacement. *(B)* Growth of tetrads on a YEPD plate, demonstrating viability of only two of the four spores of each tetrad.

A.


LEU2
Chr. IV
BRR3 ORF

B.



Characteristics of the Brr3 protein

The *BRR3* gene encodes a novel, 538 amino acid protein with a predicted molecular weight of 62 kD and pI of 9.15 (Figure 2A). A central region of the protein, spanning amino acids 150 to 240, is highly charged, with a striking prevalence of arginine, lysine, and glutamic acid residues. This lends to the strong prediction of an α-helical secondary structure throughout this region (Figure 2B). Moreover, analysis of the protein sequence using the COILS algorithm (Lupas, 1991) results in an extremely high probability of the α-helical region forming a coiled-coil structure (Figure 3A). Coiled-coil structures are defined as a series of 2 to 5 α-helices that interact with each other to form a bundle. Driving the packing of these helices is a hydrophobic core, with adjacent hydrophobic residues of one helix interacting with the corresponding hydrophobic residues of another helix. The signature primary structure of coiled-coils is a series of heptad repeats, with hydrophobic amino acids at two adjacent positions of the helix (usually residues 1 and 4 of the repeat), and hydrophilic residues at the other positions to form the solvent-exposed part of the coil (Lupas, 1996).

Coiled-coils are predominant in structural proteins, such as myosins and intermediate filaments, where they contribute to the rigidity of these fibrous proteins (Stewart, 1993). Much smaller coiled-coils mediate the dimerization of the leucine-zipper family of transcription factors (O'Shea, 1991). Most interestingly, coiled-coils have been predicted to form in a subset of nucleoporins (Doye, 1997). Of these nucleoporins, the putative coiled-coil domain of Nsp1 has been best characterized, where it is found at the C-

Figure 2: The predicted α-helical region of Brr3. *(A)* The Brr3 protein sequence, with the stretch of charged residues underlined. The NES sequence is boxed. Also marked is the *brr3-1* mutation, a single amino acid substitution at residue 517. *(B)* Graphical representation of the probablility of α-helix (in red) and β-sheet (in blue) structures in Brr3, according to the Chou and Fasman algorithm (Chou, 1978). Note the uninterrupted run of α-helix forming residues from amino acids 150 to 240.

A.

1 MRFVFDEVFNSDTDSPEFEETCSTTSSTSSQCPTPEPSPAIKLPSFTKVG
51 TKKLVNESVVILDPALENALRDLNLQSKLIPINEPIVAASSIIVPHSTNM
101 PLPRASHSSLLDNAKNSNATAPLLEAIEESFQRKMQNLVLANQKEIQSIR
151 ENKRRVEEQRKRKEEEERKRKEAEEKAKREQELLRQKKDEEERKRKEAEA
201 KLAQQKQEEERKKIEEQNEKERQLKKEHEAKLLQQKDKLGKAVTNFDKIS
251 KMFWHYKDKIAQIKQDIVLPIKKADVNVRNLLSRHKRKINPKFGQLTNSN
301 QQLFKIQNELTQLINDTKGDSLAYHWILNFIAKAVVHQAETEVRVKPESA
351 LPLGKLTLYLLVQFPELQELFMARLVKKCPFVIGFTCEIDTEKGRQNMGW
401 KRNNENKWEDNTSYDERMGGILSLFAIITRLQLPQEFITTTSHPFPIALS
451 WHILARICNTPLNLITNTHFVILGSWWDAAAVQFLQAYGNQASKLLILIG
501 EELTSRMAEKKYVGAARLRILLEAWQNNNMESFPEMSP$_{538}$

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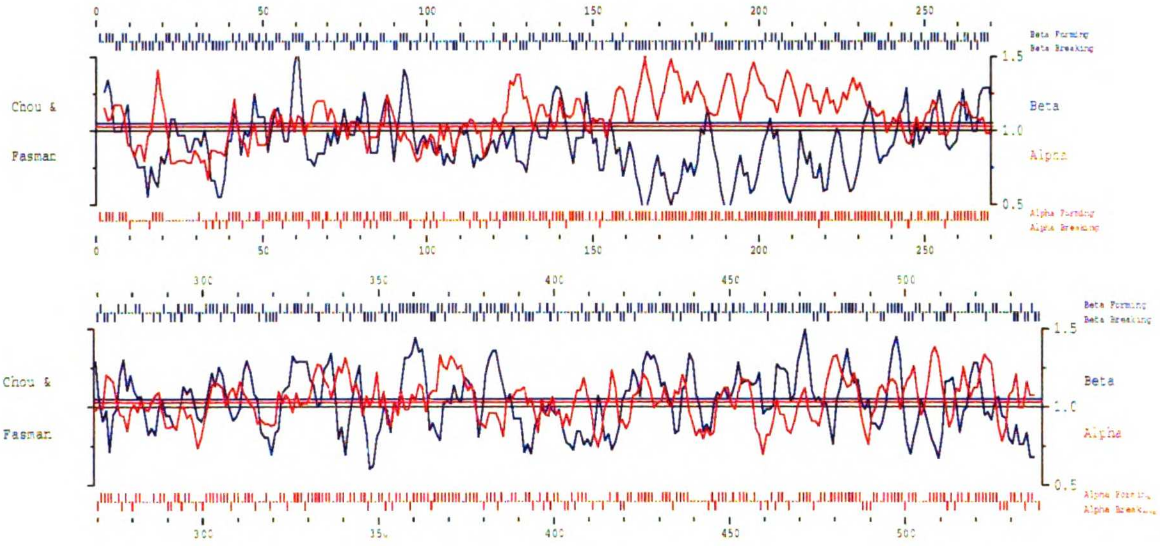
K
brr3-1

B.



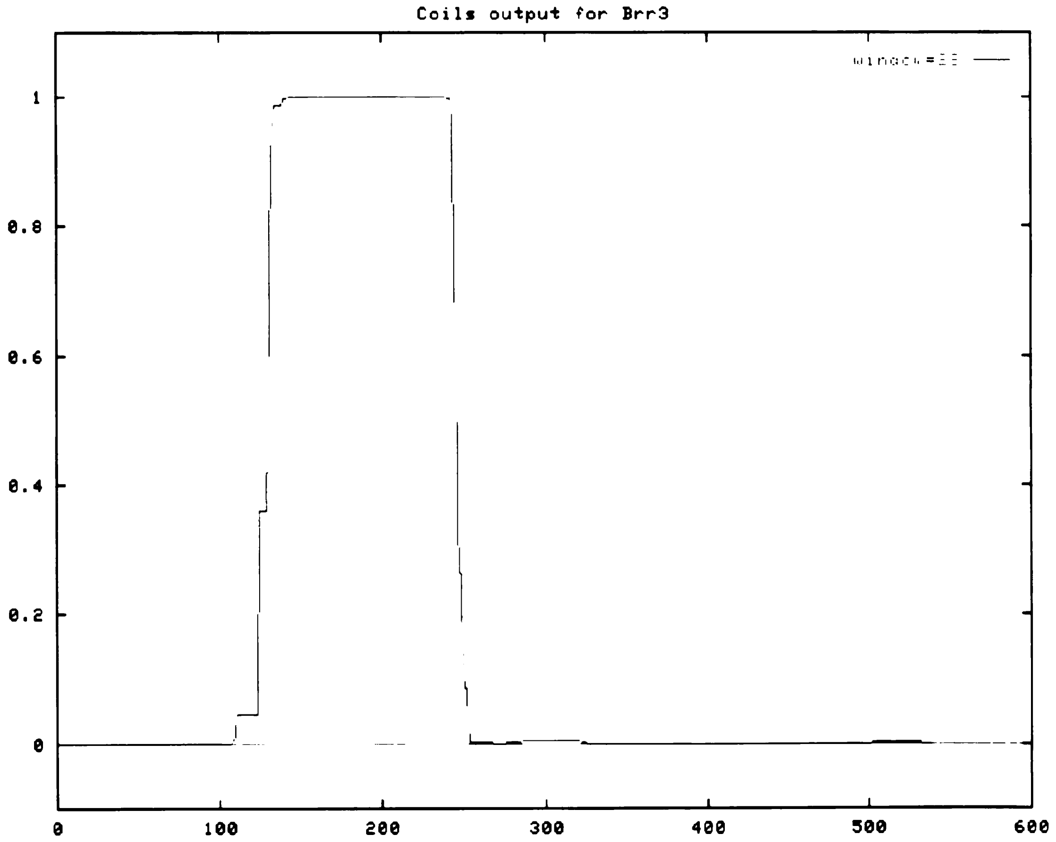
terminus (Figure 4A). Intriguingly, this domain is the only part of the protein essential for cell viability, and it mediates physical interactions with Nup49, Nic96, Nup57, and Nup82, all of which are also predicted to contain coiled-coil structures (Nehrbass, 1990; Grandi, 1993; Grandi, 1995b).

Despite this tantalizing connection to nucleoporins, a closer inspection of the potential coiled-coil region of Brr3 revealed a heptad repeat pattern inconsistent with the formation of this structure (Figure 3B). Specifically, the hydrophilic residues are dispersed throughout the helix, and there is no clear clustering of hydrophobic residues. This is in contrast to the predicted coiled-coil region of Nsp1, which distinctly follows the heptad repeat pattern of a hydrophobic face, in this case at positions 1 and 5 of the helices, flanked by hydrophilic residues (Figure 4B). The high score given to Brr3 by the COILS program is undoubtedly the result of the abundance of charged amino acids in that region. Since five of the seven positions in a coiled-coil helix are occupied by hydrophilic residues and the program gives equal weight to all positions, it has proven to be biased towards stretches of charged residues (Lupas, 1997). However, this region of Brr3 is still likely to form a long α-helical rod, which may be required for assembly into a multi-protein complex.

Thus far, searches for Brr3 homologies in any known bacterial, yeast, or metazoan proteins have yielded very little. The original hope was to find motifs consistent with a role in mRNA splicing and/or export, such as known RNA-binding domains or nucleoporin repeats, but none are apparent in the Brr3 sequence. In the course of this work, *BRR3* was identified as *GLE1* in a screen for mutants synthetically lethal with a *nup100* deletion

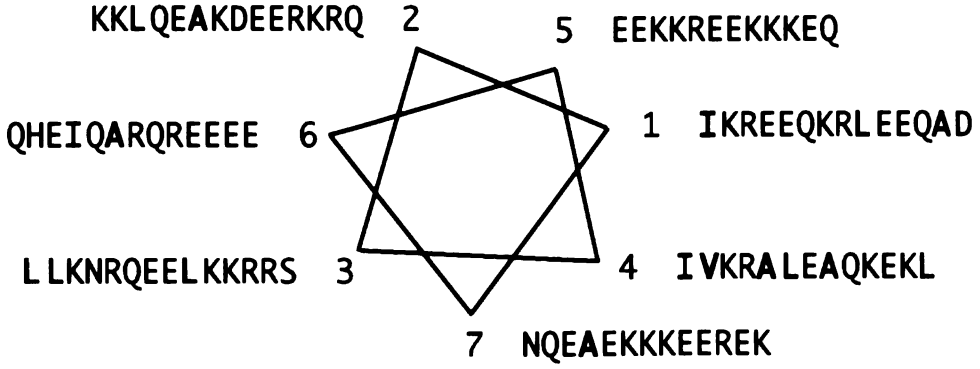
Figure 3: Brr3 coiled-coil prediction and corresponding helical wheel. *(A)* A graph of the probability of coiled-coil structures in Brr3, based on the COILS program. Probabilities are plotted on the y-axis, with the amino acid numbers on the x-axis. The numbers are based on scanning the protein in 28 amino acid windows, as recommended for predictions of new sequences. *(B)* Helical wheel of the high-scoring region of Brr3, based on heptad repeats. Hydrophobic residues are colored red.

A.

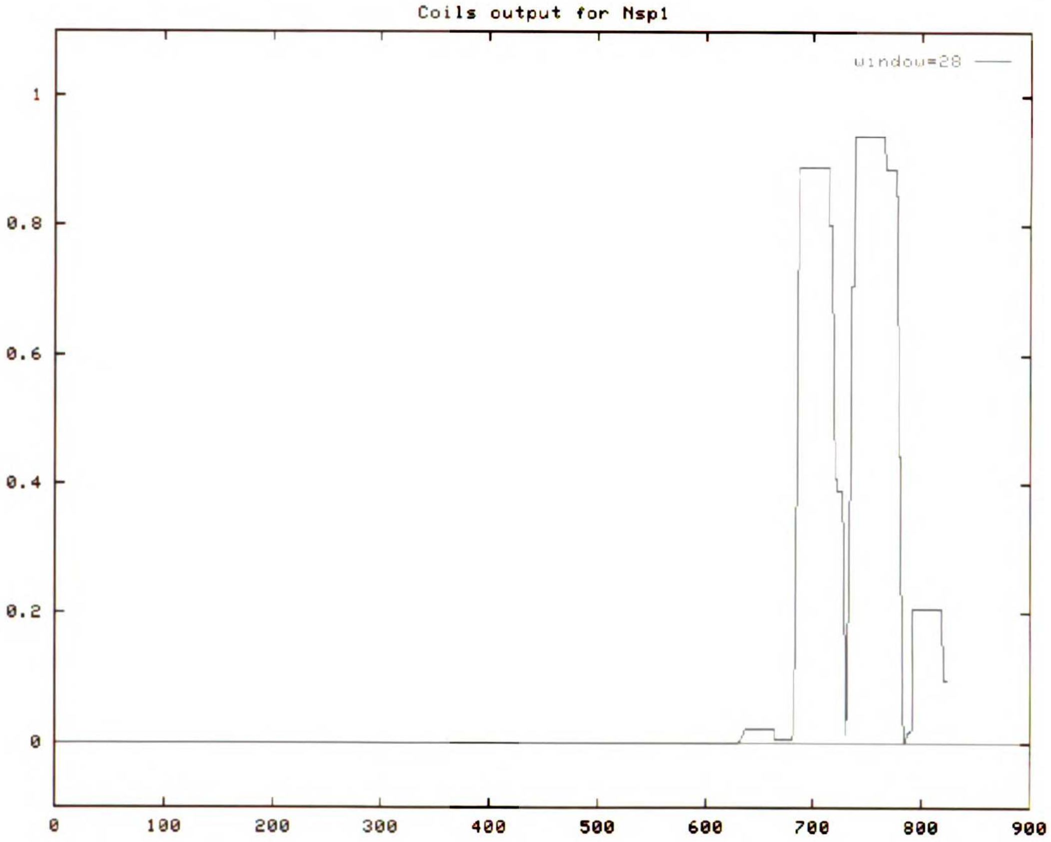


B.

Brr3 Helical Wheel: Residues 146-239

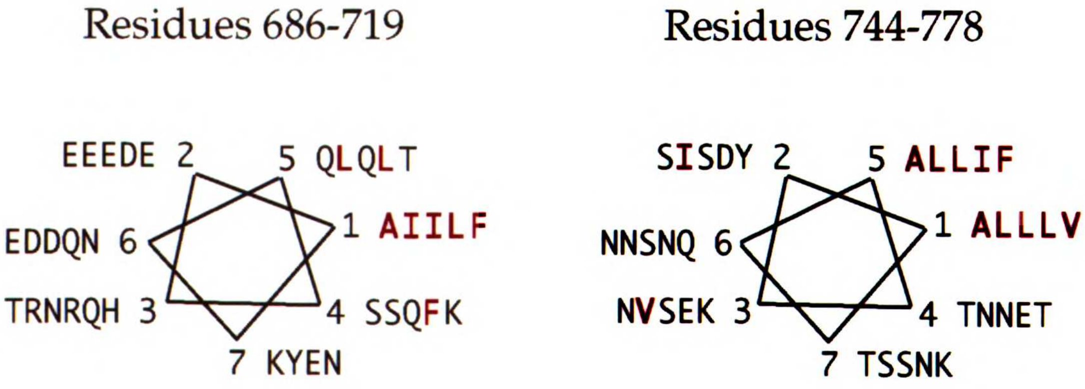

KKLQEAKDEERKRQ 2
5 EEKKREEKKKEQ
1 IKREEQKRLEEQAD
QHEIQARQREEEE 6
LLKNRQEELKKRRS 3
4 IVKRALEAQKEKL
7 NQEAEKKKEEREK

A.



B.

Nsp1 Helical Wheels


Residues 686-719
EEEDE 2
5 QLQLT
EDDQN 6
1 AIILF
TRNRQH 3
4 SSQFK
7 KYEN
Residues 744-778
SISDY 2
5 ALLIF
NNSNQ 6
1 ALLLV
NVSEK 3
4 TNNET
7 TSSNK

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(Murphy, 1996). This report included the discovery of a region in Brr3, from amino acids 351 to 358, similar to a previously identified nuclear export signal (NES, see Figure 2A and Figure 5). This NES, a short stretch of regularly spaced leucine residues, was initially identified in the mammalian protein kinase inhibitor PKI and the HIV protein Rev, and was shown to be necessary and sufficient to drive the rapid export of proteins from the nucleus (Fischer, 1995; Wen, 1995). Supporting the idea that NESs provide a signal for the nuclear export of mRNAs, Rev functions to export unspliced and partially-spliced viral mRNAs from the nucleus to the cytoplasm. This is intringuing in light of the potential role for Brr3 in mRNA export. Upholding this model, the NES peptide of Brr3 functions as an export signal in a heterologous system (Murphy, 1996), however whether it functions as an export signal in yeast, and in the context of the whole protein, has yet to be determined.

To ascertain the nature of the *brr3-1* mutation, the chromosomal mutation was first gap repaired onto a plasmid. Subsequent sequencing of the *brr3-1* mutant allele identified the mutation as a single amino acid substitution at position 517, a change from arginine to lysine (Figure 2A). The mutation lies at the extreme C-terminal end of the protein, outside the long α-helical region and the NES, in a region of unknown function. Curiously, the amino acid change is conservative, as both arginine and lysine are positively-charged. A highly interesting distinction between the two is that arginine acts as a crucial residue in RNA-binding motifs such as the arginine-rich motif in HIV Rev and Tat, and the RGG box of nucleolin and hnRNP U (Burd, 1994). Moreover, arginine is thought to allow more versatile binding to RNA than

Figur

cons

Figure 5: Comparison of the NES sequences in PKI, Rev, and Brr3. The consensus leucine residues are in pink.

PKI	L	A	L	K	L	A	G	L	D	I
Rev		L	P	P	L	E	R	L	T	L
Brr3 (aa 351-358)			L	P	L	G	K	L	T	L
consensus			L	X	L	X	X	L	X	L

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lysine, due to an increased number of hydrogen bonding atoms that could interact with the sugar-phosphate backbone and bases of RNA. This leads to the attractive hypothesis that the C-terminal end of Brr3 contains a novel RNA-binding motif which is compromised by the *brr3-1* mutation. At this point, experimental evidence is needed to substantiate this speculation, especially since a direct interaction between Brr3 and RNA has not been demonstrated. Possible ways to test this are *in vitro* RNA binding experiments using purified Brr3, or *in vivo* crosslinking of Brr3 to RNA. If these assays yield positive results, it can then be determined if the C-terminal domain is mediating RNA binding, and furthermore if the *brr3-1* mutant protein alters RNA binding.

In summary, although the Brr3 sequence is not as informative as initially anticipated, this essential protein does reveal interesting characteristics that may correlate with a role in mRNA splicing and/or export. First, the predicted α-helical rod in the center of Brr3 could mediate an interaction with a macromolecular assembly, such as the nuclear pore complex or RNA processing machineries. Second, the functional, Rev-like NES of Brr3 predicts an important role in mRNA export. Finally, the *brr3-1* arginine to lysine mutation may uncover a novel RNA-binding motif. Future experiments addressing these issues will likely lead to illuminating results in the search for the primary function of Brr3.

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Materials and methods

Subcloning, sequencing, and inactivation of the BRR3 gene

The pBRR105 plasmid contains the minimal *brr3-1* complementing fragment, consisting of the NlaIV/SpeI genomic fragment that includes the *BRR3* ORF with approximately 400 basepairs upstream and 600 basepairs downstream. It was cloned into pSE360 (*CEN-URA3*) at the SmaI/XbaI sites in the polylinker, destroying all four restriction sites. Sequencing of the *BRR3* gene was done using fast alkaline denaturation, as detailed in the protocol accompanying the Sequenase T7 DNA polymerase (Amersham).

To inactivate the chromosomal *BRR3* ORF, one step gene replacement was performed using a PCR-based method (Baudin, 1993). Primers were designed to amplify the *LEU2* gene with 60 nucleotides of 5' *BRR3* sequence at the 5' end, and 60 nucleotides of 3' *BRR3* sequence at the 3' end. The primer sequences are as follows (*LEU2* sequences in lower case): 5'brr3LEU = 5'TATAATTGATAACAAGAGAATCGCGGGCCGTTGCTACAGGATATTCTGCAGAGTCGAAGAtcgaggagaacttctagtat3', 3'brr3LEU = 5'CCATACGCCTGAAGAAACTGTACGGCAGCGGCATCCCACCACGAACCGAGTATGACAAAAtcgactacgtcgtaaggccg3'. The *LEU2* gene was amplified from pRS305 using 1uM final concentration of each primer, and 25 cycles of 65°C, 40 sec.; 72°C, 2 min.; 94°C, 60 sec. The amplified DNA was then transformed into a W303 diploid strain, and leucine prototrophs were selected. Proper recombination of the DNA with the *BRR3* locus was assayed by whole-cell PCR and Southern blotting, resulting in the heterozygous diploid strain yTA310.

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All yeast manipulations were done using standard methods (Guthrie, 1991), as were molecular biological techniques (Ausubel, 1994).

Secondary structure analysis

Chou and Fasman results were obtained using the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin.

The COILS program was accessed on the Internet at http://ulrec3.unil.ch/software/COILS_form.html. The Brr3 and Nsp1 graphs were generated using the MTIDK matrix over a 28 residue window, with unweighted scoring (no significant changes occurred when the program was run with the weighting option).

Gap repair of *brr3-1* mutation

The template plasmid used for gap repairing was pBRR117, containing essentially the same *BRR3* genomic fragment as pBRR105, in pRS358 (*CEN-TRP1*). The gap was generated by digesting pBRR117 with ClaI and SnaBI, excising a 1.5 kb fragment from the *BRR3* ORF. The remaining DNA fragment was transformed into yTA305, a *brr3-1* mutant strain [brr3-1 MATa ura his trp LEU LYS (S288C/W303 background)]. Tryptophan prototrophs were screened for a cold-sensitive growth phenotype. Plasmids were rescued from the strains that failed to grow at 16°C, and assayed for the ability to confer cold-sensitivity when present in the *brr3* deletion background. The resulting plasmid, pBRR123, harbors the *brr3-1* mutant allele.

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To determine the identity of the *brr3-1* mutation, the *BRR3* region of pBRR123 was sequenced by the Biomolecular Resource Center at the University of California, San Francisco.

CHAPTER 2

Brr3 functions in mRNA export and also affects pre-mRNA processing

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Abstract

Precursors to mRNAs undergo multiple processing steps in the nucleus before their export to the cytoplasm. The study of mRNA processing and export in yeast has led to the identification of numerous factors involved in each individual reaction, but the class of factors which are presumably necessary to direct mRNAs from the processing machineries to the export pathway has yet to be identified. This chapter describes the analysis of the yeast mutant *brr3-1*, that may normally act as one of these coupling factors. *brr3-1* is a novel, cold-sensitive allele of *GLE1/RSS1* (Del Priore, 1996; Murphy, 1996), which has been reported to encode an mRNA export factor. In support of a primary role for Brr3 in mRNA export, the *brr3-1* mutant rapidly accumulates poly(A) RNA in the nucleus when shifted to the cold, and is synthetically lethal with numerous nucleoporin mutants implicated in RNA export. Additionally, the Brr3 protein localizes to the nuclear rim in live yeast cells, and similar to nucleoporins, requires detergent to be fully extracted from cells. However, the phenotypes that distinguish *brr3-1* from other nucleoporin and mRNA export mutants are *in vivo* and *in vitro* defects in pre-mRNA splicing and poly(A) tail shortening. The synthesis of these results leads to the intriguing hypothesis that Brr3 may function in the coupling of these processes. Models addressing this possibility are presented in the discussion.

Introduction

Despite being a fundamental process in eukaryotic gene expression, the nuclear export of mRNAs is still a poorly understood phenomenon. The passage of mRNAs from the nucleus to the cytoplasm starts with transcription by RNA polymerase II. Almost immediately, the transcripts are assembled with proteins into heterogeneous nuclear ribonucleoprotein (hnRNP) particles. They acquire a 7-methyl guanosine cap at the 5' end, and most are processed at the 3' end by cleavage and polyadenylation, requiring the action of a large multi-protein complex (Keller, 1997). In addition, intron-containing transcripts are spliced by a dynamic, macromolecular structure consisting of at least 50 proteins and 5 small nuclear ribonucleoprotein (snRNP) complexes (Moore, 1993a). To reach the cytoplasm, the matured mRNA molecules must traverse the nuclear envelope through the nuclear pore complex (NPC), a highly ordered, over 100 megadalton assembly of an estimated 50 polypeptides, believed to mediate bidirectional nucleocytoplasmic transport of all cellular RNAs and nuclear proteins (Doye, 1997). Clearly an intricate process, the journey of an mRNA to the cytoplasm requires accurate recognition by a succession of macromolecular structures.

Early studies utilizing microinjection of mRNA into *Xenopus* oocytes demonstrated that mRNA export is saturable and ATP-dependent, strongly suggesting the now widely accepted concept that it is an active, receptor-mediated process (Dargemont, 1992). Specialized structural features of the mRNA, the 5' cap and the 3' poly(A) tail, contribute to the signal that determines active export, but are not absolutely required for export per se (Jarmolowski, 1994). The discovery of nuclear export signals (NESs) in proteins, such as the 10 amino acid leucine-rich sequence in mammalian PKI (Wen, 1995), has highlighted the idea that mRNA export signals reside in the protein components of mRNP complexes. Supporting this idea, NESs have been identified in several RNA binding proteins. The HIV Rev protein,

which binds unspliced viral pre-mRNAs and mediates their export, contains a PKI-like NES essential for Rev activity (Fischer, 1995). Two other types of NESs have been found in mammalian hnRNP proteins that shuttle between the nucleus and cytoplasm (Pinol-Roma, 1992; Visa, 1996). The M9 sequence of hnRNP A1 is unlike the Rev NES in that it is a longer, glycine-rich sequence, and interestingly, it appears to function as both the nuclear export and import signal for hnRNP A1 (Michael, 1995). In addition, an NES comprised of a 24 amino acid sequence unrelated to M9 or the leucine-rich motifs has been identified as the export/import signal for hnRNP K (Michael, 1997).

A picture emerges of mRNA export mediated by receptors that transport RNA via NES-containing proteins. Hypothetically, the export process can be separated into discrete steps:

1. Loading of mRNA-binding and NES-containing proteins onto the mRNA during and/or after synthesis and processing
2. Receptor-mediated movement of the mRNA from the nucleoplasm to the nuclear pores
3. Translocation through the NPC
4. Release of the mRNA in the cytoplasm
5. Recycling of export factors back to the nucleus

In addition to work in the mammalian and *Xenopus* systems, the study of mRNA export in the yeast *S. cerevisiae* has led to significant strides in understanding this process. The powerful combination of genetics and biochemistry has resulted in the identification of factors involved in the export pathway, especially at the first three steps outlined above, which will be the focus of the remainder of this discussion.

Thus far, obvious candidates for cellular proteins involved in the proposed first step of mRNA export are of the hnRNP-type. As exemplified by mammalian hnRNP A1, these proteins are highly abundant, mainly nuclear, and contain various RNA binding domains. They are thought to package newly transcribed mRNAs into hnRNP particles, and aid in pre-mRNA processing (Swanson, 1995). A role in mRNA export was demonstrated by competition experiments in which saturating amounts of hnRNP A1 protein microinjected into *Xenopus* oocyte nuclei inhibited the nuclear export of mRNA (Pante, 1997). The yeast hnRNP proteins Npl3, Nab2, and Nab4/Hrp1, have also been implicated in mRNA export. Mutant alleles of *NPL3* and *NAB2* accumulate poly(A) RNA in the nucleus (Wilson, 1994; Lee, 1996). Nab4/Hrp1 is closely connected with these proteins, as evident by two-hybrid and genetic interactions (M. Swanson, personal communication; Henry, 1996). Furthermore, there is evidence that, like hnRNP A1, all three of these proteins shuttle between the nucleus and cytoplasm (Flach, 1994; Aitchison, 1996; Lee, 1996).

Curiously, unlike hnRNP A1, the yeast hnRNP-like proteins do not appear to contain an M9-like NES sequence. One possibility is that the yeast proteins utilize a different type of NES. Alternatively, the yeast hnRNP proteins could pair with NES-containing proteins, which would act to link the RNA-binding proteins to the export machinery. Support for the second model comes from the recent identification of an essential yeast protein with a functional Rev-like NES, Mex67, which interacts with a novel protein containing multiple RNA-binding domains, Mip6 (Segref, 1997). Mex67 is likely to be an mRNA export factor; the *mex67-5* temperature-sensitive mutant displays a rapid

accumulation of poly(A) RNA in the nucleus upon shifting to 37°C, while nuclear envelope morphology and nuclear protein import remain intact. Moreover, Mex67 can be cross-linked to poly(A) RNA *in vivo*. Since the Mex67 sequence does not include any known RNA-binding motifs, Segref et al. (1997) searched for an RNA-binding partner by two-hybrid assay, and isolated Mip6. They postulate that Mex67 functions as an adaptor, linking RNA to the export machinery via its interaction with Mip6.

An important event in this early step in mRNA export is the movement of mRNAs from the processing machineries to the export pathway, which is likely mediated by factors that couple processing and export. The coupling of mRNA processing events is proving to be essential in the efficient maturation of mRNAs. This is best exemplified by the functional and physical interactions observed between the C-terminal domain (CTD) of RNA polymerase II and factors required for 5' capping, pre-mRNA splicing, and 3' processing (Mortillaro, 1996; McCracken, 1997; McCracken, 1997b). However, much less is known about how mRNAs are directed from the site of processing to the export pathway. Potential coupling factors are those involved in both mRNA processing and export; hnRNP proteins are poised for such a role. In support of this model, Nab4/Hrp1 has recently been identified as a protein essential for 3' cleavage and polyadenylation of mRNA *in vitro* (Kessler, 1997). Also, Npl3 exhibits homology to the metazoan SR-family of splicing factors, and *npl3* mutant extracts splice certain pre-mRNAs inefficiently (C. Siebel, personal communication).

The next hypothetical step in the mRNA export pathway is delivery of the RNA, in its RNP form, to the nuclear pores. The analogous step in nuclear

protein import is mediated by the importin β family of proteins (Gorlich, 1997; Nigg, 1997). The question of whether a similar receptor functions in nuclear export was recently answered by the identification of Xpo1 as the newest member of this family, since Xpo1 seems to be required exclusively for nuclear export (Stade, 1997). In yeast, a temperature-sensitive *xpo1* mutant displays a strong, rapid inhibition of the export of an NES-reporter protein when shifted to the non-permissive temperature, with only a slight protein import defect at later times. Also, the Xpo1 protein specifically interacts with both the PKI (Rev-like) NES in a two-hybrid assay, and was shown to shuttle between the nucleus and cytoplasm. Xpo1 binding to the Rev-like NES suggests a possible involvement in RNA export; consistent with this notion the *xpo1* mutant accumulates poly(A) RNA in the nucleus with the same kinetics as the protein export block.

Once the RNPs reach the nuclear pores, they are translocated through the NPC to reach the cytoplasm. Mutations in almost half of the 30 or so known yeast nucleoporins result in a poly(A) RNA export defect (Doye, 1997). However, many of these mutations also lead to aberrant nuclear envelope and NPC morphology, so it is unclear if they directly affect mRNA export (Wente, 1993; Siniossoglou, 1996). Nonetheless, there is evidence that nucleoporins could function directly in RNA export. For example, the yeast nucleoporin mutant *nup159-ts* blocks poly(A) RNA export abruptly after a shift to the non-permissive temperature (Gorsch, 1995). Furthermore, microinjecting antibodies specific to vertebrate Nup98 into *Xenopus* oocyte nuclei block mRNA, rRNA, and snRNA export without affecting protein import (Powers, 1997). Nup159 and Nup98 are among numerous repeat-containing nucleoporins located at either the nuclear or cytoplasmic face of

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the NPC that interact with Rev in two-hybrid assays; these interactions are dependent on the Rev NES (Stutz, 1995; Fritz, 1996). These data led to the model that NES-mediated translocation throught the NPC occurs by sequential interactions with nucleoporins (Fritz, 1996). Other RNA export factors that associate with nucleoporins include human Xpo1, which binds to FG-repeat nucleoporins (Fornerod, 1997). Also, the initial *mex67* mutant was isolated in a *nup85* synthetic lethal screen (Segref, 1997).

An additional factor implicated in RNA export is the essential yeast protein Gle1. *gle1* temperature-sensitive mutants were isolated in a screen for synthetic lethality with a *nup100* deletion mutant (Murphy, 1996). Consistent with an NPC association, this gene was also identified as *RSS1*, a high-copy suppresor of the *nup159-ts* mutant (Del Priore, 1996). Moreover, the Gle1/Rss1 protein localizes to the nuclear rim, coincident with nucleoporins. Also, Gle1 binds to Rip1, a nuclear pore associated protein with FG-repeats; interestingly, Rip1 was first identified as a cellular protein that interacts with Rev (Fritz, 1995; Stutz, 1995).

All the *gle1* temperature-sensitive mutants exhibit nuclear accumulation of poly(A) RNA; the onset of this RNA export defect is very fast in the most severe mutant. Because the mutants are normal for NPC morphology and protein import, Murphy and Wente (1996) postulated a specific role for Gle1 in RNA export. In support of this model, Gle1 contains a Rev-like NES, which, when mutated at a critical residue, results in a temperature-sensitive phenotype and an RNA export block five hours after a shift to 37°C. The Gle1 NES sequence is sufficient to export a reporter protein in heterologous cells, but whether it acts as an export signal in the context of the yeast protein is

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unknown. The nuclear pore association, robust RNA export defect, and Rev-like NES are characteristics shared by both Gle1 and Mex67. Nonetheless, unlike Mex67, an interaction with RNA, either directly or via an RNA-binding protein has not been demonstrated for Gle1, making its direct role in RNA export more tentative.

Since Gle1 function is less defined than the other factors described thus far, assigning it to a particular step in the export pathway would be premature. However, the identification of a novel allele of *GLE1/RSS1*, *brr3-1*, raises the intriguing possibility that it could act to link mRNA processing and export. The *brr3-1* mutant was isolated from a bank of cold-sensitive strains in two independent screens. First, it was identified as a pre-mRNA splicing mutant that displayed a strong block in the first step of splicing after a 10 hour shift to the cold (Noble, 1996). Second, it was isolated in a screen for poly(A) RNA export mutants (A. de Bruyn Kops and C. Guthrie, manuscript in preparation). The initial analysis of the RNA export defect revealed a rapid onset of nuclear poly(A) RNA accumulation after shifting to the cold, as well as an increase in poly(A) tail length. Importantly, it was determined that the splicing and export defects co-segregate with the cold-sensitivity, demonstrating that all three phenotypes are linked to the same mutation.

This chapter describes a detailed characterization of the *brr3-1* mutant, and its effects on mRNA processing and export. For simplicity, the gene and protein will be referred to as *BRR3* here. Further evidence of a direct role for Brr3 in RNA export, including association with nuclear pores, is described. Also presented is an *in vivo* and *in vitro* analysis of the mRNA processing defects of *brr3-1*, including impaired pre-mRNA splicing and poly(A) tail formation.

Finally, models regarding the potential roles of Brr3 in mRNA export and in the coupling of mRNA processing and export are discussed.

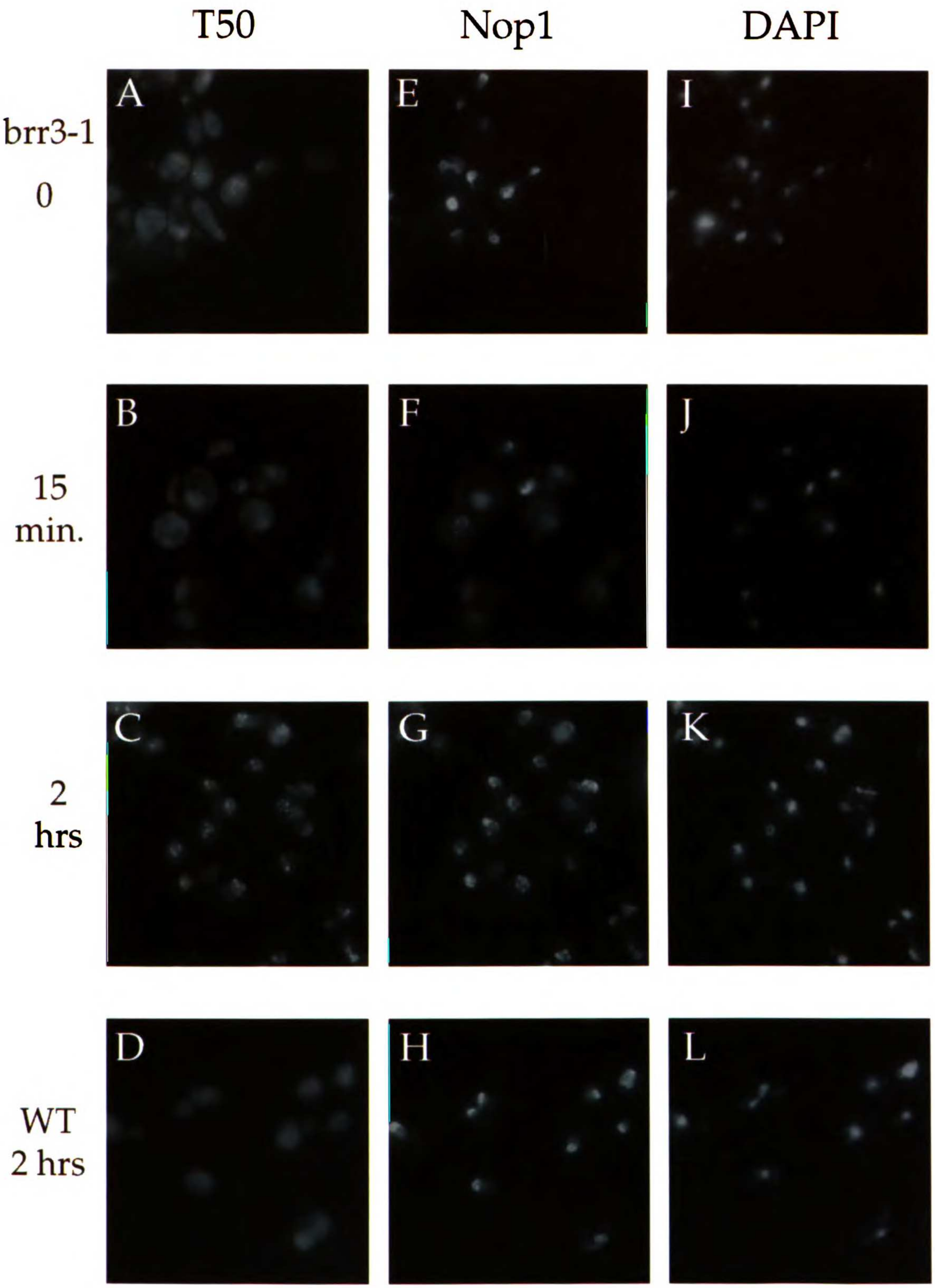
Results

brr3-1 cells rapidly accumulate poly(A) RNA in the nucleus at the non-permissive temperature

To closely analyze the poly(A) RNA export phenotype of *brr3-1* cells, isogenic mutant and wildtype strains were shifted to the non-permissive temperature of 16°C, and assayed at various times by *in situ* hybridization with an oligo dT_{50} probe. Nuclei were marked by staining with DAPI. At the permissive temperature of 30°C, nuclear accumulation of poly(A) RNA is detected in 10% of *brr3-1* cells (Figure 1, panel A). However, as early as 15 minutes after the cold shift, the proportion of mutant cells with strong nuclear staining reproducibly increases to 40% (Figure 1, panel B). This phenotype peaks 2 hours after the shift, with nearly 70% of mutant cells accumulating poly(A) RNA in the nucleus (Figure 1, panel C). In contrast, poly(A) RNA is distributed throughout the cell in the wildtype strain at both 30°C and16°C, with less than 3% of cells displaying strong nuclear staining at either temperature (Figure 1, panel D).

Further examination of mutant cells revealed an intriguing pattern in the nuclear localization of the accumulated RNA. In the initial hour after the cold shift, the RNA is localized throughout the nucleus, in a diffuse pattern. Beyond one hour after the shift, the RNA staining becomes focused in 3 to 5

Figure 1: Pattern of poly(A) nuclear accumulation in *brr3-1* cells. Cells were shifted from 30°C to 16°C, harvested at the indicated times, and probed *in situ*. Representative fields of cells are shown, probed with the following: (A-D) oligo-dT50, hybridizing to poly(A) RNA; (E-H) anti-Nop1 antibody; (I-L) DAPI stained cells, marking nuclear DNA. For comparison, staining in wildtype cells are shown in panels D, H, and L.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DAPI
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2 hrs
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punctate spots within the nucleus. These spots appear to coincide with the localization of Nop1, a nucleolar protein, as demonstrated by probing cells with both oligo dT_{50} and α-Nop1 antibodies (Figure 1, panels C and G). In wildtype cells, Nop1 staining is concentrated to one section of the nucleus, indicative of nucleolar localization (Figure 1, panel H). A change in the normal Nop1 staining pattern to punctate nuclear spots has been correlated with nucleolar fragmentation at the EM level in RNA polymerase I mutants and the RNA export mutant *mtr3-1* (Oakes, 1993; Kadowaki, 1995). Similar Nop1 mislocalization is seen in a subset of yeast mutants defective in poly(A) RNA export, including *gle1* temperature-sensitive mutants (Kadowaki, 1994; Murphy, 1996), but it is unknown how the two phenotypes relate to each other. Given that Nop1 mislocalization occurs subsequent to poly(A) RNA accumulation in cold-shifted *brr3-1* cells, it is likely to be a secondary effect of the RNA export defect. The resulting co-localization of poly(A) RNA and Nop1 could reflect trapping of RNA in nucleolar fragments, or non-specific association of RNA with mislocalized nucleolar proteins.

Brr3 genetically and physically associates with the nuclear pore complex

The rapid onset of the RNA export defect in the *brr3-1* mutant is consistent with a direct role in this process. To ascertain what part of the export pathway *brr3-1* may be affecting, the mutant was tested for genetic interactions with known mutants implicated in RNA export, including synthetic lethality with nucleoporin mutants. Synthetic lethality is defined as a lethal growth phenotype resulting from the combination of two viable mutations. At its most useful, this phenotype can reflect a physical interaction between the

proteins encoded by the two mutated genes. Otherwise, the two genes may have redundant functions or act in the same pathway. Alternatively, the genes may act in independent pathways that, when both impaired, results in cell death. Genetic screens based on synthetic lethality have proven fruitful in identifying novel nucleoporins and defining subcomplexes of the NPC (Doye, 1995). In these cases, the genetic interactions have been substantiated by biochemical evidence of physical association. To test synthetic lethality between *brr3-1* and nucleoporin mutants, a *brr3-1* strain was mated to various nucleoporin mutant strains, diploids were sporulated, at least twenty tetrads were dissected, and the resulting spores analyzed for the recovery of double mutants at the permissive temperature. A summary of the results are outlined in Table I (see Appendix II for the raw data).

Strikingly, the *brr3-1* mutant was found to be synthetically lethal in combination with a number of the nucleoporin mutants tested. Most of these *nup* mutants are also defective for RNA export. However, this cannot be the sole basis for the synthetic lethal phenotype, since *nup188-cs*, an RNA export mutant, is viable in combination with *brr3-1* (A. de Bruyn Kops, unpublished data). Furthermore, *brr3-1* synthetic lethality with *nup100Δ*, *nup159-ts*, and *nup1Δ* is substantiated by data from others, who identified *BRR3* and different conditional alleles of *BRR3* based on genetic interactions with these *nup* mutants (Del Priore, 1996; Murphy, 1996; L. Davis, personal communication). Interestingly, among the nucleoporins that interact with *brr3-1* are all of those known to contain a novel RNA binding motif, namely Nup100, Nup116, and Nup145 (Fabre, 1994). Thus, these genetic interactions imply a functional relationship between Brr3 and components of the NPC in the export of RNA.

Table I. Results of crosses between *brr3-1* and *nup* mutants

nup mutant	*nup* defective for RNA export?	Double mutants recovered?
nup116Δ[a]	yes	no
nup145ΔN[b]	ND	no
nup100Δ[c]	ND	no
nup133Δ[d]	yes	no
nup159-ts/rat7-1[e]	yes	no
nup1Δ[f]	yes	no
nup188-cs/brr7-1[g]	yes	yes
nup2Δ[h]	ND	yes

[a] Wente, 1993
[b] Wente, 1994
[c] Wente, 1992
[d] Doye, 1994
[e] Gorsch, 1995
[f] Kenna, 1996
[g] A. de Bruyn Kops, in preparation
[h] Loeb, 1993

ND, not determined

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A functional interaction between Brr3 and the NPC is supported by the observation that the subcellular localization of Brr3 is at the nuclear rim, coincident with nucleoporins. By indirect immunofluorescence of fixed cells, Murphy and Wente (1996), and Del Priore et al. (1996) detected Brr3/Gle1/Rss1 in a punctate pattern at the nuclear periphery; the latter group also reported cytoplasmic localization of the protein. To clarify this discrepancy and examine Brr3 localization in live cells, a Brr3-GFP fusion was constructed and introduced into a *brr3* deletion strain. On its own, the fusion allele supports wildtype growth in the deletion background (data not shown). In this strain, the Brr3-GFP signal is exclusively in punctate spots at the nuclear rim, consistent with a steady state localization of Brr3 at the nuclear pores (Figure 2).

To explore the possiblity of a physical interaction between Brr3 and nucleoporins, crude whole cell extracts were prepared for biochemical analysis (Figure 3A). A *BRR3* allele tagged with three copies of an influenza virus hemagglutinin (HA) peptide at the extreme C-terminus was generated and transformed into the *brr3* deletion strain. Mid-log cultures of this strain were harvested and lysed by bead-beating under native conditions, in the presence of various concentrations of salt and detergent. In buffer containing 150 mM NaCl, less than 50% of cellular Brr3-HA is extracted (Figure 3A, lanes 1 and 2). Consistent with membrane association, Brr3-HA is most effectively solubilized only when 1% or 2% Triton X-100 is present in the lysis buffer (Figure 3A, lanes 5-8). Similarly, Nup188, a nucleoporin that interacts with the integral membrane nucleoporin Pom152 (Nehrbass, 1996), requires the addition of 1% Triton to become efficiently extracted (K. Stade, unpublished data). Pom152 has been reported to require detergent for extraction from yeast

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Figure 2: Brr3-GFP localizes to the nuclear rim in live cells. Cells harboring a Brr3-GFP fusion (A, B) or untagged Brr3 (C, D) were grown at 30°C to mid-log, and cultures were examined by fluorescent microscopy. (A, C) Fluorescent images were captured by a CCD camera, with the same exposure time. (B, D) Corresponding bright field images of the cells.

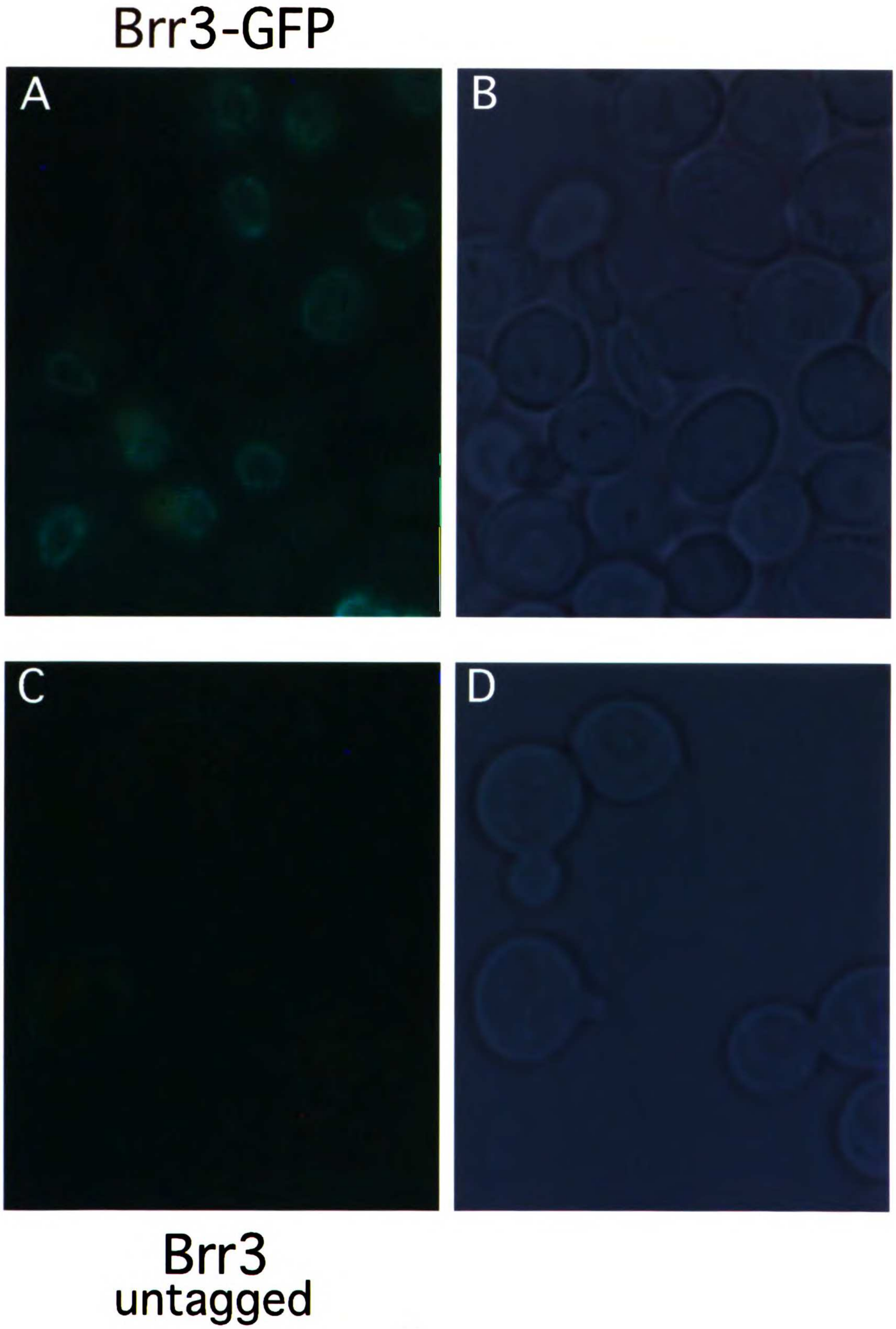
Brr3-GFP
A
B
C
D
Brr3
untagged

Figure 3: Detergent solubilization of Brr3-HA and co-immunoprecipitation with Rip1. Crude whole-cell extracts were prepared by bead-beating in the presence of detergent, and used to immunoprecipitate Brr3-HA complexes. *(A)* To assess Brr3-HA solubility, cells were lysed in the presence of increasing concentrations of the detergent Triton X-100. Equivalent amounts (same percentage of total) of the pellet (P) and supernatant (S) of each extract were Western blotted and probed with anti-HA antibodies. The open circle marks a breakdown product of Brr3-HA. *(B, top panel)* Brr3-HA immunoprecipitated from extracts containing .5% Triton X-100. Extracts from cells containing Brr3-HA (lanes 1-4) or untagged Brr3 (laned 5, 6) were incubated with protein A-sepharose beads with or without anti-HA antibodies, the beads were pelleted, and analyzed by Western blotting. Lane 1, 10% of starting extract. Lanes 2 and 5, 50% of pellet from extracts incubated with antibodies. Lanes 3 and 6, 25% of supernatant from extracts incubated with antibodies. Lane 4, 50% of pellet from extracts incubated without antibodies. The open circle marks the Brr3-HA breakdown product, which co-migrates with the IgG antibody. *(B, middle panel)* Same blot as top panel, stripped and re-probed with anti-Rip1 polyclonal antibodies. The filled circle marks the IgG band. *(B, bottom panel)* Same blot stripped and re-probed with anti-GLFG antibodies, which recognizes four GLFG-containing nucleoporins, labeled to the left of the panel. The remaining bands are breakdown products. The band in lane 2 is the IgG protein. *(C)* Extracts made from cells with the wildtype *RIP1* gene (lane 1), or a chromosomal deletion of the *RIP1* gene (lane 2), Western blotted with anti-Rip1 antibodies.

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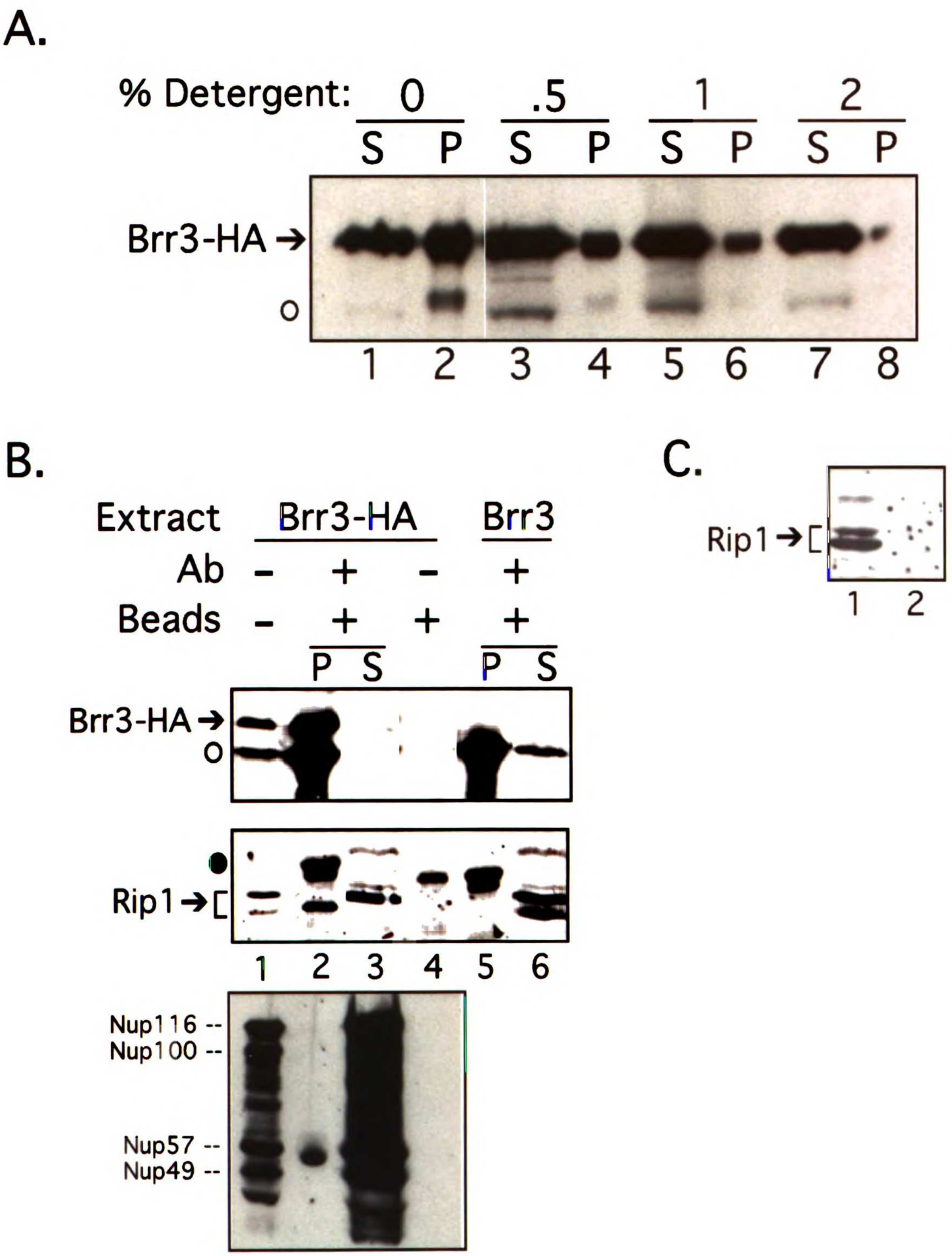
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Rip1
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nuclear envelopes, although the concentration of detergent was not specified (Strambio-de-Castillia, 1995). In striking contrast, yeast Xpo1, the putative RNA export receptor that shuttles between the nucleus and cytoplasm, is fully soluble in the absence of detergent (P. Preker and K. Stade, unpublished data).

A growing number of physical interactions between subsets of nucleoporins, and between nucleoporins and other components of the nuclear transport machinery have been identified by biochemical assays, including co-immunoprecipitation (Belanger, 1994; Doye, 1995; Iovine, 1995). To determine if Brr3 is complexed with any known nucleoporins, Brr3-HA was immunoprecipitated from whole cell extracts with antibodies recognizing the HA epitope (Figure 3B, top panel). The resulting pellet and supernatant were analyzed by Western blot using antibodies against nucleoporins, to detect any co-immunoprecipitated proteins. Of interest were the three nucleoporins that contain an RNA binding motif, all of which belong to the GLFG class. Although the GLFG nucleoporins are very abundant in the starting extract, none are detected in the Brr3-HA immunoprecipitated pellet (Figure 3B, bottom panel). The presence of 0.5% Triton X-100 in the extract could disrupt a potential interaction, but this is unlikely if it were part of a stable nucleoporin complex, since several nucleoporin complexes have been isolated under conditions as stringent as 2% Triton X-100 (Grandi, 1993) or 1M NaCl (Belanger, 1994). Furthermore, Brr3/Gle1 was not detected in a complex co-purified with Nup116 in nuclear lysates (Iovine, 1997). Taking into account the synthetic lethality results, a more plausible explanation is that Brr3 transiently interacts with the GLFG nucleoporins in the RNA export pathway. If so, only a small fraction of Brr3 may be stably associated with

these nucleoporins at a given time, thereby making an interaction difficult to detect by co-immunoprecipitation.

In contrast, a convincing co-immunoprecipitation is detected with a nuclear pore-associated protein of the XXFG class, Rip1 (Figure 3B). Using conditions where over 50% of Brr3-HA in the extract is immunoprecipitated (Figure 3B, top panel), one of two major proteins recognized by anti-Rip1 antibodies is quantitatively co-precipitated (Figure 3B, middle panel). This interaction is specific, since it depends on the presence of both anti-HA antibodies and HA-tagged Brr3 (Figure 3B, compare lanes 2, 4, and lanes 2, 5). Moreover, a Rip1-Brr3/Gle1 interaction has been demonstrated previously by two other methods, ligand-blot overlay using purified recombinant proteins and a yeast two-hybrid assay (Murphy, 1996).

The Rip1 polyclonal antibodies recognize a series of polypeptides (most clear in the supernatant samples of Figure 3B, middle panel). To distinguish between Rip1-specific and cross-reactive polypeptides, extracts were made from a *rip1* deletion strain in a similar manner, and probed with the Rip1 antibodies (Figure 3D, lane 2). Surprisingly, the two most reactive polypeptides, as well as a third less reactive one, were absent in the *rip1*-deleted extract (Figure 3D, compare lanes 1 and 2; the least reactive band is unlabeled). Intriguingly, of these three Rip1-specific polypeptides, only the smallest one is co-precipitated with Brr3; this polypeptide is approximately 43 kD, the predicted molecular weight of Rip1. The higher molecular weight species possibly represent modified forms of Rip1, which implies that Brr3 preferentially associates with unmodified Rip1. A major caveat of this interpretation is that the higher molecular weight forms of Rip1 are not

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detected in wildtype extracts where the proteins have been fully denatured by TCA (P. Preker, unpublished observation). Thus, the higher forms of Rip1 reported here may be an artifact of the non-denaturing extraction conditions used, perhaps mixing Rip1 with modifying enzymes that do not have access to Rip1 in the cell. Incubating extracts with phosphatase does not affect the mobility of the higher bands, so the putative modification is not likely to be phosphorylation (data not shown). Clarifying this discrepancy requires further investigation, however it does not detract from the main observation, that a Rip1 polypeptide of the predicted molecular weight is quantitatively co-precipitated with Brr3.

brr3-1 cells are defective in poly(A) tail shortening

As presented here and elsewhere, many lines of evidence suggest a functional association of Brr3 with nuclear pores. Additionally, the fast onset of the poly(A) RNA export defect in *brr3-1* cells, along with the report of a functional nuclear export signal (NES) present in the Brr3 sequence (Murphy, 1996), strongly predict a specific role for Brr3 in the export of RNA through the nuclear pores. A direct connection between Brr3 and RNA has yet to be demonstrated, but support for such a connection comes from the observation that the *brr3-1* mutant also affects mRNA processing events.

When poly(A) tails are examined in *brr3-1* cells, a noticeable increase in tail length is detected (A. de Bruyn Kops and C. Guthrie, manuscript in preparation). Even at the permissive temperature of 30°C, the longest species of poly(A) tails in *brr3-1* are 20 nucleotides longer than in wildtype cells. This

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defect is exacerbated at 16°C, since the percentage of poly(A) RNA with longer tails increases. Since poly(A) tail shortening is thought to take place in the cytoplasm, this phenotype had been previously interpreted as an indirect effect of impairing RNA export to the cytoplasm (Kadowaki, 1994). However, the longer poly(A) tail phenotype does not occur in all mutants that accumulate poly(A) RNA in the nucleus (A. de Bruyn Kops and C. Guthrie, manuscript in preparation).

To rigorously test whether the longer poly(A) tails are a direct or indirect effect of the *brr3-1* mutant, 3' processing of mRNA was assayed *in vitro* (Figure 4). Whole cell extracts were prepared from *brr3-1* mutant and isogenic wildtype cells grown at 30°C. Extracts were incubated at various temperatures with ^{32}P-labeled RNA corresponding to the 3' end of CYC1 mRNA, under conditions permitting cleavage and polyadenylation of the substrate. When incubated at 30°C, mutant extracts contain poly(A) tails that are 20 nucleotides longer than those in wildtype extracts, exactly paralleling the *in vivo* phenotype (Figure 4A, lanes 1, 2). Incubations at 20°C and 25°C give the same result (data not shown); the result at 16°C could not be determined, since the 3' processing reaction is inefficient at temperatures below 20°C. To rule out that the elongated poly(A) tails are due to aberrant cleavage of the substrate, downstream of the normal site, the polyadenylation step of the reaction was inhibited by substituting CTP for ATP. Under these conditions, cleavage is normal in the mutant extracts (Figure 4A, lanes 3, 4), indicating that the *brr3-1* defect occurs after the cleavage step.

Longer poly(A) tails could be a consequence of deregulated poly(A) tail length control or a failure in a subsequent poly(A) shortening step. To distinguish

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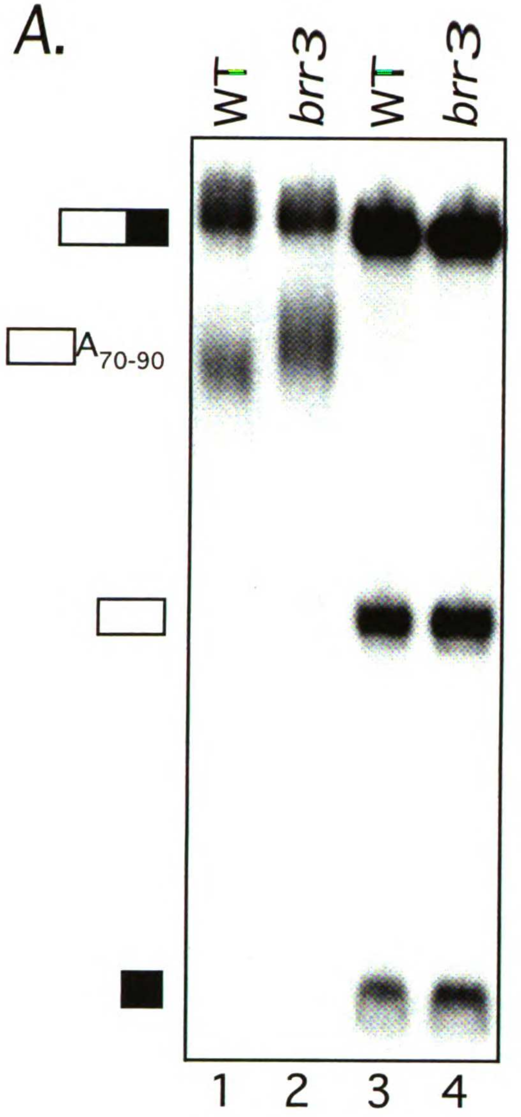
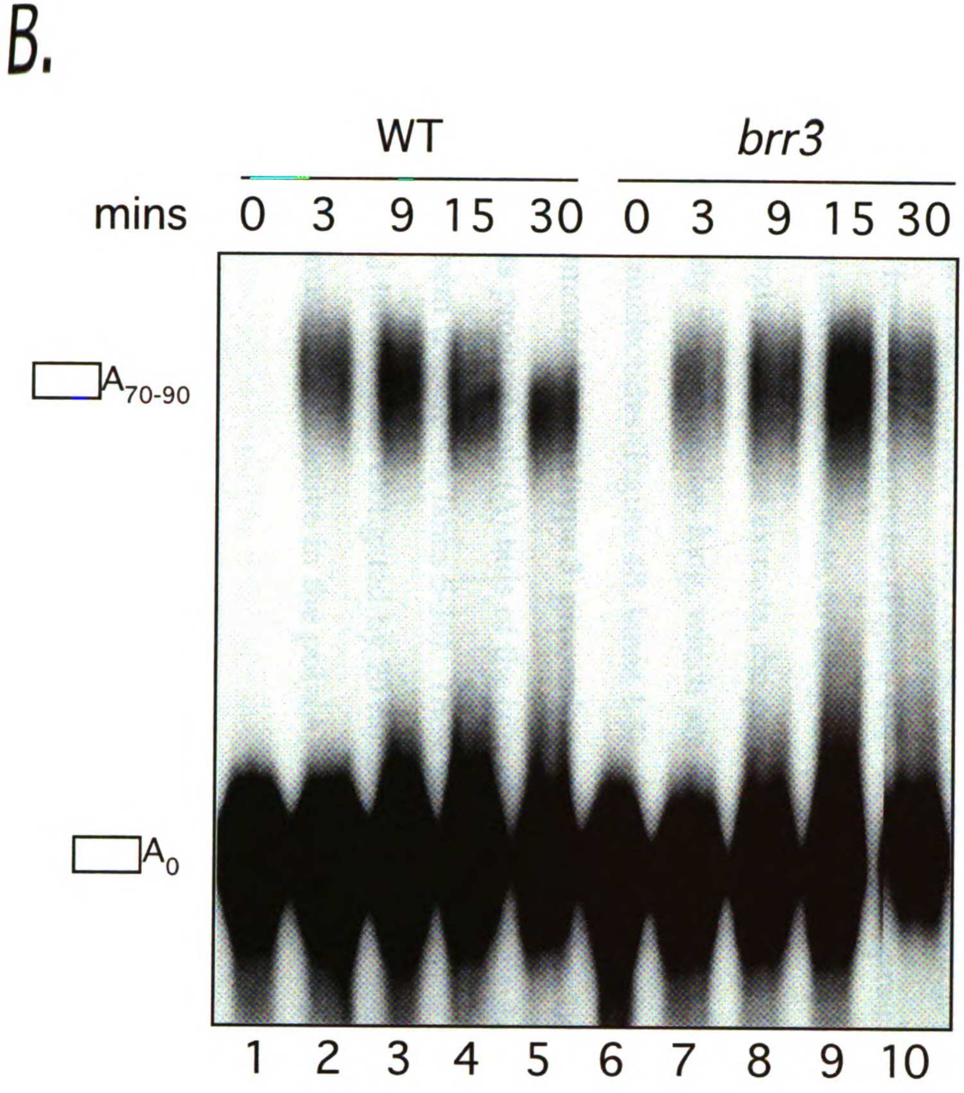
Figure 4: *brr3-1* extracts fail to shorten poly(A) tails. *(A)* Wildtype and mutant extracts incubated at 30°C with the 3' end of CYC1 mRNA, radioactively-labeled. Input RNA is at the top. Lanes 1 and 2, buffer conditions allowing cleavage of the RNA and subsequent polyadenylation of the 5' cleaved product (the 3' fragment is degraded). Lanes 3 and 4, cleavage only reactions. *(B)* Time course of the polyadenylation reaction, using a pre-cleaved CYC1 substrate. Input RNA is the unadenylated species at the bottom of the gel.


A.
WT
brr3
WT
brr3
A70-90
1
2
3
4


B.
WT
brr3
mins
0
3
9
15
30
0
3
9
15
30
A70-90
A0
1
2
3
4
5
6
7
8
9
10

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between these two possibilities, a time course of the polyadenylation reaction was performed, from 3 to 30 minutes (Figure 4B). In this case, pre-cleaved CYC1 RNA was used as the substrate, to assay the polyadenylation reaction alone. Interestingly, in wildtype extracts, the poly(A) tails initially generated are approximately 90 nucleotides long, which are then progressively shortened to 70 nucleotides (Figure 4B, lanes 1-5). This result has been previously documented in wildtype 3' processing extracts (Butler, 1990). *brr3-1* mutant extracts produce poly(A) tails of the correct length, but subsequently fail to shorten them (Figure 4B, lanes 5-10). Thus, in contrast to the wildtype case, the poly(A) tails remain elongated by 20 nucleotides. This demonstrates that the *brr3-1* mutant is defective in the poly(A) shortening reaction.

The *in vitro* data argue that the longer poly(A) tails seen in *brr3-1* cells cannot solely be a result of the RNA export block causing sequestration of mRNA from the poly(A) tail shortening machinery. However, whether or not the poly(A) shortening defect is specifically caused by the *brr3-1* mutant protein has yet to be established. To address this issue, complementation of the *in vitro* defect with purified Brr3 protein is needed; these experiments are in progress.

Complemetation of the poly(A) shortening defect by wildtype Brr3 does not directly address if Brr3 is normally required for the poly(A) shortening reaction. One way to determine this is to assay shortening in extracts depleted of Brr3. Multiple attempts to immunodeplete Brr3-HA from extracts using anti-HA antibodies were unsuccessful (3' processing extracts do not contain detergents). This likely signifies inaccessibility of the epitope to the antibodies, perhaps due to burying of the C-terminal domain in the core of

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the protein, or masking of this domain by other proteins. Immunodepletion by polyclonal antibodies raised against Brr3, or genetic depletion of Brr3 may be more successful.

brr3-1 cells are unable to splice pre-mRNAs efficiently

The *brr3-1* mutant also affects another step in mRNA processing, pre-mRNA splicing. A detailed analysis of this phenotype was carried out by shifting mutant and wildtype cells to 16°C for various times. At each time point, cells were harvested for RNA preparation, and the levels of precursor and mature forms of intron-containing mRNAs were assayed by primer extension. In *brr3-1* cells, accumulation of U3 precursor is detectable 15 minutes after the cold shift, and is saturated by 4 hours (Figure 5A). No precursor accumulation is seen in wildtype cells. As measured by this message, the onset of the *brr3-1* splicing defect is rapid; however, this is not the case for the other transcripts examined. Accumulation of RP51 pre-mRNA, with a concomitant decrease of mature mRNA, does not occur until mutant cells have been in the cold for 4 hours (Figure 5B). Similarly, loss of mature CYH2 mRNA and appearance of actin pre-mRNA are not detected until 4 hours after the cold shift (data not shown).

The question of whether the *in vivo* splicing defects are specifically caused by the *brr3-1* mutation, or are a secondary effect of the RNA export block, can be answered by analyzing splicing *in vitro*. Extracts were prepared from three different sets of mutant and wildtypes cells grown at 30°C. The extracts were incubated with either actin or U3 precursor RNA at 25°C and 15°C, and

[illegible]

Figure 5: Splicing defect in *brr3-1* cells. Primer extensions of RNA prepared from *brr3-1* and wildtype cells grown at 16°C for various times. *(A)* U3 primer extensions. The two pre-mRNA bands correspond to the two genes that encode U3. *(B)* RP51 primer extensions. The multiple mature mRNA species are due to multiple transcription start sites. In both (A) and (B), U5 serves as an internal control for RNA levels.

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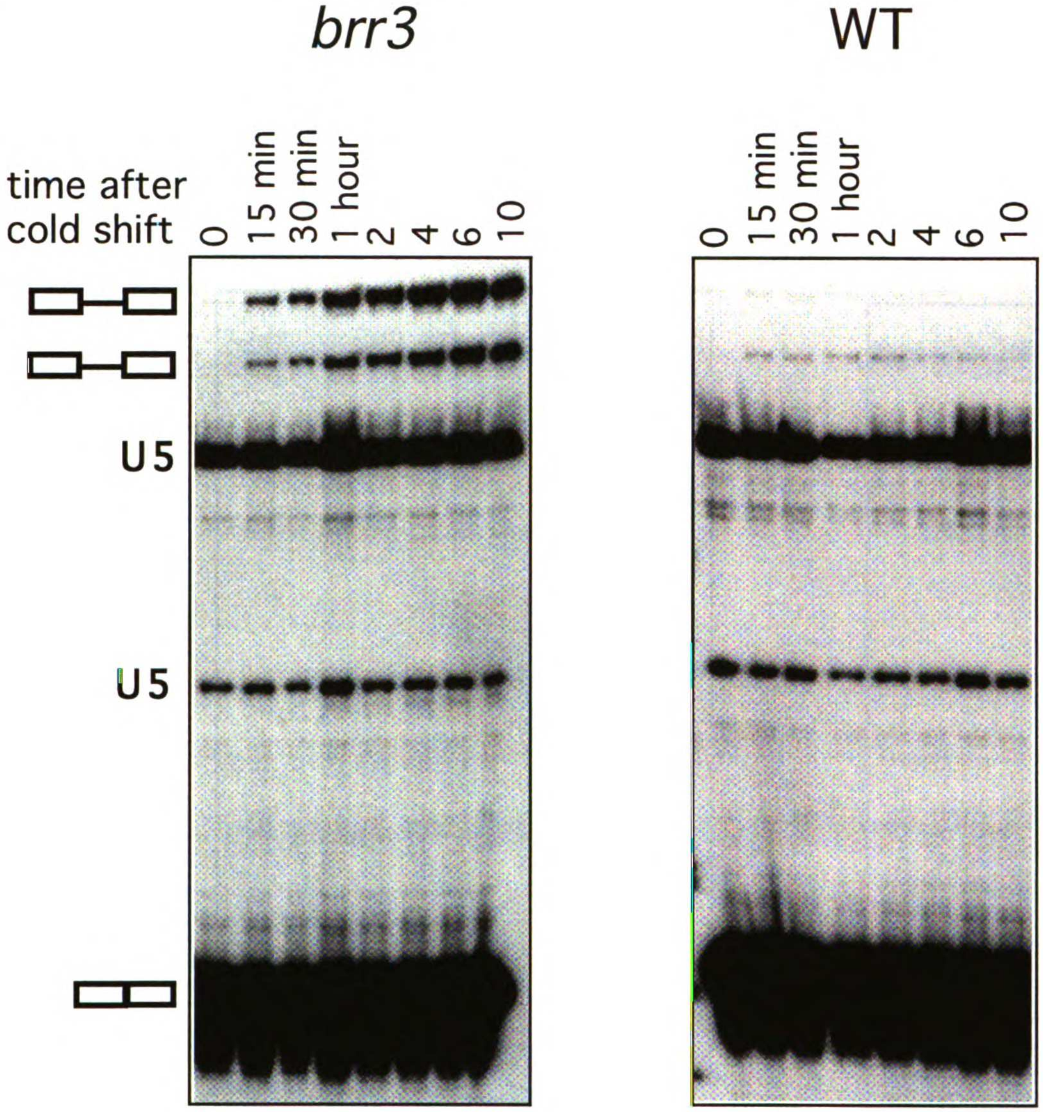
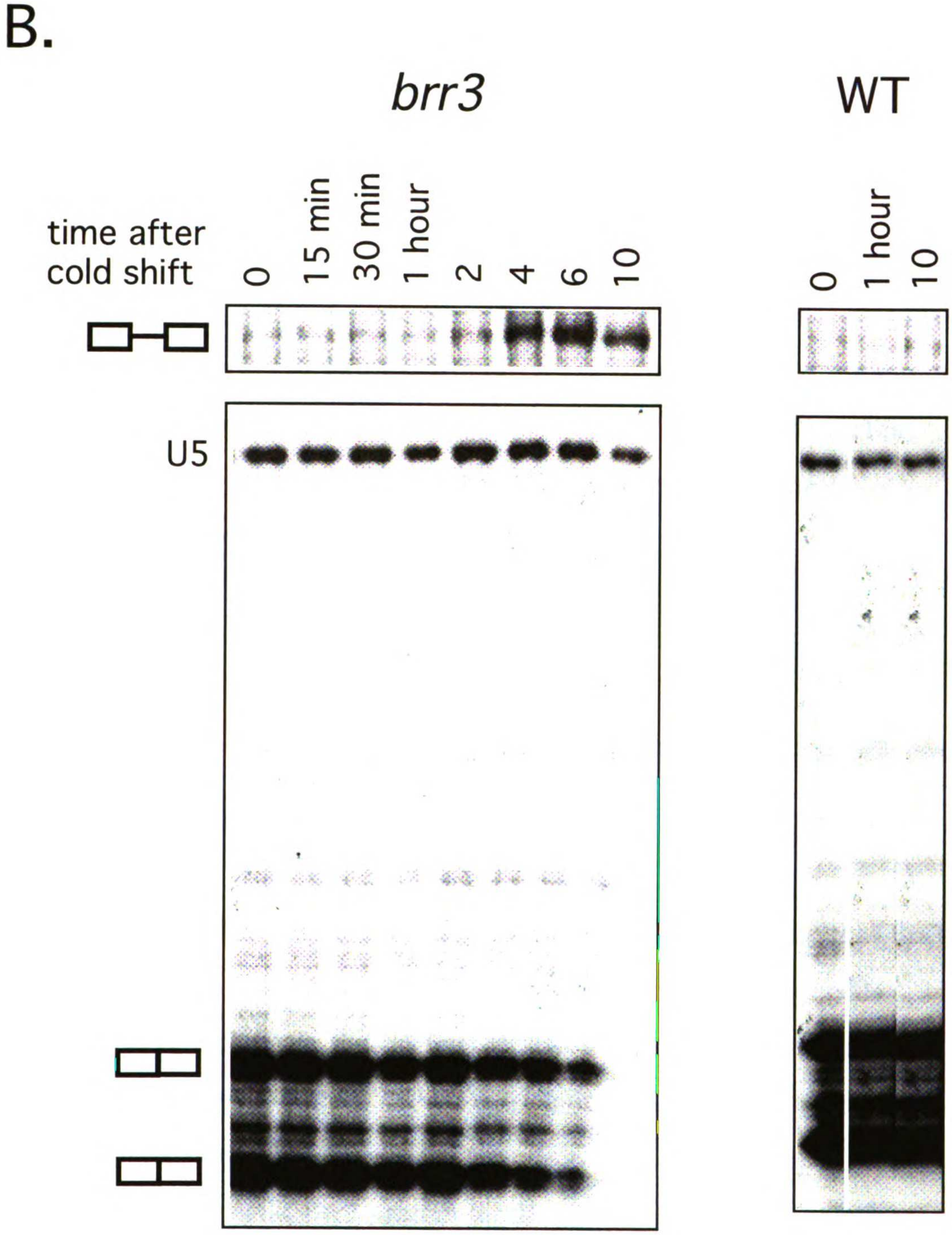
A.


brr3
WT
time after cold shift
0
15 min
30 min
1 hour
2
4
6
10
U5
U5


B.
brr3
WT
time after cold shift
0
15 min
30 min
1 hour
2
4
6
10
0
1 hour
10
U5

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splicing efficiency was quantitated. A representative result of actin splicing in *brr3-1* and wildtype extracts is shown in Figure 6; the mutant extract displays a slight decrease in splicing efficiency (Figure 6A). A quantitative comparison of actin splicing efficiency in mutant and wildtype extracts reveals that splicing is on average decreased 1.6 fold at 25°C and 3.7 fold at 15°C in mutant extracts (Figure 6B). Similarly, U3 splicing efficiency is decreased 1.6 fold at 25°C, and 3.1 fold at 15°C in mutant extracts (data not shown).

The splicing defect in *brr3-1* extracts is modest but reproducible. If the mutant protein has a direct effect on the splicing machinery, purified Brr3 protein should restore wildtype splicing efficiency in mutant extracts. These experiments are in progress.

Discussion

Possible roles for Brr3 in mRNA export

The results described in this chapter add to the mounting evidence that Brr3 plays a primary role in mRNA export. In summary, *brr3-1* mutant cells accumulate poly(A) RNA in the nucleus almost immediately after a shift to the non-permissive temperature. The *brr3-1* mutant is also synthetically lethal in combination with a number of nucleoporin mutants implicated in RNA export. In live cells, Brr3 protein localizes exclusively to the nuclear rim, and biochemical data demonstrate an interaction between Brr3 and Rip1, a nuclear pore-associated protein. Consistent with membrane association, Brr3 extraction conditions are comparable to those of Nup188, a protein that

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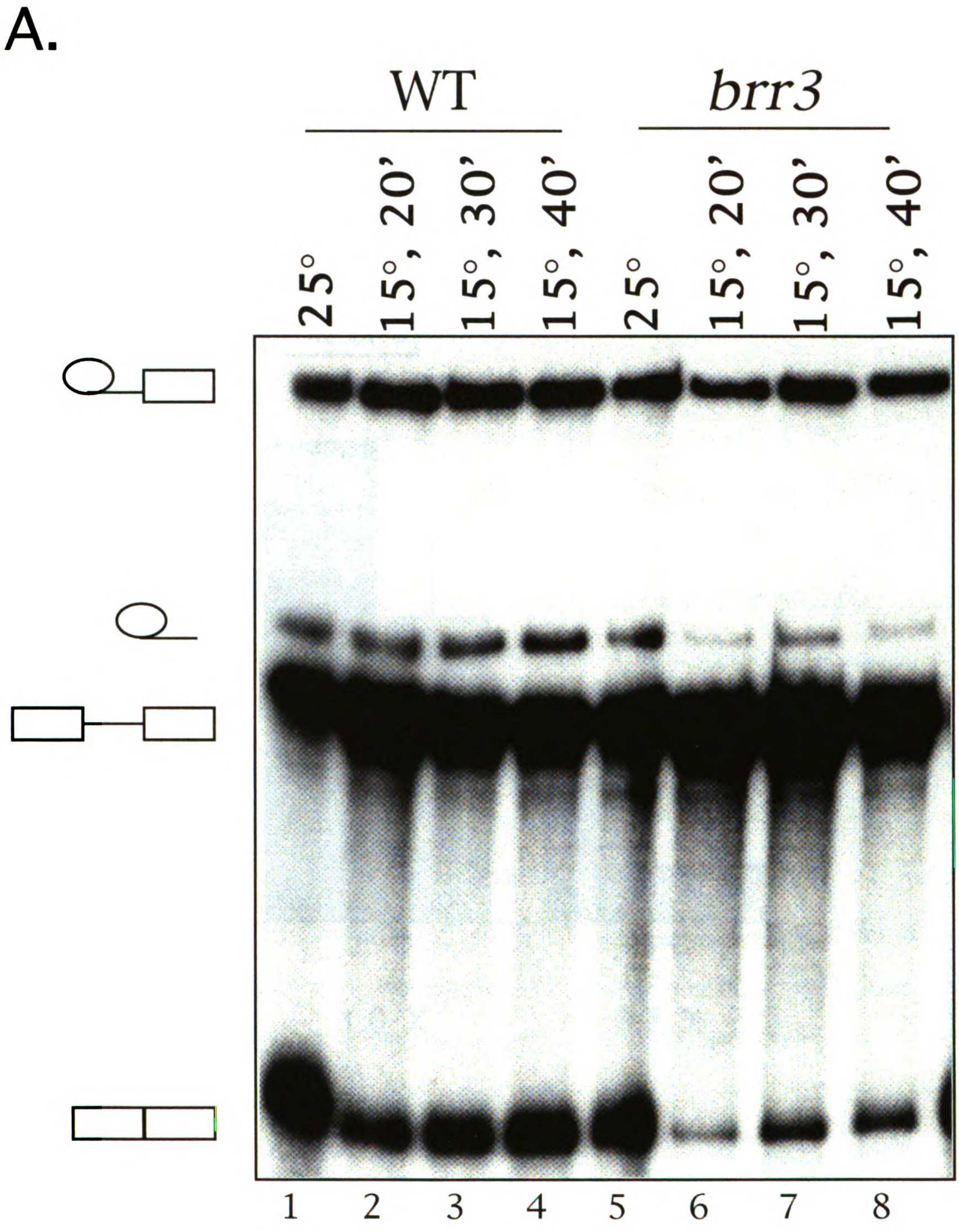
Figure 6: Decreased splicing efficiency in *brr3-1* extracts. *(A)* Wildtype and mutant extracts incubated with radioactively-labeled actin pre-mRNA, at 25°C for 30 minutes (lanes 1 and 5), or at 15°C for increasing times (lanes 2-4 and 6-8). *(B)* Quantitation of actin splicing efficiency in wildtype and mutant extracts. Precursor (P), lariat intermediate (L), and mature (M) bands were quantitated by phosphorimaging and divided by the number of labeled nucleotides for each species. Splicing efficiency was calculated as the ratio of M:P+L+M. Plotted on the graph are the average results of three independent sets of wildtype and mutant extracts.


A.
WT
brr3
25°
15°, 20'
15°, 30'
15°, 40'
25°
15°, 20'
15°, 30'
15°, 40'
1
2
3
4
5
6
7
8

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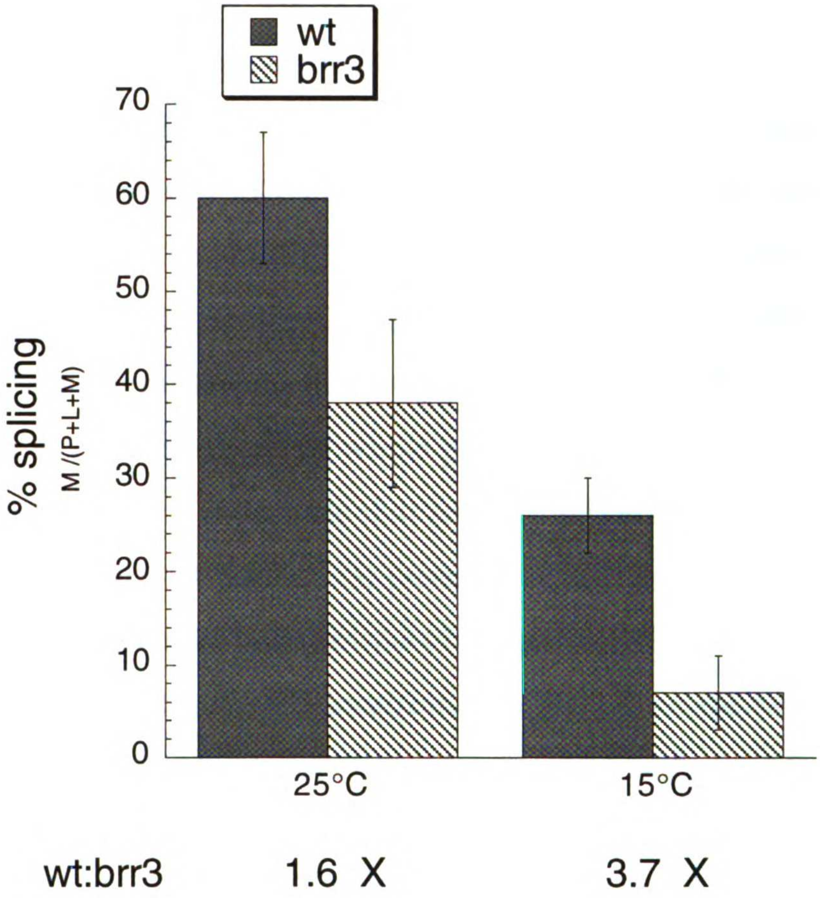
B.


wt
brr3
70
60
50
40
30
20
10
0
% splicing
M /(P+L+M)
25°C
15°C
wt:brr3
1.6 X
3.7 X

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has been detected at the NPC by immuno-electron microscopy and is part of a stable nucleoporin complex (Nehrbass, 1996).

At face value, Brr3 appears to be a conventional nucleoporin of the non-repeat class. However, a major characteristic that distinguishes Brr3 from other nucleoporins is the presence of a Rev-like NES, required for Brr3 function (Murphy, 1996). The Brr3 NES peptide, on its own, drives the nuclear export of a reporter protein microinjected into cultured mammalian cells. This type of experiment was used to delineate NES function to the leucine-rich domains of PKI and Rev. However, unlike PKI and Rev, Brr3 has not been shown to shuttle between the nucleus and cytoplasm. Thus, although the presence of an NES suggests a role for Brr3 in the escort of mRNAs from the nucleus to the cytoplasm, whether the Brr3 NES is functional in the context of the whole protein is a significant unresolved issue. For instance, it is possible that another region of the Brr3 protein, acting to target Brr3 to the nuclear pores, overrides the NES. This model predicts that deletion or mutagenesis of a putative NPC targeting signal would change the steady state localization of Brr3 from the nuclear rim to the cytoplasm. A similar but more complicated model is that the Brr3 NES is functional, but is restricted to movement from the nuclear to cytoplasmic face of the NPC, due to regions of the Brr3 protein that mediate binding to nucleoporins. In this case, disruption of Brr3-nucleoporin binding, either by deleting the relevant regions of Brr3 or deleting the nucleoporin, would result in cytoplasmic accumulation of Brr3.

Since it is unknown if Brr3 shuttles between the nucleus and cytoplasm, and careful deletion analysis of the Brr3 protein has not been done, many

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possibilites remain regarding the precise role of Brr3 in the mRNA export pathway. To facilitate a discussion of these possibilities, it is useful to revisit the proposed steps of mRNA export outlined in the introduction of this chapter:

1. hand-off of RNA from the sites of processing in the nucleoplasm and transport to the nuclear pores
2. translocation of RNA from the nucleoplasmic to the cytoplasmic face of the nuclear pores
3. egress of RNA from the nuclear pores to the cytoplasm

Hypothetically, it is plausible that an NES, in the context of Brr3, could signal the movement of RNA at any, or all, of these steps.

A function for Brr3 at the earliest step of mRNA export is an attractive hypothesis, since the *brr3-1* mutant affects pre-mRNA processing as well as export (discussed below). Three predictions of this model can be tested. First, if involved in this stage of the RNA export pathway, Brr3 is likely to work in conjunction with hnRNP-like proteins. Co-immunprecipitation of Npl3, Nab2, or Nab4 with Brr3-HA was not detected (data not shown); however, the presence of detergent in the extract could have disrupted a potential interaction. Furthermore, the RNA-binding partner of Brr3 could be a novel yeast hnRNP protein, making an open-ended screen for factors interacting with Brr3 a worthwhile endeavor. The second, and strongest prediction of this model is that although Brr3 localizes to nuclear pores at steady-state, it actually shuttles between the nucleoplasm and the nuclear pores. Therefore, blocking Brr3 from a potential docking site at the nuclear pores should lead to its accumulation in the nucleoplasm. The GLFG nucleoporins Nup116 and

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Nup100 are candidate docking factors for Brr3. Brr3 interacts with these nucleoporins genetically (Table I; Murphy, 1996), and interacts with the GLFG-repeat region of Nup100 in a two-hybrid assay (Murphy, 1996). Moreover, Nup116 and Nup100 mediate the export of Kap95, a protein import factor that is recycled back to the cytoplasm from the nucleus (Iovine, 1997); significantly, the interaction of Kap95 with these nucleoporins is dependent on the Rev-like NES present in Kap95. Thus, if Brr3 uses the same export mechanism as Kap95, it should be mislocalized to the nucleoplasm in a *nup116* or *nup100* mutant background. The third prediction of this model is that Brr3 participates in this step of mRNA export with Xpo1, a putative NES-receptor. Surprisingly, in *xpo1* mutant cells at the non-permissive temperature, the localization of Brr3 is normal, at the nuclear rim, while the mutant Xpo1 protein mislocalizes from the nucleoplasm to two discrete spots at the nuclear rim (Stade, 1997). Although normal localization of Brr3 in the *xpo1* mutant can be explained if the two proteins act in different export pathways, the more intriguing interpretation is that Brr3 functions downstream of Xpo1. For example, the mechanism of mRNA export may involve sequential, transient associations with multiple NES-containing proteins as the mRNA makes its way to the cytoplasm. Contrary to the idea of Brr3 functioning at the first step of the export pathway, this would place Brr3 at a later step, following docking at the nuclear face of the pore.

As previously discussed, several lines of evidence firmly establish the presence of Brr3 at the nuclear pores. Much more difficult to test is whether Brr3 mediates RNA export by traveling between the two faces of the NPC, transiently interacting with nucleoporins along the way. The synthetic lethality of *brr3-1* and nucleoporin mutants implicated in RNA export

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supports this model, but a crucial extension of these results is demonstrating a physical interaction between Brr3 and nucleoporins. Of the nucleoporins examined, only Rip1 has been shown to stably interact with Brr3 biochemically (Figure 3B; Murphy, 1996). This argues that Brr3 association with nucleoporins is likely to be transient, with only a small pool of total Brr3 binding to a particular nucleoporin at any given time. Thus, sensitive methods may be necessary to detect these interactions, such as directed two-hybrid assays or immunopurification of Brr3 complexes after covalent crosslinking of proteins. Another prediction of this model is that Brr3 is located at sites throughout the NPC; this could be confirmed by taking an ultra-structural look at the pores by immuno-EM, using gold particles coated with antibodies specific for Brr3.

The final stage in the export pathway consists of the delivery of the RNA from the nuclear pores to the cytoplasm. The simplest way to determine if Brr3 is involved at this step is to test if it shuttles from the pores to the cytoplasm. A standard shuttling assay is to utilize a conditional nucleoporin mutant that blocks nuclear protein import, but not export, such as *nup49-313* (Lee, 1996). Cells are shifted to the non-permissive temperature to block import, and cyclohexamide is added to inhibit new protein synthesis. If Brr3 shuttles, it should accumulate in the cytoplasm under these conditions. Importantly, resuming nuclear import by shifting the cells back to the permissive temperature should result in the return of Brr3 to the nuclear rim. In addition, a potential result in support of this model would be the mislocalization of the *brr3-1* mutant protein to the cytoplasm (or to the nucleus, if Brr3 acts at the early step of export). However, this experiment

proved to be difficult due to the instability of epitope-tagged versions of the mutant protein.

Confirming mRNA as the export substrate for Brr3

The initial report of *gle1/brr3* mutants being defective for poly(A) RNA export, in the absence of a protein import block or aberrant nuclear envelope morphology argues that Brr3 is a mediator of mRNA export (Murphy, 1996). This is supported by the rapid nuclear accumlation of poly(A) RNA seen in the *brr3-1* mutant (Figure 1). Critical evidence needed to uphold this hypothesis is the demonstration of a physical association between Brr3 and mRNA. This could be achieved by immunoprecipitating Brr3 from whole cell extracts, and assaying for the co-purification of poly(A) RNA. Conversely, to potentially increase sensitivity, poly(A) RNA complexes could be purified and examined for the presence of Brr3. Since a Brr3 interaction with mRNA is likely to be transient, it may be necessary to enrich the RNA-associated population of Brr3 by covalent crosslinking prior to purification. Since Brr3 contains no known RNA-binding motifs, its association with mRNA is likely to be via one or more RNA-binding proteins. Hence, the demonstration of a physical interaction with an RNA-binding protein would provide further evidence for a Brr3 connection with mRNA. As described above, co-immunprecipitation of Brr3 and three known yeast RNA-binding proteins, of the hnRNP class, was not detected. yCBP80, one of the yeast proteins in the complex that binds the 7-methyl guanosine cap of RNAs (Izaurralde, 1994), was also not seen in the Brr3 immunoprecipitated pellet (data not shown).

However, an interaction between Brr3 and a novel RNA-binding protein remains a likely possibility.

A related question regarding the export substrates of Brr3 is whether Brr3 is involved in a specialized export pathway. Results of competition studies in *Xenopus* oocytes argue that different classes of RNA, such as mRNA, snRNA, and tRNA, utilize export factors specific to each class (Jarmolowski, 1994; Pante, 1997). Furthermore, specialized export pathways have been shown to exist for a subset of mRNA. Heat shock treatment of yeast cells causes a rapid inhibition of bulk poly(A) RNA export, while mRNAs encoding heat shock proteins continue to be exported to the cytoplasm (Saavedra, 1996). According to a recent report, Rip1 is required for this export of heat-shock mRNAs (Saavedra, 1997). A stable and direct physical interaction between Rip1 and Brr3 has been established by multiple methods, including co-immunoprecipitation (Figure 3B) and ligand-blot overlay (Murphy, 1996). These results raise the interesting possibility of Brr3 participating in heat-shock mRNA export with Rip1. A key test of this model is to determine if the export of specific heat-shock mRNAs is blocked in *brr3-1* cells under heat shock conditions. It is important to note that unlike Brr3, Rip1 is not an essential protein and not required for bulk poly(A) RNA export; thus, a putative role for Brr3 in heat-shock mRNA export is likely to be in addition to a general role in mRNA export. Since less than 100% of detergent solubilized Brr3 is associated with Rip1 (Figure 3B), a general role in export could be carried out by the pool of Brr3 that is not bound to Rip1 (see Epilogue for further discussion of this model).

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Does Brr3 couple mRNA processing and export?

The observation that the *brr3-1* mutant affects poly(A) tail formation and pre-mRNA splicing, as well as export, leads to the intriguing hypothesis that Brr3 may act to link these events, possibly by directing mRNAs from the site of processing to the export machinery. To simplify this discussion, the coupling of splicing with export, and poly(A) tail formation with export are treated separately, although the models are not mutually exclusive.

Since defective pre-mRNA splicing is not generally seen in mRNA export mutants (Kadowaki, 1994; Sharma, 1996), it is tempting to speculate that Brr3 may be involved specifically in the export of spliced mRNAs. To date, there is no evidence for distinct factors in the export of spliced and non-spliced mRNAs. However, it is not an unreasonable hypothesis. Although less than 5% of yeast mRNAs contain introns, these mRNAs are highly abundant, coding for ribosomal proteins and structural proteins, such as actin and tubulin. Since these proteins are essential in maintaining normal cell function, coordination between the splicing machinery and the export pathway to ensure export of properly processed messages is likely to be critical. Brr3 could play a role in this coordination. The simplest prediction of this model is that the *brr3-1* mutant specifically blocks the export of spliced mRNAs, without affecting the export of non-intron containing mRNAs. This could be tested by assaying the export of distinct messages of either class *in situ*, using specific probes. An effective and reproducible method to detect a variety of specific RNAs in yeast by *in situ* hybridization is currently being developed (Long, 1995; Saavedra, 1996; M. Inada, unpublished data).

It may seem paradoxical that Brr3 could have a direct connection to splicing, given the late onset of the splicing defect after a cold shift *in vivo*, seen in three of the four intron-containing RNAs assayed (Figure 5B; data not shown). The only exception is the rapid precursor accumulation of U3 snoRNA (Figure 5A), which may reflect the fact that it is an atypical splicing substrate. Notably, a slight increase in U3 precursor has been observed in other cold-sensitive RNA export mutants that are not defective in general pre-mRNA splicing, shortly after a shift to the cold (A. de Bruyn Kops and C. Guthrie, manuscript in preparation). Thus, the early onset of the U3 splicing defect in *brr3-1* cells could be an indirect effect of the export defect. For example, U3 splicing may be sensitive to a decrease in the level of an extremely labile splicing factor encoded by an unstable mRNA; thus, a block in the export of the mRNA would result in a rapid decrease in the amount of protein synthesized. An alternative explanation for the rapid U3 splicing defect in the *brr3-1* mutant is that Brr3 is specifically involved in U3 splicing. However, U3 splicing *in vitro* is less affected by *brr3-1* than actin splicing, making this possibility unlikely. Moreover, the efficiency of actin splicing is decreased only two-fold in *brr3-1* extracts when compared to wildtype extracts (Figure 6), also arguing against an essential role for Brr3 in splicing.

Nonetheless, the splicing results are not inconsistent with Brr3 functioning as a coupling factor, coordinating the transit of mRNAs from the spliceosome to the export pathway. To elaborate on the coupling model, Brr3 may interact with a late-acting splicing factor to activate the release of fully-spliced mRNAs from the spliceosome, freeing them for export. Therefore, if the *brr3-1* mutant protein is incapable of associating with mRNA or unable to promote mRNA release from the splicing machinery, the trapping of mRNAs in the

spliceosome could inhibit the recycling of essential splicing components. This would lead to an eventual block in the first step of splicing, as is seen in *brr3-1* cells. According to this model, Brr3 should not be required for splicing reactions *in vitro*, and the slight decrease in splicing efficiency observed is not likely to be a direct effect of the *brr3-1* mutant protein. Thus, depletion of Brr3 from extracts should not affect splicing activity, and purified Brr3 may not rescue the splicing defect of *brr3-1* extracts. A strong prediction of this model is that Brr3 shuttles between the nucleus and nuclear pores or cytoplasm. Specific evidence for the model may be harder to attain. One possibility is to search for interactions with splicing factors, especially those that act late in the splicing pathway, like Prp22 and Prp27 (Vijayraghavan, 1989; Company, 1991). Interestingly, mutants in these genes lead to nuclear accumulation of poly(A) RNA (A. de Bruyn Kops, unpublished data), perhaps as a result of an inability to release mRNAs from the spliceosome. *brr3-1* does not interact with two mutant alleles of *prp22* genetically (see Appendix II), however a thorough investigation of an interaction between these proteins has not been attempted. Furthermore, Brr3 could interact with a late-acting splicing factor other than Prp22, which has yet to be tested.

Supporting a role for Brr3 in the coupling of mRNA poly(A) tail formation and export, the RNA export defect and long poly(A) tails in *brr3-1* cells are closely linked phenotypes. Both are initially observed at the permissive temperature, and are exacerbated when cells are shifted to the cold (though it is difficult to assess whether the fraction of cells with the export defect are the ones with the longer tails). Based on *in vitro* data, the long poly(A) tails in *brr3-1* cells can be attributed to a defect in poly(A) tail shortening, after the initial polyadenylation of the transcripts (Figure 4). Unfortunately, the

poly(A) shortening reaction is not well understood, especially in yeast. It has been believed to occur after mRNAs are exported to the cytoplasm, but before the deadenylation event that triggers mRNA degradation (Sachs, 1989). The idea that poly(A) shortening occurs in the cytoplasm is based on very early studies of mammalian cells pulse-labeled with radioactive adenosine, then fractionated into nuclear and cytoplasmic compartments; when compared to nuclear poly(A) mRNA, the cytoplasmic poly(A) tails are shortened by a discrete length, as rapidly as five mintutes after labeling (Brawerman, 1981). The rapid kinetics of poly(A) shortening is consistent with the shortening step taking place as the mRNAs are being exported to the cytoplasm, at the nuclear pores. RNA processing at the pores is not unprecedented; pre-tRNA splicing is likely to occur as tRNAs are exported, as suggested by the localization of tRNA splicing enzymes at the nuclear envelope, and the pre-tRNA splicing defects of certain nucleoporin mutants (Simos, 1996).

This hypothesis invites the possibility that Brr3 participates in poly(A) shortening as it exports the mRNAs through the nuclear pores or to the cytoplasm. A major prediction of this model is that Brr3 is required for the poly(A) shortening reaction. This can be ascertained by assaying poly(A) shortening in 3' processing extracts depleted of Brr3. If Brr3 is not required for the reaction, a second possibility is that proper Brr3 association with mRNA is a necessary prerequisite to poly(A) shortening. For example, Brr3 may be needed to recruit the shortening enzymes to the mRNA, and the *brr3-1* mutant protein may be unable to do so. In this case, purified Brr3 protein should rescue the shortening defect in *brr3-1* extracts. Failure to complement the *in vitro* defect could be due to dominance of the *brr3-1* mutant *in vitro*. Alternatively, the shortening defect might be an indirect effect of the block to

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export. If so, any RNA export mutant with longer poly(A) tails *in vivo* would be expected to display the same *in vitro* shortening defect as *brr3-1*. A final, but unlikely possibility is that the export defect is an indirect effect of the shortening defect; poly(A) shortening is not a requirement for export, since mRNAs with long poly(A) tails have been observed as substrates for cytoplasmic degradation (Caponigro, 1995).

A potential role for Brr3 in the coupling of poly(A) shortening with mRNA export can also be explored by looking for connections with proteins involved in poly(A) tail metabolism. The most obvious candidate is Pab1, the highly conserved, major poly(A) binding protein in eukaryotic cells (Sachs, 1986), directly involved in mRNA-related events such as translation initiation and control of mRNA decay (Caponigro, 1995; Tarun, 1995). Moreover, recent reports have demonstrated a role for Pab1 in the control of poly(A) tail length, and the addition of recombinant Pab1 to elongated poly(A) tails *in vitro* stimulates tail shortening (Amrani, 1997; Minvielle-Sebastia, 1997). Significantly, a *brr3-1pab1-53* double mutant grows much more slowly at 30°C than either single mutant alone (see Appendix II). The *pab1-53* mutant affects the production of mRNA, resulting in an overall decrease in mRNA levels (Morrissey, 1997). Therefore, this synthetic effect could simply be the result of combining defects in two distinct, essential cellular processes. However, a more attractive explanation is that it reflects a functional association between Brr3 and Pab1 in the maturation of mRNAs. Data showing a biochemical interaction between Brr3 and Pab1 is needed to support this hypothesis, but attempts to co-immunoprecipitate the two proteins from yeast extracts have not been successful (data not shown). Also, an interaction was not detected by two-hybrid analysis (P. Preker, unpublished data).

Two other proteins that could partner with Brr3 in the poly(A) shortening reaction are Pan2 and Pan3, the two subunits of the Pab1-dependent poly(A) nuclease (Boeck, 1996; Brown, 1996). Yeast cells deleted for either *pan2* or *pan3* contain longer poly(A) tails *in vivo*, similar to what is seen in *brr3-1* cells. Moreover, recent results support a role for Pan2 and Pan3 in poly(A) shortening, but not in deadenylation-dependent mRNA decay (C. Brown, personal communication). In a yeast strain deleted for *pan2* and *pan3*, transcriptional pulse-chases of individual mRNAs demonstrate a decrease in the rate of initial deadenylation (likely representing poly(A) shortening), but normal rates of deadenylation prior to mRNA decay. The *brr3-1* mutant does not interact genetically with a *pan3* deletion (see Appendix II); however, since cells deleted for both *pan* genes are viable, it may be necessary to construct the triple mutant to detect a genetic interaction. Also, testing for interactions between Brr3 and the Pans biochemically, or by two-hybrid assays would be worthwhile.

It remains to be seen whether Brr3 is specifically involved in the coupling of poly(A) tail shortening and export, and/or directing mRNAs from the spliceosome to the export pathway. Results from many experiments support a strong connection between Brr3 and the NPC, but a precise role for Brr3 in mRNA export has yet to be elucidated. Ultimately, to prove a direct role for Brr3 in mRNA export and coupling of RNA processing with export, an *in vitro* system capable of carrying out these events is required. In the meantime, further analysis of Brr3 will unquestionably lead to a better understanding of the molecular mechanism underlying these essential processes.

Materials and Methods

Yeast strains and plasmids

The following is a list of yeast strains used in these studies. All strains, except when indicated, are in the W303 background (*MATa/α ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*).

yTA302: BRR3 MATa ura his trp LEU LYS (S288C/W303 background)
yTA305: brr3-1 MATa ura his trp LEU LYS (S288C/W303 background)
yTA311: *brr3*::LEU2 {pBRR105 = *BRR3* in pSE360, *CEN-URA3*}
yTA315: *brr3*::LEU2 {pBRR109 = *BRR3-HA*$_3$ in pSE360}
yTA319: *brr3*::LEU2 {pBRR115 = *BRR3* in pSE358, *CEN-TRP1*}
yTA320: *brr3*::LEU2 {pBRR118 = *BRR3-GFP* in pSE358}
yTA326: *brr3*::LEU2 {pBRR123 = *brr3-1* in pSE358}

yTA305 was isolated by outcrossing ySN103 (Noble, 1996) to W303 twice. yTA302 is a wildtype sister spore of yTA305. yTA311 and yTA315 were made by transforming a diploid strain heterozygous for the *BRR3* chromosomal knock-out with pBRR105 and pBRR109, respectively, followed by sporulation and tetrad dissection. The resulting spores were scored for leucine and uracil prototrophy. yTA319, yTA320, and yTA326 were generated by transforming yTA311 with the plasmids listed above. Transformants were streaked onto plates containing 5-FOA to induce loss of the *URA3* plasmid. The RIP1 deletion strain is described elsewhere (Stutz, 1995). All yeast manipulations were done as in Guthrie and Fink (Guthrie, 1991).

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pBRR105 consists of the NlaIV/SpeI genomic fragment that includes the BRR3 ORF with approximately 400 basepairs upstream and 600 basepairs downstream. This fully complementing fragment is cloned into pSE360 at the SmaI/XbaI sites in the polylinker, destroying all four restriction sites. To make pBRR109, a NotI site was introduced at the extreme C-terminus of *BRR3* in pBRR105 by PCR using oligonucleotides brr3cn [5'CTTTCCGGAAATGTCTCCTGGCGGCCGCTAGAAAATTGTGAACTTATATA-3'] and brr3SalI [5'GCAGGTCGACTCTAGTAATATC-3']. The resulting PCR fragment was digested with BspEI and SalI, cloned into the corresponding sites of pBRR105, and sequenced for confirmation. Subsequently, a fragment containing three tandem sequences of the influenza virus hemgglutinin peptide, flanked by NotI sites, was cloned into the NotI site of *BRR3*. pBRR115 was generated by subcloning the EcoRI/SalI fragment of pBRR105, containing the entire *BRR3* genomic insert, into the matching sites of the pSE358 polylinker. To make pBRR118, the *GFP* (S65T, F64L) gene in a pBluescript KS+ vector was amplified by PCR with primers containing NotI sites [5'GFPNot = 5'ATAAGAATGCGGCCGCATGAGTAAAGGAGAAGAAC-3'; 3'GFPNot = 5' TATTCTTAGCGGCCGCTTATTATTTGTATAGTTCATCC-3'], digested with NotI, and cloned into the C-terminal NotI site of *BRR3* in pSE358. pBRR123 was generated by gap repairing the *brr3-1* mutation onto pSE358 (see Chapter 1).

In situ hybridization, immunofluorescence, and fluorescence microscopy

For dT_{50} in situ hybridization, 1 ml of mid-log cells were prepared as described (Amberg, 1992; Kadowaki, 1994), with modifications to be presented elsewhere

(A. de Bruyn Kops et al., manuscript in preparation). For the double-labeling experiment, 1/1000 diluted anti-Nop1 antibodies were added to cells after dT_{50} hybridization and washes, followed by incubation with rhodamine-conjugated secondary antibodies and fluorescein-conjugated anti-digoxygenin antibodies.

For Brr3-GFP localization experiments, mid-log cultures in synthetic complete media were spotted on slides, examined and imaged as described (Stade, 1997).

Bead-beat extract preparation and immunoprecipitations

30 OD_{600} units of mid-log cultures were pelleted, washed with PBS, and resuspended in 400 µl of lysis buffer consisting of 150 mM NaCl, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, .1 mM DTT, 10% glycerol, fresh protease inhibitors, and various concentrations of Triton X-100 (see Figure 3). An equal volume of acid-washed glass beads was added, the eppendorf tubes inverted and vortexed at full speed with the VWR multi-tube vortexer for 10 minutes in the cold room. A 23-gauge needle was used to puncture the bottom of the tubes, and the vortexed extracts were centrifuged into a fresh eppendorf tube at 1000 RPM for one minute. Unlysed cells were pelleted with a brief spin, and the remaining supernatant transferred to a new tube. Insoluble material was pelleted at high speed for 15 minutes, and the resulting supernatant stored at -80° C for immunoprecipitation experiments.

For immunprecipitation, extracts were pre-cleared with half-volumes of Protein A sepharose beads, pre-washed with lysis buffer. Extracts were then

incubated with half-volumes of beads alone, or beads pre-bound with 12CA5 anti-HA antibodies (1:5 ratio of antibodies to beads), and double the extract volume of IPP$_{150}$ (150 mM NaCl, 50 mM Tris pH 7.4, and .05% NP-40). The mixture was rotated for one hour in the cold room. Beads were pelleted by light centrifugation (3000 RPM, 2 minutes), washed three times with IPP$_{150}$, and resuspended in SDS-Laemmli buffer for SDS-PAGE (Laemmli, 1970). Western blots were probed with a 1/2000 dilution of 12CA5 antibodies, washed, and incubated with 1/2000 diluted horseradish-peroxidase conjugated secondary antibodies (BioRad). Rip1-probed blots were incubated with 1/2000 anti-Rip1 polyclonal antibodies overnight at 4°C. Blots were developed by ECL (Amersham).

Splicing and 3' end formation *in vitro*

Splicing extracts were prepared from one liter of late-log cultures as previously described (Umen, 1995). Standard splicing reactions were done in 20 µl (Lin, 1985), and quantitated using a PhosphorImager with ImageQuant software (Molecular Dynamics).

3' processing extracts were prepared as splicing extracts, with an additional 40% ammonium sulfate precipitation, as described (Butler, 1990). Preparation of labeled CYC1 and pre-cleaved CYC1, and 3' processing reactions were performed as in (Minvielle-Sebastia, 1994; Preker, 1995).

RNA preparation and primer extensions

RNA was prepared from mid-log cells and analyzed by primer extension according to (Lesser, 1993). The oligonucleotides used are as follows: U3 exon 2 [CCAAGTTGGATTCAGTGGCTC], RP51A exon 2 [CGCTTGACGGTCTTGGTTC], U5-7wtsmnr [AAGTTCCAAAAAATATGGCAAGC].

EPILOGUE

What's in store for Brr3?

As testimony to the nature of scientific research, the results from my experiments did not completely follow my initial predictions. Based on the strong export and splicing defects of the *brr3-1* mutant, my original hope was that Brr3 is directly involved in both export and splicing, and therefore be the first example of a protein that couples mRNA splicing and export. My work and work from others has provided ample evidence that Brr3 plays an important role in mRNA export (Del Priore, 1996; Murphy, 1996), starting with the observation that all mutant alleles of the *BRR3* gene isolated thus far cause a defect in poly(A) RNA export. At this point, the majority of the data supports the model that Brr3 functions at the nuclear pore: the Brr3 protein localizes to the nuclear rim by fluorescence microscopy, is fully soluble only in the presence of detergent, stably and directly interacts with the nucleoporin Rip1, and *brr3* mutant alleles are synthetically lethal with a number of nucleoporin mutants. The presence of a Rev-like NES in the Brr3 sequence, which functions as an NES on its own, raises the possibility that Brr3 actually accompanies mRNA molecules from the nucleus to the cytoplasm. Mutating a key leucine residue in the Brr3 NES results in a temperature-sensitive phenotype and inhibition of poly(A) RNA export; however, it has yet to be determined whether this sequence functions as an NES in the context of the Brr3 protein, as opposed to mediating a protein-protein interaction at the nuclear pore, necessary for Brr3 function. Also, it is still unknown whether Brr3 stably associates with mRNA.

As a potential splicing/export coupling factor, Brr3 may be required for both splicing and export, remaining with the mRNA after splicing is complete to

mediate its export. Alternatively, Brr3 may not be essential for splicing per se, but may interact with a late-acting splicing factor in an active hand-off of the mRNA from the spliceosome to the export pathway. The observation that *brr3* mutant extracts are able to splice pre-mRNAs, albeit at a slightly lower efficiency than wildtype, makes it unlikely that Brr3 is required for splicing. However, as I discussed in Chapter 2, an essential role in splicing cannot be ruled out without assaying splicing in the absence of Brr3 protein, and a putative interaction with a splicing factor has not been thoroughly investigated. Hence, it is still too early to abandon the hypothesis that Brr3 couples splicing with export.

To my surprise, I discovered that the *brr3* mutant has a more dramatic effect on another step in mRNA processing, poly(A) tail shortening, which is inhibited in 3' processing extracts prepared from *brr3* mutant cells. This observation led to a new hypothesis, that Brr3 participates in poly(A) tail shortening as it exports mRNAs, possibly at the nuclear pores. Pascal Preker, a post-doc in the Guthrie lab, is continuing to test this intriguing hypothesis.

Clearly, future investigations to elucidate the primary function of Brr3 can proceed from a number of starting points. Among the list of unknowns are whether Brr3 acts anywhere in the export pathway other than the NPC, whether it interacts with mRNA transcripts, and, if so, when, whether it is involved in the export of any other RNAs, and the nature of its interaction, if any, with the spliceosome and the poly(A) shortening machinery. For this final chapter of my thesis, I would like to elaborate on two models for Brr3 function that I believe represent the type of questions the mRNA export field will face next. First is the issue of Brr3 involvement in poly(A) shortening,

which addresses the question of coupling mRNA processing events with export. Second is the latest model for Brr3 function, mediating the export of heat shock mRNAs under stress conditions (Saavedra, 1997; Stutz, 1997). Until the discovery of the heat shock mRNA export pathway (Saavedra, 1996), the study of mRNA export in yeast was limited to bulk poly(A) RNA. The identification of this specialized mRNA export pathway emphasizes the importance of refining studies of mRNA export to include examination of specific mRNAs.

Brr3 and poly(A) shortening

The poly(A) shortening reaction is the first of two phases of mRNA deadenylation. In mammalian cells, it has been demonstrated that shortening of mRNA poly(A) tails by a discrete amount occurs soon after the mRNAs appear in the cytoplasm (Brawerman, 1981). In a kinetically distinct phase, mRNAs are almost completely deadenylated as a prerequisite to degradation. The inability to fractionate nuclear and cytoplasmic compartments in yeast makes it difficult to examine cytoplasmic deadenylation events. However, two distinct phases of deadenylation have been observed in yeast cells lacking the *PAN2* and *PAN3* genes; in these cells, the initial poly(A) shortening reaction is slowed, while the rate of deadenylation prior to mRNA degradation is unaffected (C. Brown, personal communication). The functional significance of poly(A) shortening is unclear, but given that it occurs soon after mRNAs appear in the cytoplasm, and appears to be distinct from the process of mRNA degradation, it is likely to constitute a step in the maturation of mRNAs.

Three proteins that are apt to play a role in poly(A) shortening are the poly(A) binding protein Pab1 and the two subunits of its associated poly(A) nuclease, Pan2 and Pan3. However, since these proteins also function in other steps of mRNA processing and metabolism, it is difficult to isolate their effect on poly(A) shortening (Caponigro, 1995; Tarun, 1995; Brown, 1996). This is best illustrated by the examination of 3'-end formation in extracts lacking either Pab1, or Pan2 and Pan3. In both cases, poly(A) tails much longer than wildtype are formed, strongly suggesting that these proteins are necessary for poly(A) tail length control (Amrani, 1997; Minvielle-Sebastia, 1997; C. Brown, personal communication). Since formation of poly(A) tails occurs before poly(A) shortening, a subsequent failure to shorten poly(A) tails due to the absence of Pab1, or Pan2 and Pan3 would not be detected by this assay.

In contrast, the *brr3-1* mutant affects only the poly(A) shortening step of 3'-end formation *in vitro* (Chapter 2, Figure 4). The next step in this analysis is to determine the consequence of depleting Brr3 from 3' processing extracts. A potentially exciting result would be if poly(A) tails fail to be shortened in the absence of Brr3, suggesting that Brr3 is required for poly(A) shortening *in vitro*. To speculate, a requirement for Brr3 in poly(A) shortening could reflect any of several mechanisms. First, Brr3 could be a poly(A) nuclease. If so, purified or recombinant Brr3 protein should exhibit nuclease activity when incubated with radiolabeled poly(A), possibly stimulated by Pab1. Second, Brr3 could be required to recruit poly(A) nucleases to the mRNA. Pan2 and Pan3 are possible targets of Brr3, and two-hybrid interactions between Brr3 and the two nuclease subunits are being tested. On the other hand, since poly(A) tail length control is normal in *brr3* mutant extracts, Pan2 and Pan3

appear to be active; thus, Brr3 may act in conjunction with a novel poly(A) nuclease. A third possibility is that Brr3 is required to make the poly(A) tail accessible to poly(A) shortening enzymes, perhaps by mediating a conformational change in the mRNA. Although challenging, one potential test of this model is to determine if RNase footprinting patterns of poly(A) tails are altered in the absence of Brr3, or in *brr3* mutant extracts, such that the poly(A) tail is in an aberrant RNP structure.

If Brr3 is directly involved in poly(A) shortening, how would that function relate to the mRNA export function of Brr3? First, it is important to note that mRNA export is unlikely to be dependent on the poly(A) shortening step per se; in *pab1* mutant cells, mRNAs with elongated poly(A) tails are substrates for cytoplasmic degradation (Caponigro, 1995). Nonetheless, it remains an intriguing possibility that Brr3 couples poly(A) shortening with export by mediating the shortening step as it exports mRNAs. Admittedly, since the exact function of Brr3 in mRNA export is still unclear, postulating specific coupling models requires further speculation on the role of Brr3 in export. For example, if Brr3 is a nucleoporin directly involved in mRNA export, it may be part of a trail of nucleoporins guiding mRNAs through the NPC by transiently associating with an mRNA then releasing it to the next nucleoporin in line. In this case, Brr3 could function in shortening the poly(A) tail as it interacts with the mRNA, by acting as the shortening nuclease, recruiting the relevent nucleases to the mRNA, or making the mRNA accessible to the poly(A) shortening enzymes. Alternatively, if Brr3 is a soluble export factor that accompanies mRNAs to the cytoplasm, it could mediate the poly(A) shortening event in the cytoplasm, after translocation through the NPC. One way to distinguish between these two models is to

determine if Brr3 shuttles between the nucleus and cytoplasm, or at least between the NPC and cytoplasm. A second way to differentiate between the two models is to figure out precisely where poly(A) shortening occurs. Fractionation studies in mammalian cells suggest that poly(A) shortening is a cytoplasmic event (Brawerman, 1981), but it is possible that shortening occurs as mRNAs are exported to the cytoplasm. Once our understanding of the distinct steps of mRNA translocation through the NPC has improved, it may be possible to block mRNA export at various stages of translocation through the NPC *in vivo*, perhaps by mutations in specific nucleoporins. Then, assaying poly(A) tail length of a specific mRNA in the various nucleoporin mutant backgrounds would reveal if poly(A) shortening is occuring at the nuclear pores or in the cytoplasm, after translocation through the NPC.

Brr3 and heat shock mRNA export

Heat shock mRNA export is the first example of a specialized export pathway for cellular mRNAs. In response to environmental stress, such as heat shock (42°C in yeast) and ethanol shock, synthesis of heat shock proteins is rapidly induced at high levels to help cells survive the stress conditions (Lindquist, 1986; Morimoto, 1993). Saavedra et al. (1996) discovered that poly(A) mRNA export is inhibited when yeast cells are stressed, while the export of mRNAs encoding two heat shock proteins proceeds normally. To determine any distinguishing features between the two export pathways, heat shock mRNA export was assayed in mutants that inhibit poly(A) RNA export. Cells harboring a temperature-sensitive mutant of *NPL3*, the yeast mRNA-binding protein, were not defective for heat shock mRNA export, suggesting that one

distinction between heat shock mRNAs and general mRNAs is at the level of RNP structure (Saavedra, 1997). Additionally, in contrast to bulk mRNA export, heat shock mRNA export does not require the Ran GTPase cycle. The two export pathways appear to converge at the nuclear pore, since heat shock mRNA export is blocked in three nucleoporin mutants which are also defective in general mRNA export.

Piquing my interest, further requirements for heat shock mRNA export were recently reported involving the nucleoporin Rip1 and Brr3/Gle1/Rss1 (Saavedra, 1997; Stutz, 1997). Previous studies had determined that Rip1 is not essential for cell growth under normal conditions, and cells deleted for the *RIP1* gene do not display a poly(A) RNA export defect (Stutz, 1995). However, the new reports describe two conditions where Rip1 is essential. First, Rip1 is required for heat shock mRNA export. In *RIP1*-deleted cells, heat shock mRNA export, and subsequently heat shock protein expression, are inhibited (Saavedra, 1997). Heat shock protein expression is also blocked in cells producing levels of Rip1 five to ten-fold lower than wildtype (Stutz, 1997). Second, Rip1 is essential for cell viability when the *BRR3* gene is mutated, as demonstrated by synthetic lethal interactions between the *rip1* deletion and a number of *brr3* mutant alleles. In a screen for mutants synthetically lethal with the *rip1* deletion, the majority of mutants isolated were alleles of *BRR3/GLE1/RSS1* (Stutz, 1997). Furthermore, the *brr3-1* mutant is synthetically lethal with the *rip1* deletion (P. Preker, unpublished data), as is the *rss1-37* temperature-sensitive mutant (Saavedra, 1997). The general model postulated to explain these results invokes differential requirements for Rip1 in mRNA export. Rip1 is proposed to function in both general mRNA and heat shock mRNA export, but to play a more critical role

in the export of heat shock mRNAs. Thus, while nuclear pores lacking Rip1 may be suboptimal for nuclear export, mRNA export proceeds at an efficiency sufficient to sustain cell growth. However, in a situation where mRNA export is further compromised, such as in a *brr3* mutant background or under stress conditions, cells can no longer tolerate the loss of Rip1.

The strong genetic link between Rip1 and Brr3 is not surprising, given the multiple observations of a stable and direct physical interaction between the two proteins. First, Murphy and Wente (1996) showed a two-hybrid interaction between Brr3 and Rip1, as well as a direct interaction by blot-overlay. More recently, Stutz et al. (1997) reported that a purified Rip1-GST fusion directly binds to *in vitro* translated Brr3 protein. I have provided further evidence for this interaction by co-immunoprecipitating Rip1 and HA-tagged Brr3 in yeast cell extracts (Chapter 2, Figure 3B). Importantly, the co-immunoprecipitation result revealed an additional finding about the Brr3-Rip1 interaction; the total population of Rip1 detectable in crude cell extracts is complexed with Brr3, whereas not all the Brr3 protein in the extract is bound to Rip1. The most interesting interpretation of this result is that two functional pools of Brr3 exist in the cell (regardless of its putative role as a soluble export factor or a nucleoporin), Brr3 complexed with Rip1 and Brr3 complexed not with Rip1, but perhaps with other nucleoporins (Figure 1). Refining the model described above, while both Brr3 complexes could function in general mRNA export, the Brr3-Rip1 complex may be the only one able to function when cells are stressed. Therefore, in the absence of Rip1, the Brr3 complex normally lacking Rip1 retains normal export function, and cells are viable. However, under conditions where Rip1 is absent and Brr3 is mutated, neither Brr3 complex is functional for export, and cells are no

Figure 1: Model for the roles of Brr3 and Rip1 in general mRNA and heat shock mRNA export. Brr3 is found in two separate complexes, one with Rip1, which functions in both general mRNA export and heat shock mRNA export, and one without Rip1, which functions only in general mRNA export. In the absence of Rip1, heat shock mRNA export is inhibited, but general mRNA export can proceed; thus, cells are viable. When Brr3 is mutated, general mRNA export is compromised, and heat shock mRNA export is likely to be inhibited; cells are conditionally lethal. When Brr3 is mutated and Rip1 is deleted, both general mRNA export and heat shock mRNA export are inhibited; cells are inviable.

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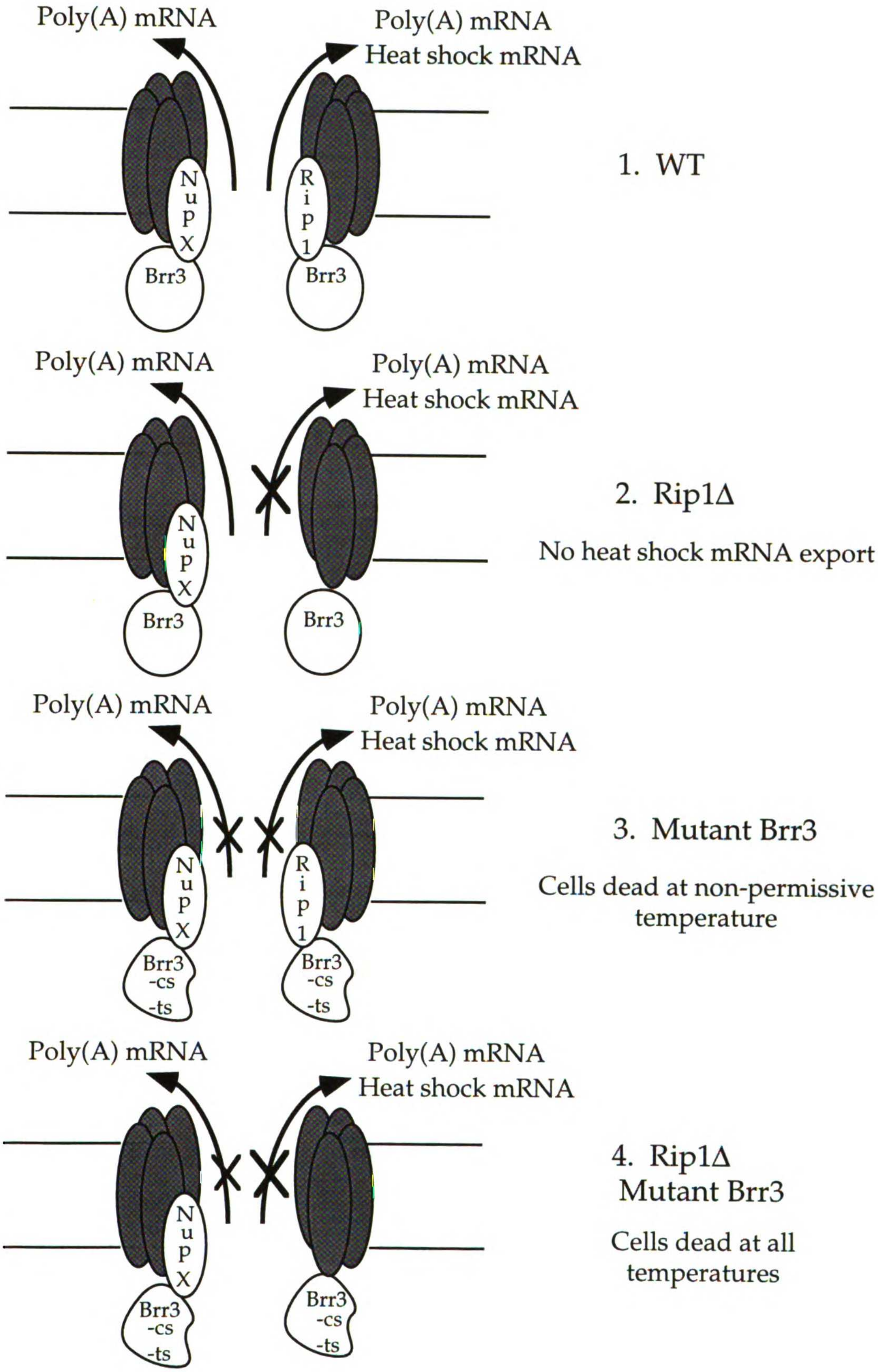
Poly(A) mRNA
Poly(A) mRNA
Heat shock mRNA
NupX
Rip1
Brr3
Brr3
1. WT
Poly(A) mRNA
Poly(A) mRNA
Heat shock mRNA
NupX
Brr3
Brr3
2. Rip1Δ
No heat shock mRNA export
Poly(A) mRNA
Poly(A) mRNA
Heat shock mRNA
NupX
Rip1
Brr3 -cs -ts
Brr3 -cs -ts
3. Mutant Brr3
Cells dead at non-permissive temperature
Poly(A) mRNA
Poly(A) mRNA
Heat shock mRNA
NupX
Brr3 -cs -ts
Brr3 -cs -ts
4. Rip1Δ Mutant Brr3
Cells dead at all temperatures

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longer viable. Finally, since the Brr3-Rip1 complex is the only one that functions under stress conditions, the absence of Rip1 is detrimental to stressed cells.

Since this model postulates that the Brr3-Rip1 complex is critical for exporting heat shock mRNAs when cells are stressed, a major prediction is that Brr3 is required for heat shock mRNA export. This is the case for the *rss1-37* mutant allele, which inhibits heat shock mRNA export at 42°C (Saavedra, 1997). However, contrary to this result, Stutz et al. (1997) found that three mutant alleles of *brr3* suppress the inhibition of heat shock protein synthesis in cells expressing low levels of Rip1. They explain their result by proposing a competition model whereby the heat shock export pathway competes with the general mRNA export pathway for shared factors; thus, mutant alleles of *brr3* depress the general mRNA export pathway, and the shared factors are free to compensate for the low levels of Rip1 and rescue the heat shock mRNA export block. This model is problematic, however, since the general mRNA export pathway is normally inhibited under heat shock conditions. Also, the mutant alleles of *brr3* were not tested for a defect in heat shock protein synthesis on their own. Therefore, more data is needed to explain this discrepancy.

The results implicating Brr3 in heat shock mRNA export underscore a more general issue, the existence of specialized RNA export pathways. Besides heat shock mRNA export, there are likely to be distinct export factors for the other classes of cellular RNAs: tRNA, snRNA, and rRNA. An alternative explanation for the *brr3-rip1* synthetic lethal data is that Brr3 participates in the export of another class of RNA, in addition to mRNA export, that is more

sensitive to mutations in Brr3 in the absence of Rip1. Once methods to assay the export of tRNA, snRNA, and rRNA in yeast cells are developed, this model can be tested. Moreover, with these assays, the field will benefit from another explosion in the discovery of novel export factors, and it will become increasingly clear where the different pathways overlap.

Final Thoughts

I am amazed when I chart the progress of the mRNA export field since the time I joined the Guthrie lab, over six years ago. Back then, the only factors implicated in mRNA export were a few nucleoporins. Now, close to 30 nucleoporins have been identified, as well as numerous mRNA-binding proteins which are important for export, and even a potential mRNA export receptor. Furthermore, the signals that direct mRNA export are being defined. Despite this impressive progress, the mRNA export field has hit a roadblock: without the use of an *in vitro* system, we cannot definitively test which factors are required for export. So, as we anxiously await the arrival of this assay, what can be done? As I mentioned at the beginning of this epilogue, I believe the next wave of interesting results will come from two areas. First, with the development of *in situ* assays to probe specific RNAs in yeast, distinct RNA export pathways will be better defined, and novel export factors will be identified. Second, the increasing attention being given to the question of how mRNA processing and export are coupled will lead to an improved understanding of the coupling mechanism, and which factors are involved. It is my hope that Brr3 will play a prominant role in these new areas, if not as a central figure then at the very least as a stepping stone to the

key factors. With the continuation of the Brr3 project and countless others like it, the RNA export field holds the promise of many more exciting years of discovery.

APPENDIX I

Searching for the cellular function of eIF5A: the effects of eIF5A depletion on pre-mRNA splicing, mRNA export, and mRNA stability in yeast

Abstract

The eIF5A protein was originally isolated as a translation initiation factor, based on its ability to stimulate methionyl-puromycin synthesis *in vitro*. However, an essential role in translation initiation is unlikely, since it was shown that yeast cells depleted of eIF5A do not display dramatic defects in protein synthesis or polysome formation. eIF5A has also been identified as a cellular co-factor of the HIV Rev protein, which promotes the cytoplasmic accumulation of unspliced and partially spliced viral pre-mRNAs. Models for the mechanism of Rev function include disruption of spliceosome assembly, activation of viral pre-mRNA export, and stabilization of viral pre-mRNAs in the nucleus. Thus, as a cellular target of Rev, eIF5A could potentially function in pre-mRNA splicing, mRNA export, and/or mRNA decay. To investigate these possibilites, eIF5A-depleted yeast cells were examined for defects in pre-mRNA splicing, poly(A) RNA export, and mRNA stability. No significant effects on any of these processes were detected. New models for the cellular function of eIF5A are discussed, in the context of more recent reports addressing the mechanism of Rev function and its association with eIF5A.

Introduction

The eukaryotic translation initiation factor 5A (eIF5A) is a protein with many interesting features: it is abundant, highly conserved, essential for cell viability, and is the only known protein to contain the amino acid hypusine (Schnier, 1991; Park, 1993). These features suggest an important cellular

function for eIF5A, however the nature of that function is unknown. As its name implies, eIF5A was originally purified as a translation initiation factor from a ribosomal high salt wash fraction of rabbit reticulocytes (Benne, 1978). The purification was based on its ability to stimulate the synthesis of methionyl-puromycin, representing formation of the first peptide bond in a reconstituted translation initiation assay. Raising suspicions about a direct role for eIF5A in translation initiation, high concentrations of the protein are required for the reaction, in excess of the amount of methionyl-puromycin formed, and the stimulation is only 2 to 3-fold. Also, unlike most other translation initiation factors, eIF5A is very abundant and mainly present unbound to ribosomes in the cytoplasm (Thomas, 1979).

To investigate the cellular function of eIF5A further, Hershey and co-workers cloned the eIF5A gene in yeast (Schnier, 1991). Two genes were found to encode yeast eIF5A, *TIF51A* and *TIF51B*. They are 90% identical and functionally redundant; under aerobic conditions, *TIF51A* is predominantly expressed, but when deleted, *TIF51B* expression is induced. Deletion of both genes results in cell death. In addition to being essential, the eIF5A genes are highly conserved. The yeast genes are 63% identical to the human gene, and more significantly, the human eIF5A cDNA can restore growth to yeast cells deleted for their endogenous *TIF51* genes (Schwelberger, 1993). Of particular interest is an especially conserved region of twelve amino acids surrounding lysine-50 (lysine-51 in yeast). This lysine is the residue that is post-translationally modified to hypusine in eIF5A, by a two-step enzymatic mechanism that transfers an aminobutyl group from spermidine to the ε-amino group of lysine, then hydroxylates the number 2 carbon of the butyl group (Park, 1993). The hypusine modification is presumed to be essential for

cell viability in yeast, since replacing lysine-51 with arginine results in a null allele (Schnier, 1991). Remarkably, the single lysine residue in eIF5A is the only amino acid found to be modified to hypusine in the cell, as determined by labeling various eukaryotic cells with radioactive spermidine (Park, 1993b).

To test if eIF5A truly plays a role in translation initiation, yeast cells depleted for eIF5A were examined (Kang, 1994). The *TIF51A* gene was placed under the control of the *GAL1, 10* promoter on a plasmid, in a strain deleted for the endogenous copies of the *TIF51* genes. Since the wildtype protein is very stable, the *GAL-TIF51A* gene was tagged with protein destabilizing elements, notably an N-terminal arginine residue, to allow for rapid depletion of the protein by the ubiquitin degradation pathway (Varshavsky, 1992). In this context, the half-life of the protein is 10 minutes, and in glucose, cells arrest after three generations. When grown in glucose for up to five hours, well after cells are depleted for eIF5A, the rate of protein synthesis is still 60-70% of wildtype. Moreover, polysome profiles, which drastically change when translation initiation is blocked, were examined in eIF5A-depleted cells; the profiles were found to be essentially similar to those of wildtype cells, displaying only slight differences. Thus it was concluded that eIF5A is not required for global protein synthesis or general translation initiation, leaving the search for its primary function wide open.

Subsequently, an intriguing clue about eIF5A function came from experiments identifying it as a cellular co-factor of the HIV-1 protein Rev (Ruhl, 1993). Rev is necessary for the cytoplasmic appearance of unspliced and partially spliced viral mRNAs, which are translated into proteins essential for reproduction of the virus (Cullen, 1991). Rev, a 116 amino acid

phosphoprotein, multimerizes and binds to a region of the viral pre-mRNAs called the Rev response element, or RRE. Binding to the RRE is mediated by a 15 amino acid arginine-rich region in the N-terminal half of Rev; this basic domain is also responsible for Rev multimerization and nuclear localization (Olsen, 1990; Malim, 1991). A second region essential for Rev function is a 10 amino acid stretch of regularly spaced leucines, termed the Rev activation domain. Mutations in this region result in a dominant negative phenotype without altering Rev-RRE binding, and is therefore thought to interact with a cellular protein that aids in the cytoplasmic accumulation of viral pre-mRNAs (Malim, 1989).

To search for the cellular proteins that interact with Rev, Ruhl et al. (1993) chemically cross-linked the Rev activation domain to polypeptides in HeLa nuclear extracts. Of the seven polypeptides that cross-linked to Rev, the one that was most sensitive to competition by excess Rev peptide was identified as eIF5A. Evidence for a physical interaction between eIF5A and the Rev activation domain was demonstrated by an anti-idiotypic response, meaning that antibodies specific for the Rev peptide bound to antibodies that recognize eIF5A. More significantly, inactivating eIF5A by antisense inhibition was shown to abolish Rev activity in mammalian culture cells, and also block Rev-dependent cytoplasmic appearance of RRE-containing RNAs.

Despite being the first identified cellular co-factor of Rev, the undetermined function of eIF5A clouded the significance of this result. Nonetheless, I saw it as an opportunity to test new ideas about the role of eIF5A. Many models explaining how Rev promotes the cytoplasmic appearance of viral pre-mRNAs have been postulated (at the time I initiated this project, its exact

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function was still controversial). First, Rev is thought to actively dissociate the spliceosome, resulting in the release of viral pre-mRNAs for export to the cytoplasm. This model originates from studies of an RRE-containing β-globin intron reporter in cultured mammalian cells; export of the reporter pre-mRNA to the cytoplasm is not only dependent on the presence of Rev, but also requires weakening of either the 5' or 3' splice site by mutation (Chang, 1989). Since pre-mRNAs with mutated introns are inefficiently spliced and thus retained in the nucleus, presumably by the spliceosome, this suggests that Rev functions to override the nuclear retention of unspliced pre-mRNAs. Furthermore, a Rev basic domain peptide was shown to specifically inhibit splicing of the RRE-containing β-globin pre-mRNA *in vitro* by interfering with spliceosome assembly (Kjems, 1991). A second model postulates that Rev directly activates the export of viral pre-mRNAs, independent of an effect on splicing. Supporting this idea, the RRE can be moved to various locations in the intron and exon of viral pre-mRNAs without affecting its function (Malim, 1989b). Moreover, the same study demonstrated that a non-spliceable version of a viral pre-mRNA is still exported to the cytoplasm in the presence of Rev. Finally, a third model proposes that Rev stabilizes viral pre-mRNAs in the nucleus, in addition to promoting their export. In human T-cells with stably integrated HIV provirus (the natural environment for HIV), Rev increases the stability of viral RRE-containing pre-mRNAs in the nucleus at least six-fold (Malim, 1993).

In sum, Rev appears to affect multiple mRNA processing events: pre-mRNA spliceosome assembly, mRNA stability, and mRNA export. Given the minimal repertoire of HIV viral proteins, Rev undoubtedly utilizes host co-

factors to function. Thus, cellular factors such as eIF5A are likely to operate at one or more of the above mRNA processing steps. For example, eIF5A could be a spliceosome assembly factor that Rev antagonizes for the release of viral pre-mRNAs, or an mRNA export factor that Rev recruits to export the viral messages, or a protein in the mRNA degradation pathway that Rev inhibits to stabilize its pre-mRNA substrates. Despite the simplicity of these models, a role for eIF5A in any of these cellular pathways has never been tested.

The following report describes a preliminary investigation to determine whether eIF5A functions in mRNA export, pre-mRNA splicing, or mRNA degradation. Specifically, yeast cells depleted for eIF5A were assayed for: (1) inhibition of poly(A) RNA export, (2) a defect in the splicing of intron-containing mRNAs, and (3) an increase or decrease in the half-life of specific mRNAs. No dramatic changes were observed in any of these processes, when compared to cells with normal levels of eIF5A, ruling out a role for eIF5A in bulk mRNA export, general pre-mRNA splicing, and mRNA degradation. More refined models to explain the cellular function of eIF5A, in light of the most recent reports on Rev function and eIF5A, are discussed.

Results

Cells depleted for eIF5A are not defective for poly(A) RNA export and pre-mRNA splicing

To rapidly deplete eIF5A protein from yeast cells, the UBHY-R strain was utilized, which harbors a plasmid copy of the *TIF51A* gene tagged with N-

terminal protein destabilizing elements, under the control of the *GAL1, 10* promoter. This strain is deleted for the endogenous copies of the *TIF51* genes, however when expression of the destabilized eIF5A protein is induced by growth in galactose-containing medium, cells grow as well as wildtype (Kang, 1994). When transcription of the tagged *TIF51A* gene is repressed by shifting cells to glucose medium, protein levels are diminished within 30 minutes, and are barely detectable beyond one hour (Figure 1A). Under these conditions, cell growth slows after one generation and arrests after three doublings, about 16 hours (Kang, 1994).

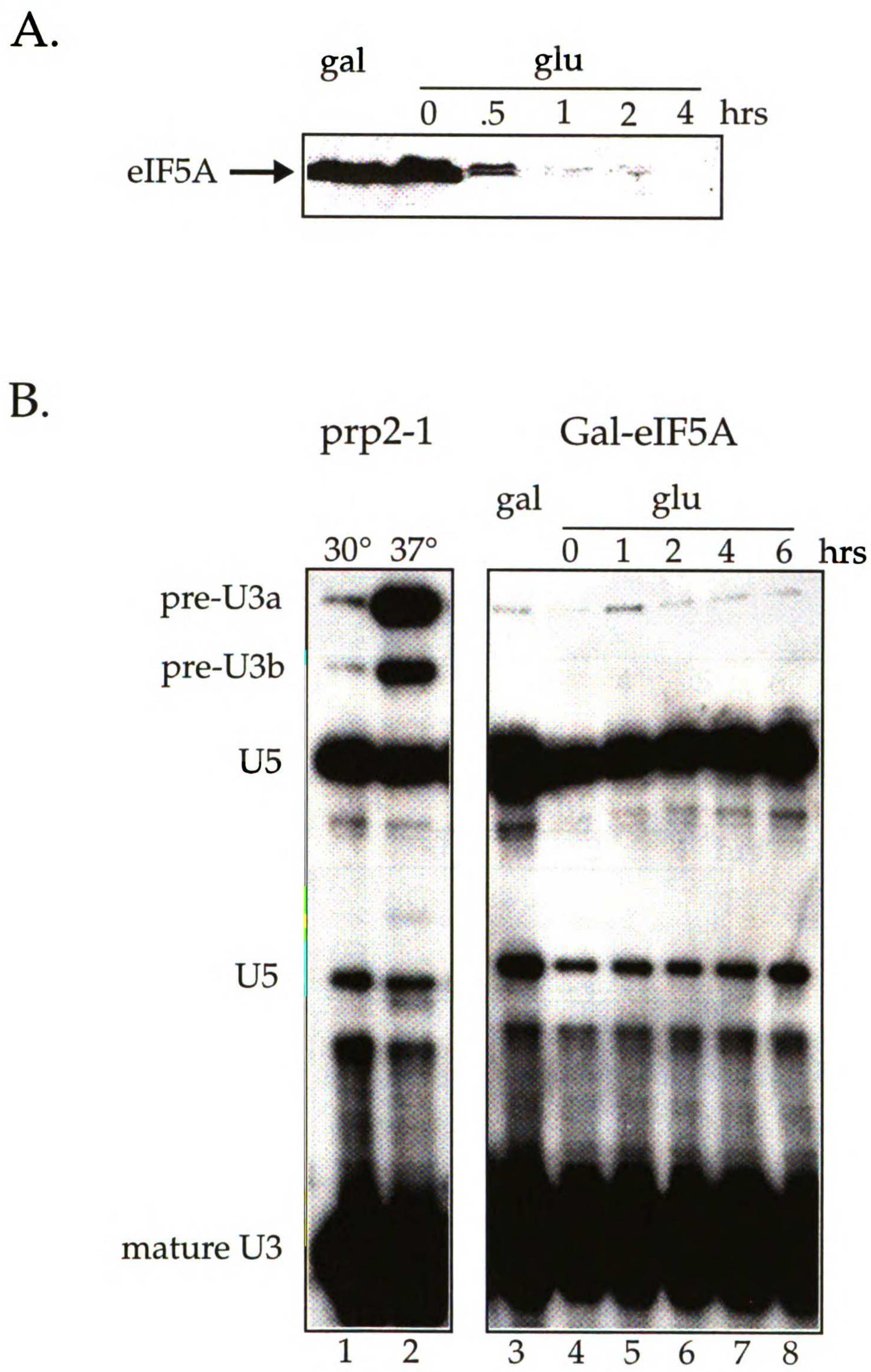
To test the hypothesis that eIF5A is an mRNA export factor, cells grown in galactose medium, or shifted to glucose medium for .5, 1, 2, and 4 hours were assayed by *in situ* hybridization with an oligo dT_{50} probe. In this assay, cells defective for poly(A) RNA export display nuclear accumulation of the T_{50} signal, whereas the signal is detected throughout the cell in wildtype strains (Amberg, 1992). At all time points examined, in cells grown in galactose or glucose, poly(A) RNA localizes throughout the cell (data not shown). Thus, both the cells containing eIF5A (grown in galactose) and depleted for eIF5A (grown in glucose for more than one hour) exhibit normal poly(A) RNA export, demonstrating that eIF5A is not required for bulk mRNA export.

A second model for eIF5A function is that it is a spliceosome assembly factor, which Rev antagonizes to release RRE-containing viral pre-mRNAs from the splicing machinery. If so, cells depleted for eIF5A should fail to properly assemble spliceosomes on cellular pre-mRNAs, resulting in inhibition of the first catalytic step of splicing. To detect this phenotype, intron-containing messages are assayed for precursor accumulation and/or loss of mature

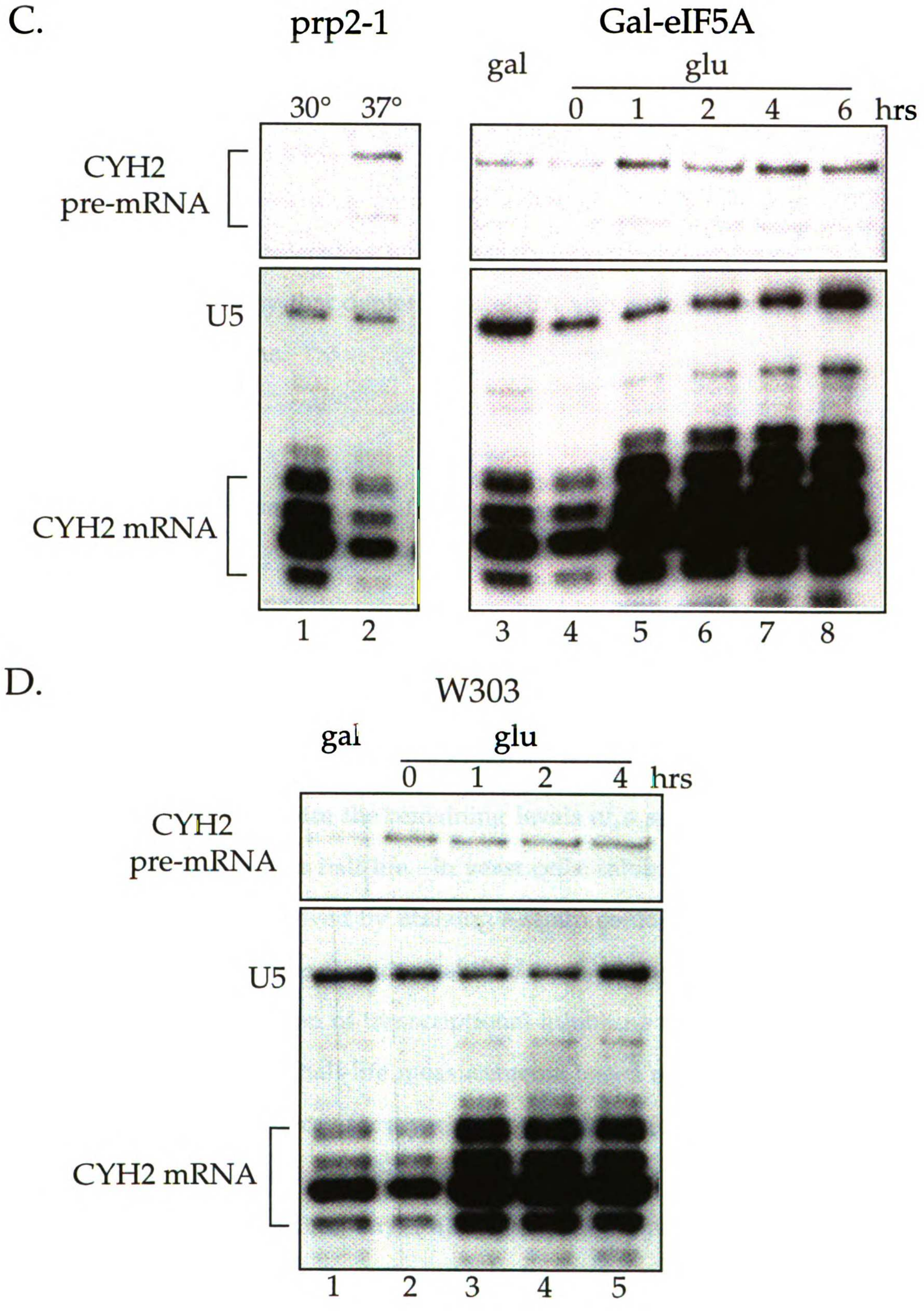
mRNA by primer extension (Noble, 1996). Cultures of the UBHY-R strain were grown in galactose medium, then diluted in either galactose or glucose medium and grown for 1, 2, 4, and 6 hours; at each time point, cells were harvested for RNA preparation, and the RNAs were primer extended to assay the levels of precursor and mature forms of intron-containing messages (Figure 1B, C). As a positive control, RNA from the prp2-1 strain, a temperature-sensitive mutant of a first step splicing factor, was also examined (Vijayraghavan, 1989). One of the messages assayed, U3, is a small nucleolar RNA containing an intron especially sensitive to splicing defects. In cells grown in glucose and thus depleted for eIF5A, U3 precursor levels are low, similar to undepleted cells (Figure 1B, lanes 3-8). In striking contrast, RNA from prp2-1 cells shifted to the non-permissive temperature contains a large increase in U3 precursor (Figure 1B, lane 2). A more conventional intron-containing message, CYH2 mRNA, was assayed for loss of mature mRNA. This phenotype is detected in shifted prp2-1 cells (as well as a slight increase in pre-mRNA), indicative of the splicing defect (Figure 1C, lane 2). Surprisingly, the opposite is seen in eIF5A-depleted cells; when shifted to glucose medium, there is a marked increase in mature CYH2 mRNA, in addition to an increase in pre-mRNA levels (Figure 1C, compare lane 4 with lanes 5-8). This pattern is also observed for another intron-containing mRNA, RP51A (data not shown). Thus, although eIF5A does not appear to be required for pre-mRNA splicing, the increased levels of mRNA seen in eIF5A-depleted cells raises the possibility that eIF5A normally functions in the degradation of mRNAs.

A more trivial explanation of the increased RNA levels in eIF5A-depleted cells is that higher rates of transcription occur in cells grown in glucose, the

Figure 1: Rapid depletion of eIF5A and the effect on precursor and mature RNA levels. The UBHY-R strain, containing the destabilized version of eIF5A, was grown in galactose medium, then shifted to glucose medium for up to six hours. Cells were harvested at various times for protein and RNA analysis. *(A)* eIF5A protein levels in cells grown in galactose (lane marked gal), and in cells shifted to glucose (marked glu) for the length of time indicated. Protein levels were assayed by Western blotting with anti-eIF5A polyclonal antibodies. *(B)* Detection of U3 precursor levels by primer extension of RNAs from UBHY-R cells grown in galactose (lane 3), and shifted to glucose for 0 to 6 hours (lanes 4 - 8). The positive control reactions are primer extensions of RNA samples from the prp2-1 splicing mutant grown at the permissive temperature of 30°C (lane 1) and the non-permissive temperature of 37°C (lane 2). Two genes encode U3, and hence two precursor bands are detected. *(C)* Primer extensions of the same RNA samples as in (B) using a primer complementary to CYH2 mRNA. The multiple mature mRNA species are due to multiple transcription start sites. *(D)* CYH2 pre-mRNA and mRNA levels, also assayed by primer extension, in W303 cells grown in galactose (lane 1) and shifted to glucose for 0 to 4 hours (lanes 2 - 5). In *(B-D)*, U5 snRNA serves as an internal control for RNA levels.



A.
gal
glu
0
.5
1
2
4
hrs
eIF5A
B.
prp2-1
Gal-eIF5A
gal
glu
30°
37°
0
1
2
4
6
hrs
pre-U3a
pre-U3b
U5
U5
mature U3
1
2
3
4
5
6
7
8


C.
prp2-1
Gal-eIF5A
gal
glu
30°
37°
0
1
2
4
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hrs
CYH2
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CYH2 mRNA
1
2
3
4
5
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7
8
D.
W303
gal
glu
0
1
2
4
hrs
CYH2
pre-mRNA
U5
CYH2 mRNA
1
2
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5

preferred carbon source for yeast. To test this possibility, RNA levels were assayed in the parental wildtype strain, W303, grown in glucose versus galactose medium. Indeed, shifting cells from galactose to glucose results in increased levels of both CYH2 (Figure 1D) and RP51A mRNAs (data not shown). Therefore, growth on glucose contributes to the increase in mRNA levels seen in eIF5A-depleted cells. However, this experiment does not eliminate the possibility that depletion of eIF5A also contributes to the stabilization of mRNAs.

Depletion of eIF5A results in slight stabilization of mRNAs

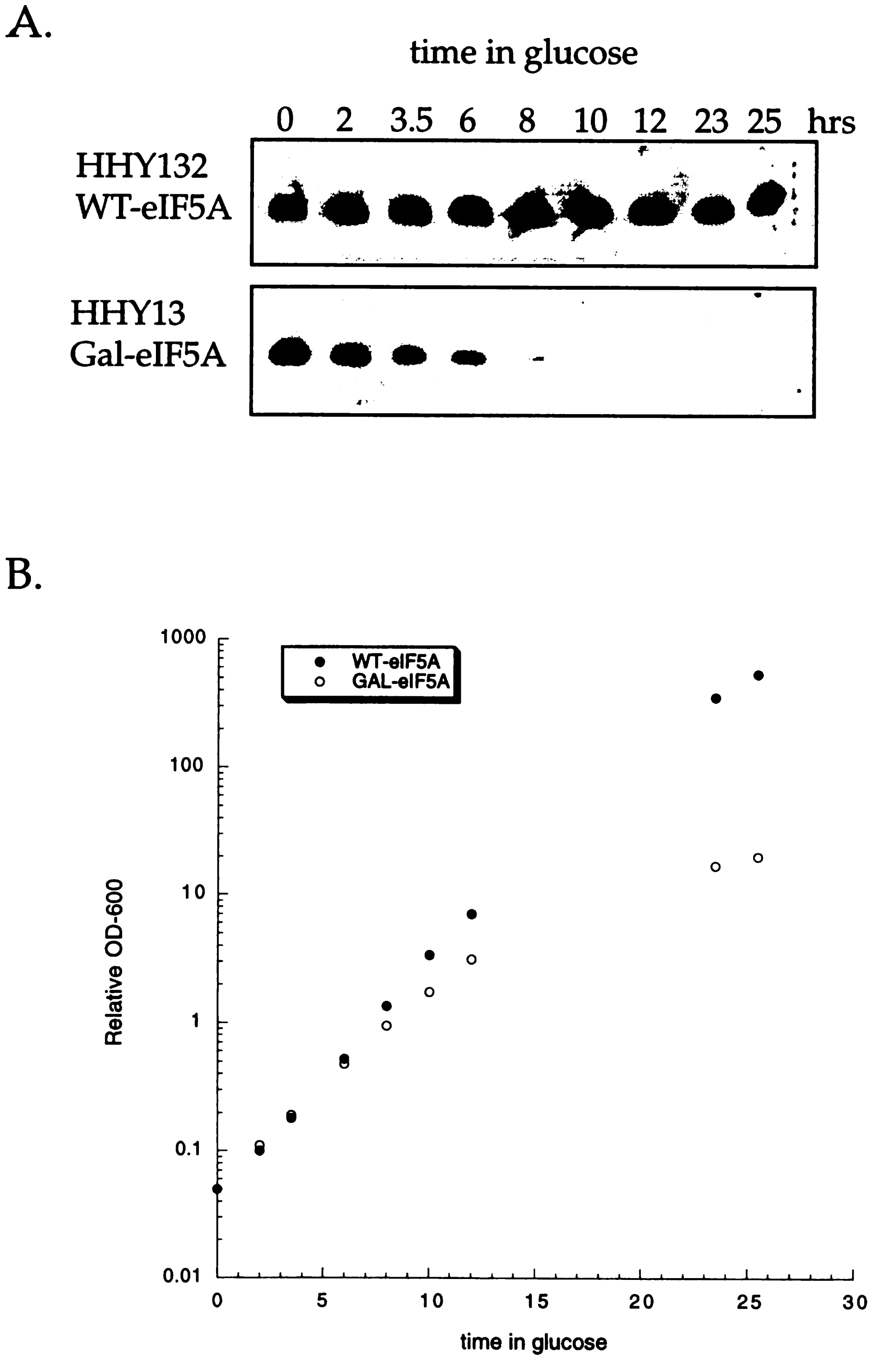
The hypothesis that eIF5A is a protein involved in mRNA degradation, which Rev antagonizes to stabilize viral pre-mRNAs, makes the possible effect of eIF5A depletion on mRNA stability a potentially exciting result. A direct way to assay mRNA stability is to inhibit transcription by RNA polymerase II, then measure the remaining levels of a specific mRNA over a time course to calculate its half-life. In yeast cells, inhibition of RNA polymerase II can be achieved by utilizing a strain containing a temperature-sensitive RNA polymerase II mutant shifted to the non-permissive temperature, or by addition of transcriptional inhibitors to cultures. A careful study comparing mRNA half-life measurements based on these two methods yielded similar rates of decay for a variety of mRNAs (Herrick, 1990). The two most common transcriptional inhibitors used to measure mRNA decay rates, thiolutin and phenanthroline, target all three RNA polymerases in yeast, and thus potentially affect cell metabolism in multiple ways (Tipper, 1973; Santiago, 1986). For example, high concentrations of phenanthroline cause a

decrease in protein synthesis rates as well as inhibiting transcription; however, titration experiments identified a concentration range that effectively inhibits transcription with minimal effects on protein synthesis (Santiago, 1986). In the following experiments, phenanthroline was chosen as the transcription inhibitor over thiolutin, because it was determined to be more effective over a time period longer than one hour (data not shown).

If eIF5A is involved in mRNA degradation, mRNAs are predicted to be significantly stabilized in eIF5A-depleted cells, with longer half-lives than in wildtype cells. To avoid complications due to carbon source effects, a strain that expresses wildtype levels of eIF5A in glucose medium was used as a control, for comparison to cells depleted of eIF5A by glucose repression. In this strain, HHY132, chromosomal copies of the *TIF51* genes are deleted, and the source of wildtype eIF5A is a copy of the *TIF51A* gene on a plasmid, under the control of its own promoter (Kang, 1994). By Western analysis, eIF5A protein levels do not change when these cells are shifted from galactose to glucose medium (Figure 2A, top panel). In addition, to circumvent any potential effects due to artificially destabilizing eIF5A, the HHY13 strain, harboring a plasmid copy of the wildtype *TIF51A* open reading frame fused to a *GAL* promoter, minus any destabilizing elements, was utilized (Schwelberger, 1993). This strain is otherwise isogenic to HHY132, and when GAL repression is induced by shifting cells to glucose medium, eIF5A protein levels are barely detectable eight hours after the shift (Figure 2A, bottom panel). At this point, cell growth begins to diminish (Figure 2B).

To measure mRNA half-lives in the presence or absence of eIF5A, logarithmic cultures of HHY132 and HHY13 were shifted to glucose medium

Figure 2: Glucose depletion of wildtype eIF5A and the resulting inhibition of cell growth. HHY132 cells, containing the wildtype *TIF51A* gene, and HHY13 cells, containing wildtype *TIF51A* under the control of a *GAL* promoter, were grown in galactose medium and shifted to glucose medium for up to 25 hours. Aliquots of cells were taken at various times for protein analysis and to monitor cell density. *(A)* eIF5A protein levels in HHY132 cells (top panel) and HHY13 cells (bottom panel) shifted to glucose medium for the length of time indicated. Protein levels were assayed by Western blot. *(B)* Growth curves of HHY132 cells (filled circles) and HHY13 cells (open circles) after dilution into glucose medium. Cells were kept in logarithmic phase. Growth was monitored by measuring optical density at 600 nm.


A.
time in glucose
0 2 3.5 6 8 10 12 23 25 hrs
HHY132
WT-eIF5A
HHY13
Gal-eIF5A
B.
● WT-eIF5A
○ GAL-eIF5A
1000
100
10
1
0.1
0.01
Relative OD-600
0
5
10
15
20
25
30
time in glucose

for eight hours, phenanthroline was then added to block transcription, and cells were harvested at various times after transcription inhibition. Addition of phenanthroline did not affect wildtype eIF5A protein levels in HHY132 cells, or interfere with eIF5A depletion in HHY13 cells (data not shown). To measure the levels of mRNAs at each time point, cellular RNA was prepared, and specific mRNAs were primer extended and quantitated by phosphorimaging. Half-life values were then calculated from semi-log plots of normalized mRNA levels over time, with decay rates generally following first-order kinetics (Belasco, 1993). Half-lives were determined for mRNAs of different classes: intron-containing versus intronless mRNAs, and unstable, moderately stable, and very stable mRNAs, as defined by previous reports (Santiago, 1986; Herrick, 1990). Results from a typical experiment are shown in Figure 3; in this case, the mRNA being measured is RP51A, an intron-containing, moderately stable message. A list of all the mRNAs examined, sorted by class, and the resulting half-life measurements are presented in Table I. Overall, eIF5A depletion has only a slight effect on the stability of mRNAs, regardless of class type, with mRNA half-lives at most two-fold greater in depleted cells than in undepleted cells. Therefore, it is unlikely that eIF5A plays a primary role in mRNA degradation.

Discussion

The results presented here argue against a general role for eIF5A in pre-mRNA splicing, mRNA degradation, or mRNA export. To determine if eIF5A functions in pre-mRNA splicing, precursor and mature levels of three intron-containing messages, typically examined to diagnose a splicing defect,

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Figure 3: RP51A mRNA half-life measurement in wildtype and eIF5A-depleted cells. Cultures of cells containing either wildtype TIF51A (HHY132) or Gal-TIF51A (HHY13) were shifted to glucose medium for eight hours to allow for depletion of eIF5A in HHY13 cells, at which point phenanthroline was added to block transcription. Aliquots of cells were taken at various times, up to 60 minutes after phenanthroline addition, for RNA analysis. *(A)* RP51A mRNA levels, assayed by primer extension, in wildtype and eIF5A-depleted cells grown in the presence of phenanthroline for the length of time indicated. The multiple mRNA bands are due to multiple transcription start sites. U5 serves as an internal control for RNA levels. *(B)* Semi-log plot of mRNA levels over time, in wildtype cells (filled circles) and eIF5A-depleted cells (open circles). mRNA levels were quantitated by phosphorimaging, and normalized to U5 levels. The slope of the each curve corresponds to the decay rate constant, used to calculate the mRNA half-life.

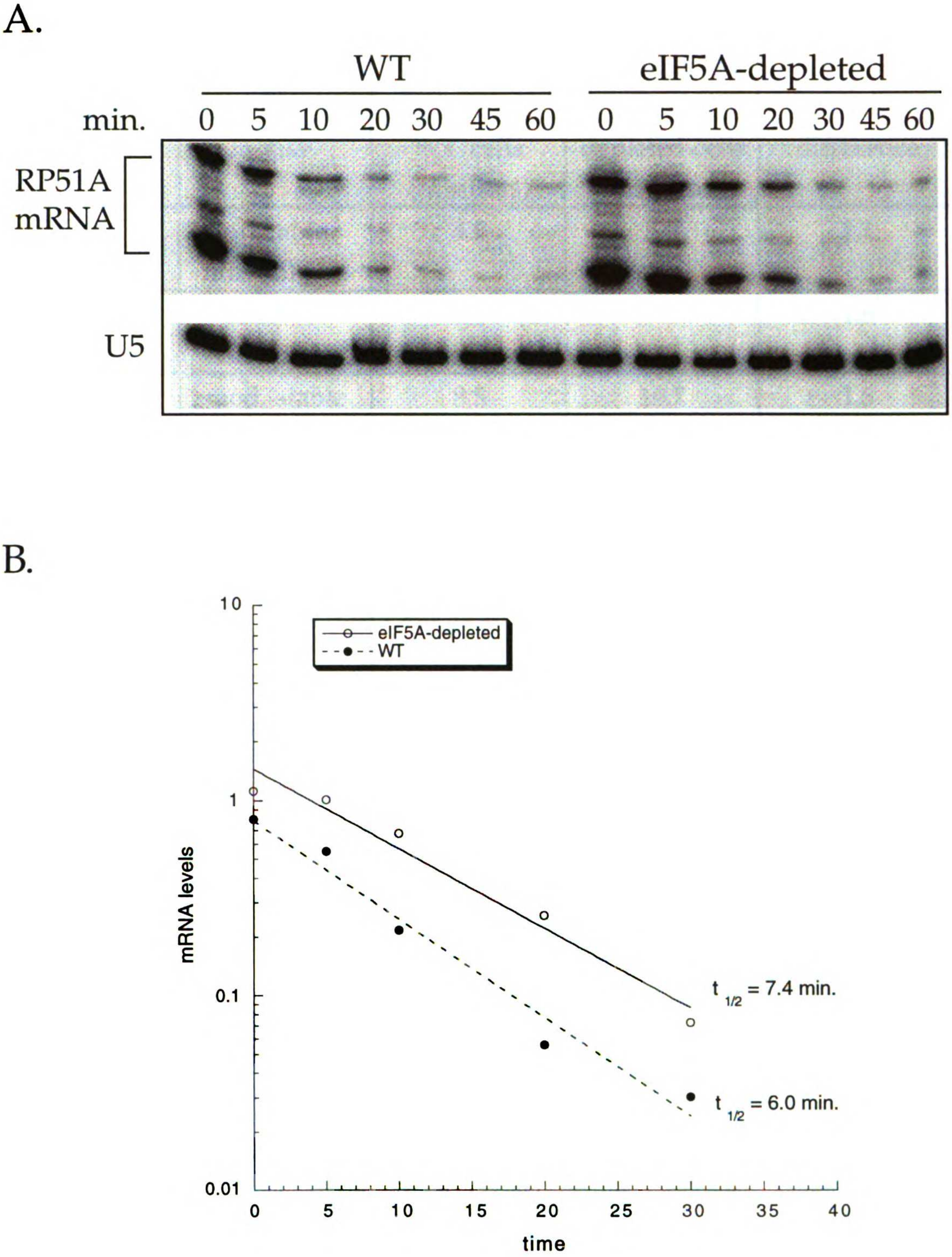
A.
WT
eIF5A-depleted
min.
0
5
10
20
30
45
60
0
5
10
20
30
45
60
RP51A mRNA
U5
B.
eIF5A-depleted
WT
mRNA levels
10
1
0.1
0.01
time
0
5
10
15
20
25
30
35
40
t 1/2 = 7.4 min.
t 1/2 = 6.0 min.

Table I: mRNA half-life measurements in wildtype and eIF5A-depleted cells

mRNA	Class	Half-life in WT cells (minutes)	Half-life in eIF5A-depleted cells (minutes)	Fold difference
MFA2	unstable non-spliced	4.8	5.3	1.1
STE2	unstable non-spliced	2.8	4.4	1.6
PAB1	mod. stable non-spliced	8.5	10.7	1.3
CYH2	mod. stable spliced	6.3	10.5	1.7
RP51A	mod. stable spliced	6.0	7.4	1.2
PGK	stable non-spliced	69.3	99.0	1.4
ACT1	stable spliced	49.5	99.0	2.0

were assayed in eIF5A-depleted cells. All three messages were spliced efficiently, strongly suggesting that eIF5A is not an essential part of the general pre-mRNA splicing mechanism. In addition, depletion of eIF5A had a nominal effect on the stability of multiple mRNAs, making a role for eIF5A in the mRNA degradation pathway unlikely. Nonetheless, it is important to note that mRNA half-lives were measured from total cellular RNA, and therefore reflect predominantly cytoplasmic degradation events (Decker, 1994). It remains a possibility that eIF5A acts in the nuclear degradation of pre-mRNAs, which is a more direct prediction of the model that Rev antagonizes eIF5A to stabilize viral pre-mRNAs in the nucleus. However, little is known about nuclear degradation of pre-mRNAs. One substrate for nuclear degradation is thought to be pre-mRNAs that are aberrantly spliced (Burgess, 1993). Since these pre-mRNAs are presumably a small percentage of the total population in cells that are not defective for splicing, they are difficult to measure. Furthermore, it is especially difficult to study nuclear decay events in yeast cells due to the lack of a method to cleanly fractionate nuclear and cytoplasmic compartments. Thus, for the time being, this hypothesis for the cellular function of eIF5A will likely remain untested in yeast.

At the time the work described in this chapter was performed, only total poly(A) RNA could be detected in yeast cells by *in situ* hybridization. By this measure, eIF5A-depleted cells are normal for mRNA export, suggesting that eIF5A is not involved in this process. However, a role for eIF5A in RNA export should be reconsidered in light of new evidence that Rev functions primarily as an export factor, possibly utilizing an export pathway other than

the general one for mRNA, and that eIF5A mutants interfere with the export function of Rev.

Convincing data that Rev can facilitate the export of RRE-containing pre-mRNAs independent of an inhibitory effect on splicing was shown by studying Rev function in *Xenopus* oocytes (Fischer, 1994). RRE-containing pre-mRNAs microinjected into oocyte nuclei are spliced with equal efficiency in the presence or absence of Rev. Moreover, it was demonstrated that Rev stimulates the export of not only the pre-mRNA, but also an end product of the splicing reaction, the excised intron lariat (still harboring the RRE). This is strong evidence against the hypothesis that splicing inhibition by Rev is a necessary prerequisite for the export of viral pre-mRNAs. Unequivocal evidence that Rev directly promotes the export of RRE-containing mRNAs came with the identification of the Rev activation domain as a nuclear export signal [NES] (Fischer, 1995; Wen, 1995). In both *Xenopus* oocytes and HeLa cells, microinjection of the Rev activation domain conjugated to BSA directs the rapid nuclear export of the reporter protein, in a temperature-dependent and saturable manner. When a non-functional mutant version of the Rev activation domain is used, export of the reporter is abolished. Further supporting a direct role for Rev in nuclear export, two-hybrid interactions are detectable between the Rev protein and numerous nucleoporins, located at both the nuclear and cytoplasmic faces of the nuclear pore complex; these interactions are dependent on the Rev NES (Stutz, 1995; Fritz, 1996). This leads to the current model of Rev directing the nuclear export of viral pre-mRNAs in part by mediating translocation through the nuclear pore complex via its NES.

Additional data suggest that the cellular export pathway accessed by Rev is not the one used for the general export of mRNAs. It has been established that RNA export is very likely to be mediated, in part, by distinct factors that are specific for each class of RNA; this was determined by competition experiments in *Xenopus* oocytes showing that tRNA, snRNA, 5S rRNA, and mRNA molecules can specifically inhibit the export of the same class of RNA, but not the export of the other classes of RNA (Jarmolowski, 1994). The same type of experiment later demonstrated that saturating amounts of the Rev NES peptide inhibits snRNA and 5S rRNA export, but not the export of two different mRNAs (Fischer, 1995). Notably, a protein implicated in 5S rRNA export, TFIIIA, contains a Rev-like NES; thus, Rev and TFIIIA possibly interact with the same cellular factor to direct the export of their respective cargoes. Another specialized cellular export pathway recently discovered in yeast concerns mRNAs encoding heat shock proteins. Under conditions of stress, such as heat shock or ethanol shock, bulk poly(A) mRNA is retained in the nucleus, while rapidly induced heat shock mRNAs are efficiently exported to the cytoplasm (Saavedra, 1996). To date, the only known cellular protein exclusively required for heat shock mRNA export is Rip1, one of the nucleoporins that associates with Rev (Fritz, 1995; Stutz, 1995; Fritz, 1996; Saavedra, 1997; Stutz, 1997). Furthermore, high level expression of Rev in yeast cells partially blocks heat shock mRNA export following stress (Saavedra, 1997). Hence, Rev may also access factors in the heat shock mRNA pathway for its own export, including Rip1 and possibly other cellular proteins.

In addition to the recent advances in the understanding of Rev function, the functional relevance of the interaction between eIF5A and the Rev activation

domain/NES has been demonstrated by the isolation of eIF5A mutants that inhibit Rev activity, and specifically Rev export (Bevec, 1996). Taking advantage of the fact that human and yeast *TIF51A* are functionally interchangeable in yeast cells (Schwelberger, 1993), mutant alleles of a human eIF5A cDNA were tested for failure to rescue a yeast strain deleted for the *TIF51* genes. Of the 10 null mutants, two were determined still capable of binding Rev:RRE complexes in gel shift experiments, and assumed to be dominant negative. Indeed, constitutive expression of these mutant alleles in human T cells inhibits HIV replication, and Rev activity. Moreover, coinjection of Rev and the mutant eIF5A proteins into HeLa cells blocks the nuclear export of Rev, while wildtype eIF5A stimulates it, suggesting that eIF5A acts to facilitate Rev export.

Considering the elucidating results on the export function of Rev and the possible involvement of eIF5A in Rev export, new models for the cellular function of eIF5A come to mind. Thus far, it appears that Rev utilizes cellular factors necessary for snRNA, 5S rRNA, and heat shock mRNA export. Rev may interact with one factor common to these export pathways, or, more likely, with multiple export factors that play distinct roles in one or more of these pathways. eIF5A could be one of these substrate-specific export factors. For example, it may be required for heat shock mRNA export; the simplest prediction of this model is that eIF5A-depleted yeast cells are defective in the export of heat shock mRNAs under stress conditions. Alternatively, eIF5A may be involved in snRNA or 5S rRNA export. These models are more difficult to test in yeast, since *in situ* hybridization methods for reliable detection of these specific RNAs are still in development (M. Inada, unpublished results). However, snRNA and 5S rRNA export can be

examined in mammalian cells in the presence of the eIF5A dominant negative mutants to test for an inhibitory effect. In general, export factors are predicted to interact with nucleoporins, such as Rip1; putative interactions between eIF5A and nucleoporins can be tested in yeast by genetic or biochemical methods. All in all, these experiments are relatively straightforward, and could potentially lead to answers about the function of this essential, but elusive protein.

Materials and Methods

Yeast strains

The yeast strains utilized in these studies are listed below, the first three were kindly provided by J. Hershey:

UBHY-R: MATα tif51A::LEU2 tif5IB::HIS3 ade2-1 ura3-1 his3-11,15 trp1-1
leu2-3,112 can 1-100 {pGal-UB-Arg-TIF51A}
HHY13a: MATa tif51A::LEU2 tif51B::HIS3 ade2-1 ura3-1 his3-11,15 trp1-1
leu2-3,112 can 1-100 {pBM272-TIF51A (Gal promoter)}
HHY132: MATa tif51A::LEU2 tif51B::HIS3 ade2-1 ura3-1 his3-11,15 trp1-1
leu2-3,112 can 1-100 {pHSTC-TIF51A (wildtype promoter)}
W303: ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100

Yeast manipulations and growth media are described in Guthrie and Fink (Guthrie, 1991).

In situ hybridization

For dT_{50} in situ hybridization, 1 ml of mid-log cells were prepared as described (Amberg, 1992; Kadowaki, 1994), with modifications to be presented elsewhere (A. de Bruyn Kops et al., manuscript in preparation).

RNA preparation and primer extensions

Total RNA was prepared from mid-log cells and 10 µg was analyzed by primer extension according to (Lesser, 1993). RNA from prp2-1 cells grown at 30°C and 37°C provided by C. Siebel. Sequences of the oligonucleotides used are as follows:

U5-7wtsmnr [5'-AAGTTCCAAAAAATATGGCAAGC-3']
U3 exon 2 [5'-CCAAGTTGGATTCAGTGGCTC-3']
RP51A exon 2 [5'-CGCTTGACGGTCTTGGTTC-3']
CYH2 exon 2 [5'-ACCGGCCATACCTCTACCACC-3']
PGK-4 [5'-ACTCTGATGAAGACACGCT-3']
ACT1-1 [5'-CCGGCTTTACACATACCAGA-3']
PAB1-2 [5'-CACCAACATATAATGATGCAG-3']
MFA2-1 [5'-TTAAGCGATAACACAGGCG-3']
STE2-4 [5'-GAGTGCAGAATGCAAAATGAT-3']

Protein analysis

Proteins were extracted from cell pellets by TCA precipitation, separated on a 12% acrylamide gel, and prepared for Western blotting by standard methods

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(Ausubel, 1994). Blots were probed with a 1/10,000 dilution of crude serum containing anti-eIF5A polyclonal antibodies for one hour at room temperature, washed, and incubated with 1/2000 diluted horseradish-peroxidase conjugated secondary antibodies (BioRad). Blots were then developed by ECL (Amersham).

mRNA stability assay

Cells were grown at 30°C in galactose medium to mid-logarithmic phase, diluted into glucose medium, and grown for eight hours to deplete eIF5A. Phenanthroline was then added to cultures to a final concentration of 100µg/ml. Aliquots of cells were taken at the point of phenanthroline addition, and at various times thereafter. Harvested cells were pelleted for extraction of total RNA, RNAs were primer extended and separated on a 6% polyacrylamide/7M urea gel. mRNA levels were quantitated using a PhosphorImager with ImageQuant software (Molecular Dynamics).

APPENDIX II

Results of genetic interactions tested between *brr3-1* and various mutants

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Table I: Segregation data from genetic crosses between *brr3-1* (Cs$^-$) and various mutants

Genotype of mutant crossed to *brr3-1*	Marker	Number of viable spores	Number of inviable spores
nup116::HIS3	His$^+$	9	7
	Cs$^-$	14	2
	His$^+$Cs$^-$	0	8
nup100::URA3	Ura$^+$	22	1
	Cs$^-$	23	0
	Ura$^+$Cs$^-$	0	17
nup145::URA3	Ura$^+$	19	0
	Cs$^-$	19	0
	Ura$^+$Cs$^-$	0	15
nup133::HIS3	His$^+$	14	0
	Cs$^-$	4	10
	His$^+$Cs$^-$	0	14
nup159/rat7^{ts}	Ts$^-$	17	0
	Cs$^-$	17	0
	Ts$^-$Cs$^-$	0	19
nup1::LEU2	Leu$^+$	16	4
	Cs$^-$	18	2
	Leu$^+$Cs$^-$	0	18
nup2::URA3	Ura$^+$	14	0
	Cs$^-$	14	0
	Ura$^+$Cs$^-$	13	5
prp22^{ts*}	Ts$^-$	18	0
	Cs$^-$	7	12
	Ts$^-$Cs$^-$	7	6
brr1::LEU2 (Cs$^+$ by patching)	Leu$^+$	15	0
	Cs$^-$	15	0
	Leu$^+$Cs$^-$	19	0

**prp22^{cs}* crossed with *brr3-1* produced 4 tetrads with 3Cs$^-$:1Cs$^+$ spore ratio

Table II: Results of genetic interactions assayed by plasmid shuffle

		Colony size on plates grown at:			
Strain backgrnd	Plasmid present	16°C	25°C	30°C	37°C
brr3::LEU2	*pbrr3-1*	--	++	+++	++
brr3::LEU2 *brr5-1*^{cs}	vector	--	--	--	--
	p*BRR3*	--	++	+++	--
	pbrr3-1	--	+	+++	--
brr3::LEU2 *mud13::HIS3*	vector	--	--	--	--
	p*BRR3*	--	++	++	++
	pbrr3-1	--	++	++	++
brr3::LEU2 *pan3::HIS3*	vector	--	--	--	--
	p*BRR3*	++	++	+++	++
	pbrr3-1	--	++	+++	++
pab1::HIS3 *brr3-1*^{cs}	vector	--	--	--	--
	p*PAB1*	--	+	++	++
	ppab1-53	--	--	+/–	--
pab1::HIS3	*ppab1-53*	++	+	++	--

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