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AN ANALYSIS OF MER1 FUNCTION DURING MEIOTIC SPLICING REGULATION IN SACCHAROMYCES CEREVISIAE

Frederick William Scherrer
University of Missouri-St. Louis, leadkeelfred@msn.com

AN ANALYSIS OF *MER1* FUNCTION DURING MEIOTIC SPLICING REGULATION IN *SACCHAROMYCES CEREVISIAE*

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FREDERICK W. SCHERRER, JR.
A.B., Biology, Harvard College, 1988

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Marc Spingola, Ph.D.
Chairman

Wendy Olivas, Ph.D.
Teresa Thiel, Ph.D.
Wesley Harris, Ph.D.

Abstract

The transition from mitosis to meiosis in the yeast *Saccharomyces cerevisiae* requires a significant change to gene expression profiles. Regulation of pre-messenger RNA splicing patterns during meiosis assists in this transition by fine tuning expression of essential meiotic genes. Produced only during meiosis, Mer1p is linked to the splicing of at least three mRNAs: *MER2*, *MER3*, and *AMA1*. Previous evidence suggests that Mer1p activates splicing by directly recruiting snRNPs or stabilizing intermediate splicing complexes formed on pre-mRNA that contains an intronic Mer1p enhancer element. However, some splicing factors, especially accessory/non-snRNP factors, have critical roles in retaining unspliced pre-mRNAs in the nucleus. I tested if Mer1p may indirectly regulate splicing by preventing the export of pre-mRNAs to the cytoplasm and also demonstrated that a second subunit of the Retention and Splicing (RES) complex, Bud13p, has transcript-specific effects on Mer1p-activated splicing.

The results indicated that Mer1p can retain unspliced pre-mRNA in the nucleus; however, nuclear retention could not be uncoupled from splicing activation. In the absence of Mer1p, the *AMA1* pre-mRNA is exported to the cytoplasm, translated, but not subjected to nonsense-mediated decay (NMD) despite a premature stop codon in the intron. A novel role for the Mer1p activation domain was revealed by a two-hybrid interaction with Prp39p, an essential U1 snRNP protein. This suggests the initial contact between Mer1p and the spliceosome occurs during commitment complex assembly. Collectively, these data imply that Mer1p can retain pre-mRNAs in the nucleus only by facilitating their interaction with the spliceosome and support models for cytoplasmic degradation of unspliced pre-mRNAs that fail to assemble into spliceosomes in yeast. A two-hybrid analysis of U1 snRNP proteins and other early splicing factors tested 460 possible interactions and the several novel interactions reported here indicate a revised model for U1snRNP structure.

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Chapter One
Introduction

Saccharomyces cerevisiae or baker's yeast is a single cellular eukaryote whose study has dramatically contributed to the understanding of human biology. Many of the principal enzymes, complexes and processes common to metazoans were first identified in yeast. Though humans and yeast may be distantly related in evolutionary terms, their similarities at the cellular and molecular levels are remarkable. The subtle differences that do exist between yeast and humans add revealing contrast when the organisms are compared side by side. Yeast research provides distinct advantages over human and plant studies because of low cost, rapid growth, as well as the ease of culturing and genetic manipulation. As such, yeast was the first eukaryote sequenced and is now considered a model organism. Genetic manipulations are simplified since yeast naturally tolerate plasmids and can function normally as a haploid or a diploid. Yeast have approximately 6000 genes spread over 16 chromosomes and during mitosis they reproduce every 90 minutes by budding. Upon starvation, diploid yeast enter meiosis where tetrads containing four spores are produced. Because recombination is also naturally occurring, disruption, modification or replacement of chromosomal genes occurs via homologous recombination (Sherman *et al.*, 1986).

Beyond scientific advances in understanding eukaryotic gene expression and cell biology, the yeast microbe benefits humans by its significant commercial application in baking and ethanol production. When fed glucose or sucrose and deprived of oxygen, yeast will ferment sugar and release carbon dioxide and ethanol. With respect to baking, carbon dioxide release causes dough to rise. Beer and wine are the direct by-products of yeast fermentation, while spirits or ethanol are fermentation products concentrated by distillation. Recently, an intense search for alternatives to gasoline has focused on utilizing yeast to create alcohol from corn. Also, creating alcohol from cellulosic sources such as wood or grasses has become a priority of the U.S. Energy Department. As a result, a significant effort is underway to supplement the yeast genome with transgene cellulases and enzymes required to ferment the five carbon sugars xylose and arabinose that accumulate during hemicellulose hydrolysis (D.O.E., 2005). Thus, continued study of yeast biology will likely benefit humans for years to come.

In the following chapters, I present research that uses yeast to better understand pre-mRNA splicing, which is an important step in eukaryotic gene expression. During splicing a large ribonucleoprotein complex called the spliceosome specifically identifies and removes RNA sequences from transcripts before their translation into proteins. In doing so, it adds both regulation and diversity to gene expression. Chapter Two questions whether the meiotic splicing factor, Mer1p, contributes to splicing efficiencies by retaining pre-mRNA in the nucleus. Chapter Three uses the two-hybrid assay to test interactions between Mer1p and many of the first proteins that are attracted to a pre-mRNA undergoing splicing. Chapter Four tests whether the newly identified splicing factor, RES, plays an important function during meiosis. In the final chapter, I discuss

my results in a broader context and consider the fate of pre-mRNAs that fail to undergo splicing. The remainder of this introduction serves as a brief overview that places splicing in the nuclear context where it occurs.

Splicing

Pre-mRNA splicing occurs in the nucleus during post-transcriptional processing of primary transcripts just prior to their export to the cytoplasm. Splicing is the removal of introns or intervening sequences from pre-mRNAs via dual transesterification reactions, which are catalyzed by a large ribonucleoprotein complex, termed the spliceosome. In yeast there are over 80 proteins that comprise the spliceosome, but the catalytic core of the spliceosome is comprised of small nuclear RNAs or snRNAs. Each of the five snRNAs (U1, U2, U4, U5, and U6) associates with a specific subset of proteins. Together they form the small ribonucleoprotein particles or snRNPs. The five snRNPs assemble in a step-wise sequence until the active spliceosome is complete and bound to a pre-mRNA. This assembly process involves extensive rearrangements among these snRNAs and also with the primary transcript. The snRNPs and the splicing process are conserved throughout eukaryotes (Staley and Guthrie, 1998; Ryan *et al.*, 2004).

Identification and removal of an intronic sequence within a pre-mRNA requires spliceosome interaction with three conserved sequences contained within an intron. These sequences are called the 5' splice site sequence, the branchpoint sequence, and the 3' splice site sequence. The 5' splice site sequence consists of the first six nucleotides of the intron. In yeast, a majority of introns share the consensus 5' splice site sequence GUAUGU. The first step of splicing is the formation of the "commitment complex" where the U1 snRNA binds to the 5' splice site. The branchpoint sequence is located roughly in the middle of the intron and in yeast has the conserved sequence of UACUAAC. After commitment complex formation, the U2 snRNA binds to the branchpoint sequence and the "pre-spliceosome complex" forms. The last adenosine of the branchpoint sequence is exceptionally important for splicing because it provides the 2' OH group required for the first transesterification reaction of splicing. The 3' splice site defines the end of the intron and in yeast it has the conserved sequence YAG (Staley and Guthrie, 1998; Tardiff and Rosbash, 2006; Ares and Weiser, 1995).

An active spliceosome is formed once the pre-spliceosome complex and the tri-snRNP (U4, U5, and U6 snRNPs) interact with splicing helicases. Here the U6 snRNA undergoes a dramatic rearrangement as it disassociates with U4 snRNA and binds the U2 snRNA. The U6 snRNA also binds the pre-mRNA at the 5' splice site causing the U1 snRNA to disassociate. This restructuring brings the branchpoint adenosine in close contact to the 5' splice site. The resulting transesterification reaction between the adenosine 2' OH group and phosphate group linking the 5' exon and intron, cleaves the pre-mRNA at the 5' splice site and forms a lariat intermediate. A second reaction exchanges the 5' exon's 3' OH group for the phosphate at the 3' splice site and the intron is released (Staley and Guthrie, 1998; Ares and Weiser, 1995). See Figure 1.

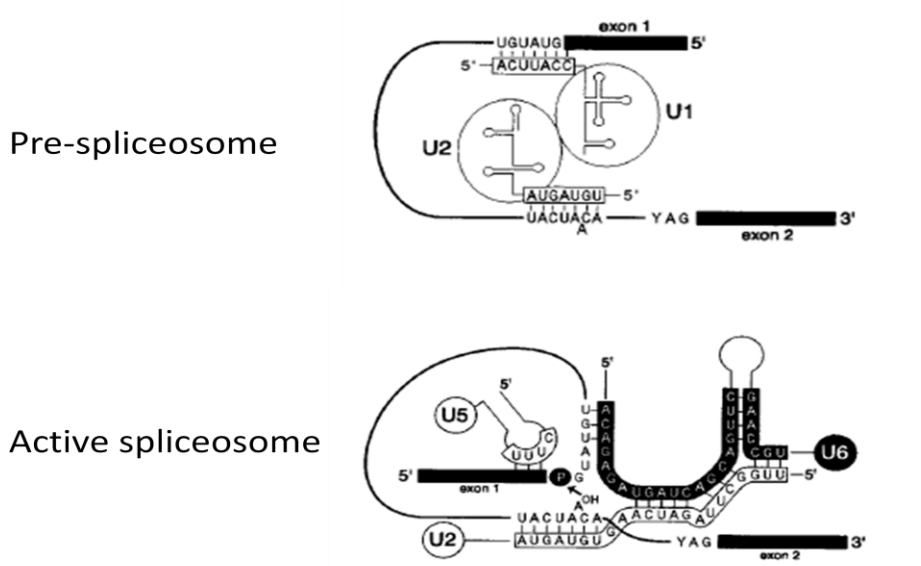

Figure 1. Model of yeast splicing complexes (adapted from Nagai *et al.,* 2001).

More recently, splicing research has utilized new techniques such as microarrays and chromatin immunoprecipitation (ChIP) assays to explore global splicing patterns and the co-transcriptional nature of splicing. Microarrays analyzing mutant or deleted splicing factors have identified specific subsets of intron-containing transcripts for which they are required (Clark *et al.,* 2002; Sapra *et al.,* 2004; Pleiss *et al.,* 2007). The transcript specificity for a certain splicing factor, but not another suggests splicing offers another level of regulation during gene expression. For example, it was recently observed that the 13 meiotic-specific intron-containing transcripts splice with low efficiencies if expressed during mitosis. It is therefore likely that splicing factors expressed during meiosis act to regulate the splicing of these meiotic pre-mRNAs and serve to minimize their unintended and possible harmful expression during mitosis (Juneau *et al.,* 2007). Microarrays in combination with bioinformatics have also served to identify more and more yeast introns. Whereas, in 1999, 228 yeast introns were recognized (Spingola *et al.,* 1999), today the Saccharomyces Genome Database recognizes 297 intron containing transcripts (Hong *et al.,* 2008). This total includes 3 dubious ORFs and 24 transcripts with introns in their 5' UTRs. It does not include 11 mitochondrial transcripts or 13 predicted, but not confirmed transcripts with introns: SNT1 (Juneau *et al.,* 2007) and BDF2, YEL023C (Zhang *et al.,* 2007) and PRP5, PES4, IRC18, YJR005C-A, YKL133C, YLR049C, YLR173W, YML053C, YMR147W, and YNL194C (Miura *et al.,* 2006).

In contrast to years of genetic and *in vitro* evidence that supported a step-wise assembly of the spliceosome, a biochemical report announced discovery of a penta-snRNP or completely assembled spliceosome that included the U1snRNP and U4 snRNP (Stevens *et al.,* 2002). This surprising discovery opened the possibility that instead of the predicted *de novo* spliceosome formation around every intron containing transcript, a spliceosome, once assembled in the nucleus, remained assembled and intact as it transferred between pre-mRNAs. However, ChIP assays have recently reaffirmed the earlier *in vitro* studies supporting the sequential model of spliceosome assembly for each splicing event (Gornemann *et al.,* 2005). This technique features *in vivo* formaldehyde

treatment that serves to crosslink nascent transcripts to adjacent chromatin. Upon chromatin shearing and co-immunoprecipitation, small and discrete gene segments can be analyzed for splicing factor enrichment (Lei *et al.,* 2001). ChIP assays have also demonstrated that splicing occurs co-transcriptionally (Lacadie *et al.,* 2006).

Both the Neurgebauer and Rosbash labs have contributed to these recent findings. While previous work had suggested that the yeast U1 snRNP is recruited to an intron during transcription, Nerurgebauer and colleagues used the ChIP technique to demonstrate the U1 snRNP is highly enriched in a chromatin region corresponding to the middle of an intron (Gornemann *et al.,* 2005). Here, there was a 5-20 fold increase in bound U1 snRNP compared to the promoter or other upstream regions. Also, the U2 snRNP becomes enriched in a chromatin section coding for the 3' splice site. Further downstream, the U5 snRNP becomes enriched and this corresponds with the departure of the U1 snRNP, which is consistent with the step-wise spliceosome assembly model (Gornemann *et al.,* 2005). Studies from the Rosbash lab have reported similar results of the U1, U2 and U5 snRNP distributions along chromatin downstream of sequences coding for the branch point. Also, using a depleted U1 snRNA strain, they found that neither U2 nor U5 snRNP will bind to the chromatin (or crosslinked mRNA). If U2 snRNA is depleted, then the U5 snRNP will not bind and the level of U1 snRNP enrichment increases, which suggests an accumulation of arrested splicing complexes (Lacadie and Rosbash, 2005; Tardiff and Rosbash, 2006).

By demonstrating that Prp19p, a member of the NTC particle, binds to chromatin at the point of U5 snRNP enrichment it was concluded that an active spliceosome assembled cotranscriptionally. So rather than mere loading of splicing factors during transcription, the Prp19p accumulation (a putative indicator of spliceosome assembly and activity) indicated actual splicing during transcription (Gornemann *et al.,* 2005). Any objections to this conclusion were placated when ChIP assays utilizing an intron-based ribozyme demonstrated significant activity for the ribozyme in a splicing mutant construct, but not other control constructs. Because the substrate for the intronic ribozyme was the 3' exon, the lack of ribozyme activity in the control constructs indicated splicing was occurring during transcription and served to remove the ribozyme from the substrate. The intact substrate recovered during the ChIP assay demonstrated cotranscriptional splicing (Lacadie *et al.,* 2006). Interestingly, a subsequent study concluded that while splicing can occur cotranscriptionally this is primarily dependent on the length of the 3' exon. A long 3' exon permits splicing to occur during transcription due to the increased time necessary to complete transcription. Yet because most yeast 3' exons are short, it was reported that the majority of yeast splicing events occur posttranscriptionally (Tardiff *et al.,* 2006).

When yeast and human splicing mechanisms are compared, the similarities are remarkable. For example, the core set of snRNPs are conserved and the assembly order is nearly identical (Staley and Guthrie, 1998). Two noteworthy differences between yeast and human splicing are the exon junction complex and the frequency of alternative splicing. The exon junction complex forms in metazoans just 20-24 nucleotides upstream of each splice juncture (Le Hir *et al.,* 2000). It consists of four core proteins (eIF4AIII,

Y14, Magoh, MLN51) that export to the cytoplasm bound tightly to the transcript. Because this tetrameric core serves as a docking platform for more than twelve other proteins, the EJC has been linked to roles in alternative splicing, export, nonsense-mediated decay (NMD) and translation (Tange *et al.,* 2005). More likely, however, the EJC core's main function consists primarily as a very stable docking point. eIF4AIII is a DEAD-box ATPase and makes the primary contact with the mRNA in a sequence and structure independent manner. While MLN51 also binds to the mRNA, the Y14-Magoh heterodimer acts to prevent conformational rearrangements to eIF4AIII by inhibiting ATP hydrolysis. This serves to create a stable docking platform by locking the core to the mRNA (Shibuya *et al.,* 2006; Stroupe *et al.,* 2006). Yeast, on the other hand, do not have an EJC or any other group of proteins that remains bound to the mRNA after a splicing event. Though this may be an artifact of a diminished role for splicing in yeast, it is interesting to note that Drosophila possess an EJC, but it does not play a role in NMD as it does in humans. The EJC's regulatory duties appear to increase as organisms become more complex (Gatfield *et al.,* 2003).

Alternative splicing occurs in yeast and humans. If a transcript contains two introns, splicing regulators can bind to either exonic or intronic sequences and vary the splicing pattern to generate four isoforms. Three introns in a transcript allow for nine potential isoforms. Virtually all human transcripts contain introns and it is estimated that 60% of these transcripts splice in alternative patterns. Alternative splicing can increase protein diversity with a minimal impact on genome size. It offers another form of regulation during gene expression and may be particularly useful for periods of development and multiple tissue types (Black, 2003). On average each human pre-mRNA transcript contains ten introns (Ares *et al.,* 1999). However, in yeast only 10-13 transcripts contain two introns and none are reported to have three introns except in the mitochondria (Hong *et al.,* 2008; Davis *et al.,* 2000; Miura *et al.,* 2006). A difference between yeast and humans for the reliance on alternative splicing may be lack of tissue types necessary for yeast survival.

Splicing Regulation During Meiosis

When properly spliced, the *AMA1* mRNA codes for a protein critical for the formation of the anaphase-promoting complex (APC), which allows the meiosis cell cycle to progress beyond metaphase. Correct expression of the spliced *AMA1* transcript is essential for spore production (Cooper *et al.,* 2000). *AMA1* pre-mRNA splicing requires the splicing factor Mer1p, which is also expressed during meiosis. Placing both of these meiotically expressed genes into expression plasmids and transforming these plasmids into vegetative cells results in a significant increase of splicing to the plasmid based *AMA1* transcript (Davis *et al.,* 2000). In addition to *AMA1* splicing, Mer1p also regulates the pre-mRNA splicing of two other meiotic proteins, Mer2p and Mer3p (Engebrecht *et al.,* 1991; Nakagawa *et al.,* 1999).

The exact mechanism whereby Mer1p regulates the splicing of the three meiotic transcripts is currently under investigation, but many critical elements of this splicing regulation have already been revealed and reported. For example, the *MER2* and *MER3* transcripts do not contain the ideal or consensus 5' splice site sequences, while *AMA1*

contains a silencer element immediately adjacent and downstream of the 5' splice site. As a result, these three transcripts may experience difficulty forming stable commitment complexes between the U1 snRNP and the 5' splice sites (Spingola *et al.*, 2000; Nakagawa *et al.*, 1999; Nandabalan *et al.*, 1993). Second, the three Mer1p regulated transcripts contain a conserved intronic enhancer sequence AYACCCUY. Mutation of this sequence will abolish the Mer1p splicing activation. Third, Mer1p contains an RNA binding domain (KH motif) that is essential for Mer1p splicing activity (Spingola and Ares, 2000). Taken together, these observations support a splicing model where Mer1p binds to the enhancer sequence in an intron via its KH domain. Mer1p may then interact with the U1 snRNP to provide extra affinity that compensates for the weak 5' splice sites of AMA1, MER2, and MER3 (Spingola and Ares, 2000). Several additional observations support and refine this splicing model.

If the KH domain of Mer1p is replaced with a different RNA binding motif (MS2 Coat), this modified Mer1p will activate splicing of transcripts containing an appropriate RNA sequence (MS2 operator) in its intron (Spingola *et al.*, 2004). Furthermore, the conserved intronic enhancer sequence for *AMA1*, *MER2*, and *MER3* can be placed into the intron of a modified actin pre-mRNA and establish Mer1p-regulated splicing. As a result, this enhancer is both necessary and sufficient (Spingola and Ares, 2000). These observations provide evidence that the Mer1p KH domain specifically binds the enhancer sequence of Mer1p regulated transcripts and it is not needed to recruit splicing factors. Yet, experimental evidence does suggest that splicing factors are recruited by an activation domain in Mer1p's N-terminal region (Spingola *et al.*, 2004).

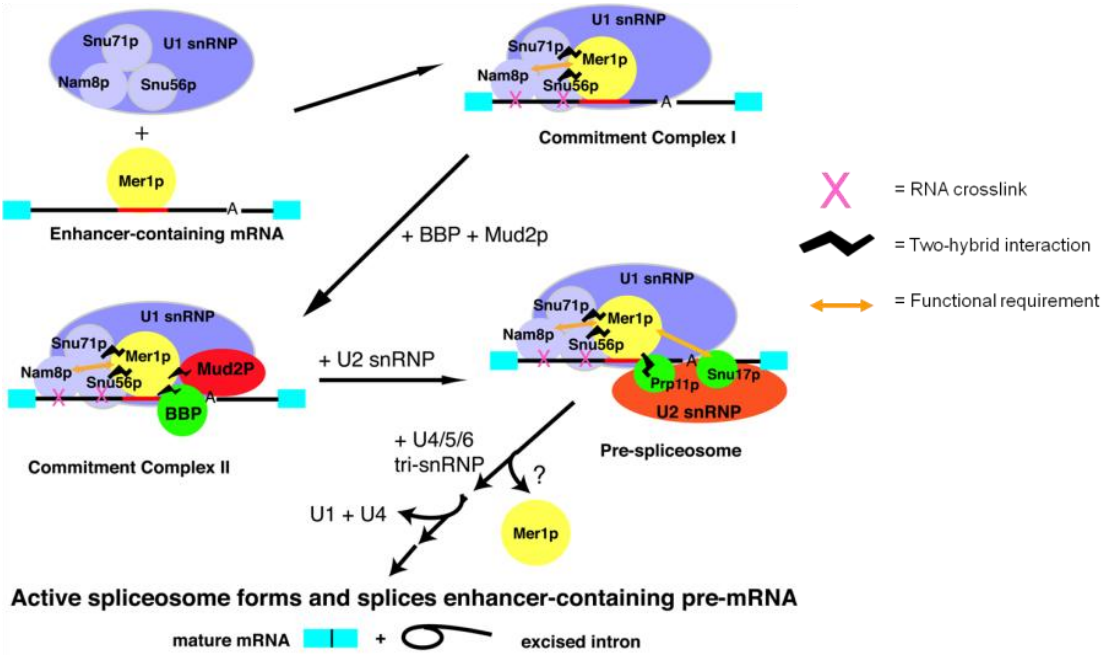

Figure 2. Mer1p-regulated splicing model as described by Spingola *et al.* (2004).

After co-immunoprecipitation experiments revealed binding between Mer1p and the U1 snRNP, it was suggested that Mer1p functions to stabilize commitment complex

formation (Spingola and Ares, 2000). Subsequent two-hybrid experiments and gene deletion studies served to reinforce this hypothesis. For example, by two-hybrid interactions Mer1p was linked to the U1 snRNP proteins Snu56p and Snu71p. Also indicated by two-hybrid tests are interactions between Mer1p and the yeast branch point protein (BBP) and the U2 snRNP protein Prp11p. By measuring Mer1p-regulated splicing efficiencies in numerous knockout strains, it was observed that Mer1p-activated splicing requires both the splicing factors Nam8p and Snu17p. While these factors contribute to early splicing complexes, they are non-essential for yeast growth and general splicing, but they are required for Mer1p-regulated splicing (Spingola and Ares, 2000; Spingola *et al.,* 2004). Taken collectively, this data suggests that Mer1p with its numerous spliceosome interactions provides enhancer-containing transcripts additional cohesion with the assembling spliceosome, which leads to increased splicing efficiencies. Figure 2 provides a model of the splicing factor interactions with Mer1p.

Nuclear Components of Eukaryotic Gene Expression

The Central Dogma of molecular biology first proposed by Francis Crick is now celebrating its golden anniversary. While it has been subjected to some scrutiny by the discovery of reverse transcriptase and prion diseases, it has largely survived the advent of biotechnology. In its simplest form the Dogma neatly summarizes gene expression by stating that DNA codes for RNA and RNA codes for protein (Crick, 1970). It predicts a one-way flow of genetic information such that protein does not code for RNA or DNA. The discovery of mRNA splicing by Phil Sharp in 1977 served to strengthen the Dogma's predictions because the splicing event removes genetic information from the mRNA and helps ensure a downhill flow of information (Berget *et al.,* 1977).

The theory was correct describing the direction of genetic information flow, but it did little to describe the mechanics and quality control required for the information transfer. Now fifty years later many of the details of eukaryotic gene expression are well known. Consider, for example, the transition from DNA to RNA. It has been long understood that during transcription RNA polymerases read a strand of the DNA double helix and create an equivalent RNA molecule. Yet additional research revealed that modifications such as capping, splicing and polyadenylation of the RNA are also part of this transition (Neugebauer, 2002). Once details of mRNA export were revealed in the 1990s, it became clear that "naked" mRNAs do not enter the cytoplasm. Instead, mRNAs are converted to messenger ribonucleoproteins (mRNPs) as proteins cover the mRNAs to protect mRNAs from decay and negotiate the nuclear pores. Also, a nuclear quality control system controlled by the exosome was discovered that works to eliminate improper mRNAs (Saguez *et al.,* 2005).

Over time, as these various steps of nuclear gene expression were discovered, they were studied individually and viewed as independent modifications to transcripts. While it is true they can be dissected as discrete steps and can be reproduced using *in vitro* systems, some of the latest research suggests post-transcriptional modifications are entwined together and regulate each other (Orphanides and Reinberg, 2002). Examples of this regulation abound: defects to nuclear pore components cause mRNA accumulation at transcription foci and enhanced exosome activity; capping improves cotranscriptional

splicing; polyadenylation and capping are influenced by the RNA polymerase CTD; and nuclear pores are linked to chromatin remodeling and positioning (Hilleren *et al.*, 2001; Gornemann *et al.*, 2005; Proudfoot *et al.*, 2002; Brown and Silver, 2007). So while at one time, the events of nuclear gene expression were strung together as discrete steps in a linear progression, the emerging model suggests co-regulation, feedback, and enzymes with multiple functions. The appropriate analogy for nuclear gene expression is now more akin to a "barn raising" rather than a "Henry Ford assembly line" (Orphanides and Reinberg, 2002).

In addition to the advances in understanding the interrelationships among transcription and the classic post-transcriptional events (capping, splicing, and polyadenylation), the recent discoveries of the SAGA and TREX complexes now clearly link early transcription factors to nuclear export (Rodriquez-Navarro *et al.*, 2004; Sträßer *et al.*, 2002). Also, while enhanced understanding of mRNP export and quality control portray the nuclear pores as selective gatekeepers, the most recent research now suggests that nuclear pores control transcription activation and repression (Tran and Wente, 2006). Though the statement "transcription controls export and export controls transcription" hints of a paradox, in reality it may mean yeast biology has come full circle.

Nuclear Pores

The existence of the nuclear membrane and the nuclear pores is the defining difference between prokaryotes and eukaryotes and explains the rise of the metazoans. Yet only during the last few years has the significance of this formidable partition between transcription and translation been realized. The separation allows for enhanced regulation through quality control exerted together by the exosome and the nuclear pores and for enhanced gene diversity by splicing. New evidence now links the inner nuclear membrane to chromatin silencing, while the nuclear pore complex (NPC) mediates transcription activation (Akhtar and Gasser, 2007).

Macromolecular import to and export from the nucleus of a eukaryotic cell is a tightly controlled process. Dual bilayer membranes of the nuclear envelope provide sufficient resistance to the passive diffusion of complex molecules. As a result, the gatekeeper of the nucleus, the nuclear pore complex (NPC), controls entry and exit of the nucleus. An average nucleus in a cultured cell contains approximately 4000 NPCs. Metazoan NPCs consist of more than 50 components (nucleoporins) and have a mass of 120 Mda. In yeast, multiple copies of 30 different proteins comprise the NPC of 60 Mda (Rout *et al.*, 2000; Vasu and Forbes, 2001; Cullen, 2003).

An NPC has 8 fibrils, which reach 50 nm into cytoplasm and a nuclear basket that extends 100 nm into the nucleus. It is believed these extensions (fibrils and basket) serve as docking sites for proteins. The NPC, when closed, is 9 nm in diameter, but 25 nm when open. It can accommodate proteins or RNA less than 40 kDa when closed; yet this passive diffusion is slow, such that these small molecules are usually actively transported anyway (Cullen, 2003). Many nucleoporins lining the NPC have domains containing phenylalanine-glycine repeats (FG repeats). These FG repeats serve as temporary hydrophobic docking sites for export factors. For example, Nup116p, a nucleoporin

located on the cytoplasmic side of the NPC, contains FG repeats (Rout *et al.*, 2000; Sträßer *et al.*, 2000). Most nucleoporins remain stationary at the NPC, yet some migrate nearby the complex. These mobile nucleoporins may serve to attract export factors to the NPC. Deletion studies have identified several nucleoporins that are essential for mRNA export (Thomsen *et al.* 2003; Cullen, 2003).

Several theories explain how the FG rich nucleoporins control export. The Brownian Affinity Gate model argues that hydrophobic export factors (export receptors) bind nucleoporins at the entrance of the pore complex. The pore itself is so narrow that only nucleoporin-bound receptor proteins have access to it. Thus, non-hydrophobic molecules are blocked from the aqueous channel. Alternatively, the Selective Phase model proposes a wide channel. Here, the numerous nucleoporins form a mesh-like network throughout the channel. Very small molecules can passively diffuse, but larger molecules are blocked by the mesh. Only molecules with a sufficient hydrophobic nature i.e. numerous hydrophobic pockets, can mimic the nucleoporin interactions. These hydrophobic receptor proteins (and bound cargoes) can literally melt the mesh and efficiently migrate the channel. In this model, the size and hydrophobic nature of the cargoes become important determinants for export. Supporting both proposed models is the observation that all known export and import receptor proteins are efficiently captured on a phenyl-Sepharose column (Ribbeck and Gorlich, 2002). The Virtual Gating model reasons that the high entropy of a freely moving macromolecule is curtailed upon entering the pore. Loss of the entropy in this closed system results in a positive ΔG and translocation will not take place. However if binding occurs between the macromolecule and the NPC, the change in enthalpy can overcome the loss of entropy caused by the restrictive pore and the translocation will proceed (Rout *et al.*, 2003).

The models discussed above predict that export or import factors will bind to macromolecules (i.e. RNA, protein or mRNP) and together negotiate the NPC. In general, these factors are either adapter or receptor proteins. For example, during import, adapter proteins bind target macromolecules containing a nuclear location signal (NLS). The receptor proteins bind the adapter proteins carrying cargo and also interact with the FG repeats of nucleoporins. Protein import signals to the nucleus were determined in the early 1980s and they are commonly short basic sequences rich with lysine. The receptors that recognize these lysine signals are in the karyopherin protein family (importins and exportins). In fact, a majority of the nuclear traffic is controlled by receptors in the karyopherin family (Gorlich and Kutay, 1999).

During export, macromolecules with a nuclear export signal (NES) interact directly with a karyopherin receptor protein. This receptor/cargo complex also requires RanGTP to mediate the transit to the nucleus. Again, the FG repeats of the nucleoporins likely play a key role allowing movement through the pores. For export, the signals recognized by the karyopherin receptors are short and leucine rich. However, some macromolecules to be exported do not contain a leucine rich NES. In this case, these molecules must bind an adapter protein that contains an NES before joining the karyopherin. For this reason, most types of RNA require adapter proteins. Also, most types of RNA require the karyopherin adapter protein Crm1p; see Figure 3. In the case of tRNA no adapter protein

is required and tRNA binds directly to its receptor exportin-t (Fornerod *et al*, 1997; Gorlich and Kutay, 1999; Cullen, 2003).

Amazingly, missing from this collection of RNA species in Figure 3 is cellular mRNA. Messenger RNAs are unusual because they require neither the Crm1p nor Ran-GTP for their export (Neville and Rosbash, 1999; Clouse *et al.*, 2000). Instead, Mex67p serves as the primary receptor protein for yeast mRNAs. Experiments revealed that mutation of *MEX67* would block mRNA export and that Mex67p binds to poly (A) + RNA and nuclear pore proteins. Mex67p also shuttles between the nucleus and the cytoplasm (Segref *et al.*, 1997). Mex67p forms a heterodimer with Mtr2p, a shuttling protein that does not bind mRNA but does physically and genetically interact with the nuclear pore complex. Formation of this heterodimer is essential for stable interaction with the nuclear pore and mRNA export (Santos-Rosa *et al.*, 1998). The Mex67p-Mtr2p mRNA receptor complex is conserved throughout eukaryotes and in metazoans the homolog is TAP-p15, which can complement a lethal *MEX67*Δ/*MTR2*Δ yeast knockout (Katahira *et al.*, 1999). Fluorescent *in situ* hybridization (FISH) analysis, using three different mRNA species, demonstrated export blocks for specific transcripts in *MEX67* mutants and suggested that Mex67p was a general export factor (Hurt *et al.*, 2000).

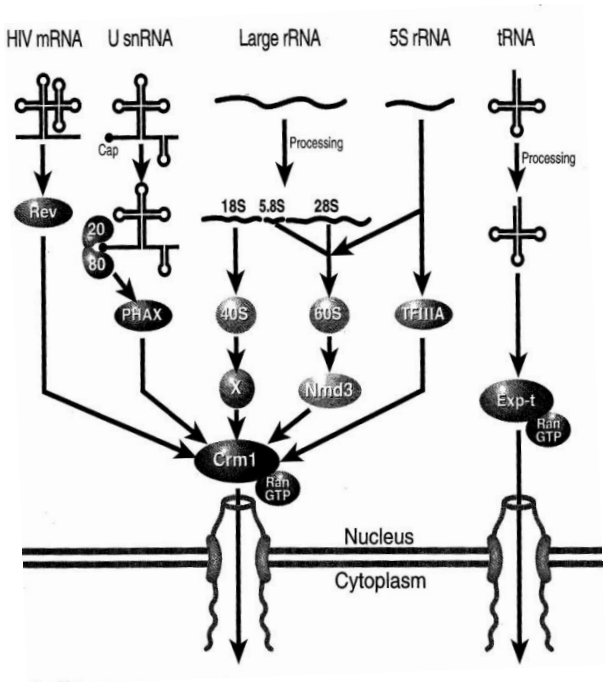

Figure 3. Majority of RNA species utilize the Ran-dependent Crm1p as the receptor protein for nuclear export. REV, PHAX, Nmd3, and TFIIA function as adapter proteins for various RNA types. tRNA is an exception. Notably absent from Ran-dependent export is cellular mRNA. Adapted from Cullen (2003).

While the Mex67p-Mtr2p heterodimer can bind mRNA, considerable evidence suggests this contact is mediated by the essential mRNA export factor Yra1p, which has RNA-RNA annealing activity and binds mRNA. Like Mex67p, Yra1p is evolutionary conserved as ALY in mice and REF in humans. *YRA1* and *MEX67* are synthetically lethal and *YRA1* mutants cause nuclear poly (A)+ accumulation. By a variety of

biochemical assays Mex67p and Yra1p physically interact (Sträßer and Hurt, 2000). Furthermore, a binding region on Yra1p for Mex67p was identified and it was recognized that the Mex67p association with mRNPs is weakened in *YRA1* mutants (Zenklusen *et al.,* 2001). Thus, a yeast export model developed where Mex67p-Mtr2p functioned as the mRNA export receptor that interacted with the NPC and Yra1p with its mRNA binding and Mex67p binding activities served as the general mRNA export adapter protein (Reed and Hurt, 2002). However, a global analysis of yeast transcripts conducted in the Silver lab concluded that these putative export factors, Mex67p and Yra1p, may only transport mRNA for 20% of the genome. Their work suggests that multiple export factors function to guide yeast mRNPs through nuclear pores (Heironoymus and Silver, 2003). See Export Factors below.

Transcription

Transcription of protein-coding genes is catalyzed by RNA polymerase II. However, the polymerase requires many additional proteins in order to efficiently initiate transcription and recognize a variety of promoters and activation sequences in yeast. Collectively called the polymerase holoenzyme this transcription machinery includes: RNA Pol II, Mediator, Swi/Snf complex, Srb10p CDK complex, and the general transcription factors (Myer and Young, 1998). Upon binding of TBP, a subunit of TFIID, to the TATA box in the promoter sequence, TFIIB along with RNA Pol II and other transcription factors rapidly assemble around the promoter to form the pre-initiation complex (PIC). Transcription initiation follows the promoter melting and once 20-25 nucleotides of RNA have assembled, 5' end capping takes place. After promoter clearance, rapid and processive RNA synthesis occurs during transcription elongation (Woychik and Hampsey, 2002; Neugebauer, 2002). Once the polymerase creates the entire mRNA, it continues transcribing downstream beyond the 3' end of the 3'UTR. The cleavage/polyadenylation complex assembles on the poly (A) signal and cleaves the transcript with an endonuclease. The poly (A) polymerase then adds a long poly (A) tail to the mRNA. At the same time, the exonucleases Xrn1p and Rat1p degrade the cleaved and un-capped mRNA that is still being transcribed. The Torpedo model suggests the exonucleases degrade the mRNA so rapidly they catch the RNA Pol II and dislodge it from the DNA. The Allosteric model of termination instead suggests a conformational change to RNA pol II occurs as it transcribes the poly (A) signal and as a result it disassociates from the DNA soon thereafter (Luo *et al.,* 2006; Rosonina *et al.,* 2006).

Cap Acquisition

Just prior to transcription elongation, a methylated guanosine monophosphate is added to the 5' end of the nascent transcript just emerging from the holoenzyme. It covalently bonds to the transcript in an unusual and essentially backwards 5'-5' manner. Capping requires three enzymes; first, the nascent transcript's 5' phosphate is removed by the active triphosphatase Cet1p. Next, a guanosine monophosphate is added by guanyltransferase Ceg1p. Finally, the methyltransferase Abd1p adds a methyl group to the guanosine (Neugebauer, 2002). In higher eukaryotes, capping activity causes a pause to transcription, whereas in yeast, the capping enzyme, Cet1p, downregulates nearby transcription initiation, thus ensuring greater resources for transcripts possessing caps. (Jensen *et al.,* 2003).

Not only does the cap and its unusual bond protect the transcript from exonucleases, but it permits formation of the cap binding complex or CBC. In the nucleus, this consists of Cbp20p and Cbp80p, while in the cytoplasm, these proteins are exchanged for eIF4E. While capping is not essential for mRNA export, it is required for export of snRNAs in metazoans (Neugebauer, 2002; Aguilera, 2005). Electron micrographs of Balbiani ring granule mRNA transcripts in *Chironomus tentans* demonstrate that the CBC remains bound to the transcript and is first to enter the cytoplasm as the 5'end of the transcript leads export (Daneholt, 1997). The cap complex also promotes splicing efficiency, translation efficiencies and protects the 3' end of the transcript via circularization of the mRNA (Aguilera, 2005; Cougot *et al.*, 2004). The importance of the CBC for the formation of export ready mRNPs may not be yet fully appreciated. For example, a Cbp80p deletion is synthetically lethal with Hmt1p an arginine methylase that modifies key hnRNP export factors. Also *CBP80* is synthetically lethal with the mRNA export factor *NPL3*, as well as, numerous splicing factors. (Shen *et al.*, 1998; Fortes *et al.*, 1999).

CTD Subunit

Evidence supporting the cotranscriptional nature of "posttranscriptional" mRNA processing events continues to grow. As discussed above, capping and polyadenylation, as well as, splicing occur during transcription. In addition, export factors are loaded onto the mRNA as transcription proceeds (Lei *et al.*, 2001); see Export Factors below. In light of data pointing to transcription occurring at the nuclear pores, cotranscriptional mRNA processing and packaging may be necessary to prevent incompletely processed mRNA from exporting to the cytoplasm. Experiments from several research efforts suggest the C-terminal domain (CTD) subunit of RNA Pol II plays an important role in coordinating and catalyzing cotranscription processing of mRNA (Neugebauer, 2002).

The CTD or C-terminal Domain of RNA Polymerase II is conserved from yeast to humans and likely regulates transcription and post-transcriptional events by modifications to its phosphorylation levels (Hirose and Manley, 2000). In yeast the CTD consists of 26 heptad repeats of YSPTSPS, while in humans the CTD contains 52 repeats. During transcription initiation, serine #5 of the heptad repeat becomes phosphorylated, which serves to attract transcription factors. Regulated by TFIIH, this phosphorylation event also recruits the capping enzymes Ceg1p and Abd1p (Lacadie *et al.*, 2006; McCracken *et al.*, 1997b). Later after capping, the CTD phosphorylation state shifts to Serine #2 and elongation proceeds. This modification to serines in the CTD is associated with transcription of coding regions and 3'end formation. As transcription proceeds, the CTD recruits enzymes and functions as a binding platform for termination and poly (A)+ tail formation (Lacadie *et al.*, 2006; McCracken *et al.*, 1997a; Neugebauer, 2002).

The CTD may also enhance or regulate cotranscriptional splicing by its interaction with the U1 snRNP protein Prp40p (Nuegebauer, 2002). Because the CBC is important for contranscriptional splicing, the CTD may indirectly influence splicing by interaction with the CBC (Gornemann *et al.*, 2005). Also, a study that compared the splicing efficiencies between transcripts created by T7 polymerase or RNA Pol II concluded that the CTD of RNA Pol II positively influences splicing efficiencies and minimizes degradation of pre-

mRNA (Hicks *et al.*, 2006). Finally, removal of the CTD from RNA Pol II will impair splicing efficiency compared to splicing nearby a wild-type polymerase (McCracken *et al.*, 1997a).

SAGA

The SAGA complex contains a variety of transcription factors including Gcn5p that has histone acetylation activity. The complex enhances expression for a subset of the yeast genome and it is estimated that Gcn5p alone is required for 5% of the genome's transcription (Holstege *et al.*, 1998). With respect to *GAL1* gene expression, it has been demonstrated that Gal4p, when bound to a UAS, will recruit SAGA to the promoter region. A subunit of SAGA, Spt3p, then interacts with the TATA binding protein at the TATA box as transcription initiates (Larschan and Winston, 2001). Surprisingly, the Hurt lab identified a link between the SAGA complex and mRNA export. They not only identified Sus1p as an essential mRNA export factor, but also demonstrated that it physically associates with SAGA and the export factors Sac3p and Thp1p, which bind to the nuclear pores. Additionally, SUS1 was linked genetically to several other export factors including YAR1, SUB2 and MEX67. Yet because a physical association between these export factors could not be demonstrated, it suggested that Sus1p may function as a tethering protein that brings the transcription machinery in close contact to the nuclear pores; see Figure 4 (Rodrigues-Navarro *et al.*, 2004; Sommer and Nehrbass, 2005).

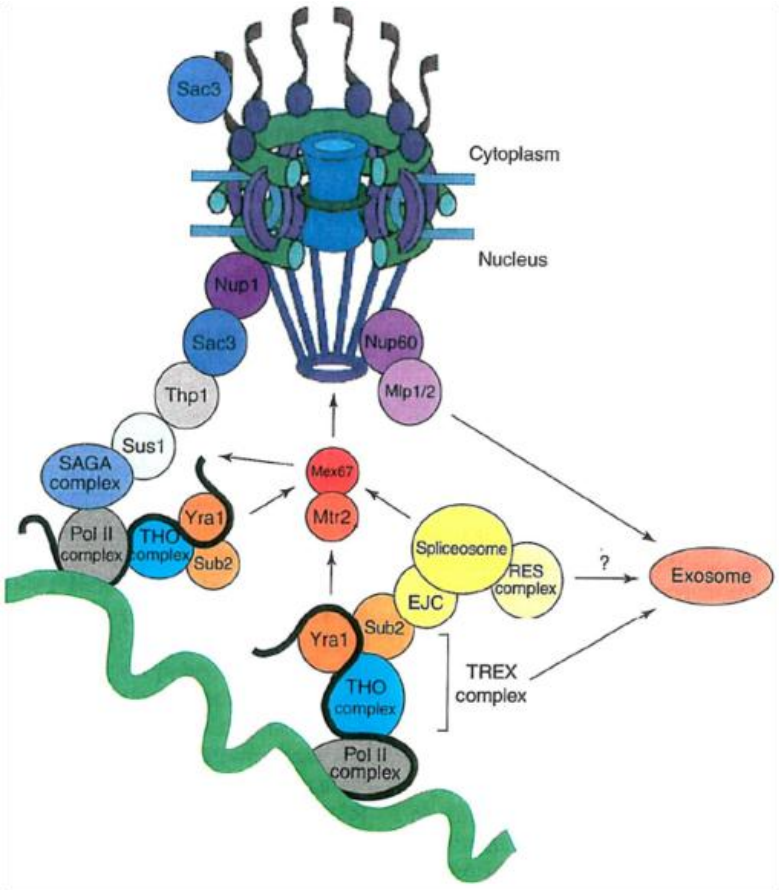

Figure 4. Current mRNA export model presented by Sommer and Nehrbass (2005).

Two recent studies have validated this model and served to extend the growing body of evidence that ties the nuclear pores to chromatin remodeling and transcription activation. Nehrbass and colleagues monitored movement of a *GAL1* –TetO construct using a TetR-GFP protein while simultaneously viewing GFP labeled nuclear pores. They observed a correlation between transcriptional activity and proximity to the nuclear pore in a wild-type strain. Also using mutant strains they observed this tight positioning of active genes at the nuclear pores was mediated by SAGA components including Sus1p (Cabal *et al.*, 2006). A second report using a series of biochemical assays including ChIPs demonstrates physical interactions during active transcription between SAGA components and the nuclear pore basket proteins Mlp1p and Mlp2p (Luthra *et al.*, 2007). The Mlp proteins are large filamentous proteins that have a number of proposed activities including serving as docking platforms for mRNPs preparing for nuclear export (Strambio-de –Castillia *et al.* 1999; Green *et al.* 2003).

TREX

The TREX protein complex was first described in 2002 and consists of seven proteins that influence both TRanscription and EXport. Four of these proteins, Tho2p, Hpr1p, Mft1p, and Thp2p were previously identified as the THO complex by the Aguilera lab in 2000. The THO complex has a role in transcription elongation. While the individual THO components are not essential, deletion or mutation to any particular THO component results in a matching phenotype of lowered gene expression, hyperrecombination and defects in mRNA export. Furthermore, THO mutants suffer from increased RNA-DNA hybrid molecules, which suggests the THO complex serves to prevent the nascent transcript from binding to melted DNA (Chavez *et al.*, 2000; Sträßer *et al.*, 2002; Zenklusen *et al.*, 2002).

Concerning the other 3 components of TREX, Tex1p is not particularly well known, but the remaining proteins Sub2p and Yra1p were previously recognized as vital for splicing and export (Sträßer *et al.*, 2001; Sträßer and Hurt, 2000; Zenklusen *et al.*, 2001). Before isolation of the seven proteins as a complex, genetic interactions were established between THO and *SUB2* and *YRA1*. For example, synthetic lethal interactions were noted between *SUB2* or *YRA1* and each individual THO component. Also the human homologues of Sub2p and Yra1p (UAP56 and REF) were linked to the human THO complex, which suggests TREX is conserved. Interestingly, Sub2p will not bind to the THO complex or Tex1p, if any one of the four THO complex members is deleted. Furthermore, Sub2p appears to bind tighter to the THO complex or Tex1p than Yra1p. These results collectively suggest that early during transcription initiation, THO recruits Sub2p, which in turn recruits Yra1p (Sträßer *et al.*, 2002; Zenklusen *et al.*, 2002). ChIP assays from several labs have demonstrated consistently that TREX components are recruited to active transcription sites. The recruitment of THO components appears dependent on RNA Pol II, while Sub2p and Yar1p recruitment depends more on RNA sequence than choice of a polymerase (Lei and Silver, 2002; Zenklusen *et al.*, 2002; Abruzzi *et al.*, 2004).

Export Factors

The Balbiani ring mRNA experiments conducted by Mehlin *et al*. (1992) and Visa *et al*. (1996) yielded quality pictures of very large mRNPs barely squeezing through the nuclear pore complex. These pictures gave a strong indication that an mRNA particle does not exit the nucleus without multiple protein escorts. In yeast and metazoans the surprising variety of proteins that coat mRNAs are generally referred to as heterogeneous nuclear ribonuclear proteins (hnRNPs). For humans approximately 30 different hnRNPs have been identified (Green *et al.,* 2002). Some of these proteins assist with cotranscriptional processing and offer protection to the mRNA from the nuclear exosome. Other hnRNPs, as the Balbiani ring pictures demonstrate, travel with the mRNA to the cytoplasm and then shuttle back to the nucleus. These export factors make contact with nucleoporins on both sides of the membrane and likely assist the primary adapter (Yra1p) and receptor (Mex67p) with mRNA export (Rodriguez *et al.,* 2004). Many of the hnRNPs appear to have dual roles such that they are essential for export and some other processing event such as splicing, polyadenylation or nonsense-mediated decay. hnRNPs are loaded onto the mRNA during transcription and several specific binding motifs have been determined. For example, yeast Nab2p favors AAAAAG, while Hrp1p prefers TATATAA (Guisbert *et al.,* 2005; Lei *et al.,* 2001; Hector *et al.,* 2002).

Post translational modifications play an important role with hnRNPs. They are subject to glycosylation, phosphorylation and methylation. These modifications likely alter binding status and the ability to shuttle. Methylation of arginines, in particular, is important in yeast. The methyltransferase Hmt1p modifies the hnRNPs: Nab2p, Npl3p, and Hrp1p (Shen *et al.,* 1998; Green *et al.,* 2002). Hmt1p is recruited cotranscriptionally and also methylates the export factor Yra1p; an additional role for Hmt1p in maintaining silenced chromatin has been identified (Yu *et al.,* 2004; Yu *et al.,* 2006).

The classic characterization for an mRNA export factor has relied on FISH analysis that shows nuclear accumulation of pre-mRNA in the event of a deletion or mutation to a potential export factor. By this test more than 20 yeast proteins are essential for mRNA export (Stutz and Rosbash, 1998; Zenklusen and Stutz, 2001; Rodriguez *et al.,* 2004). Thomsen and colleagues suggest classifying these essential factors in three categories: early, intermediate, and late. The late export factors are nucleoporins such as Nup116p, Nup133p, Gle1p, Sac3p, Thp1p and the cytoplasmic Dbp5p. Intermediate factors would comprise Mex67p and Mtr2p, which serve as receptor proteins that interact with both the mRNP and the nuclear pore. Finally the various hnRNPs that load cotranscriptionally are the early factors. These proteins bind either to RNA motifs or nonspecifically and protect the mRNP but they also dock the mRNP at the nuclear pores as Mex67p and other middle acting factors are loaded. These include: Npl3p, Nab2p, Hrp1p, Gbp2, and TREX components among others (Thomsen *et al.,* 2003; Windgassen and Krebber, 2003; Rodriguez *et al.*, 2004; Fischer *et al.*, 2002). The observation that some early export factors bind to specific RNA sequences and that the middle factors Mex67p/Mtr2p binds only 20% of the genome suggests that each mRNA is packaged uniquely and that multiple pathways exist for mRNAs to exit the nucleus (Guisbert *et al.,* 2005; Hieronymus and Silver, 2003). Supporting this possibility are experiments that now suggest Npl3p in addition to Yra1p can serve as an adapter protein for the mRNA export

receptor Mex67p/Mtr2p. After cotranscriptional loading of Npl3p to an mRNA, the phosphatase Glc7p acts to dephosphorylate Npl3p allowing Mex67p to bind to Npl3p. Once in the cytoplasm Npl3p is phosphorylated by Sky1p and Npl3p shuttles back to the nucleus (Gilbert and Guthrie, 2004).

Nuclear Pores Control Transcription

A number of yeast and metazoan studies have established a relationship between telomeres or silenced chromatin and the nuclear membrane (Brikner and Walter, 2004). In yeast, discrete telomere clusters form adjacent to the nuclear envelope and generate a silenced heterochromatin structure that represses transcription of nearby genes. The silencing is known as the telomere position effect (TPE) (Taddei *et al.,* 2004). The silencing initiates upon Rap1p binding to telomere $TG_{1\text{-}3}$ repeats. The *SIR* genes are also required for repression. Sir3p and Sir4p interact with Rap1p and also bind to histone tails. FISH assays have identified the Sir proteins and Rap1p with telomere clusters at the nuclear envelope. (Maillet *et al.,* 1996). Gasser and colleagues created a *HML* silencer reporter construct and determined that silencing is dependent on chromosomal location. Insertion of the construct near a telomere greatly enhanced repression of the reporter. When the reporter was inserted into the chromosome distant from a telomere, overexpression of the Sir proteins induced silencing. From their experiments they concluded that the Sir proteins normally localize near telomere clusters at the nuclear envelope and create silencing compartments, yet when overexpressed they can silence more distant internal genes (Maillet *et al.,* 1996). Subsequent studies identified Esc1p as a chromatin anchor at the nuclear envelope that works in conjunction with Sir4p to sequester and silence telomeres. Additionally, a second tethering mechanism between Yku70p/Yku80p and Sir4p can also generate telomere clusters. Once chromatin is anchored in the SIR silencing compartments, Rap1p or Sir2p and Sir3p interact with Sir4p and histone tails to induce repression (Taddei *et al.,* 2004).

Surprisingly, the nuclear pores have been implicated in chromatin repression by acting as an anchoring point for the Yku70p/Yku80p heterodimer that binds teleomeres and has activities involving both telomere maintenance and DNA double-strand break repair. A report from the Nehrbass group concluded the filamentous nuclear pore protein Mlp2p secures Yku70p to the nuclear pore complex via Nup145p. Also this report demonstrated loss of Mlp1p and Mlp2p caused a notable decrease in double-stranded break repairs, as well as, a release of telomere clusters from the perinuclear compartment. This telomere migration resulted in a loss of chromatin silencing. Because this work was completed prior to an understanding of Esc1p function it concluded the nuclear pores were responsible for the telomere silencing long observed at the nuclear envelope (Galy *et al.,* 2000). A subsequent study also by Nehrbass *et al.* determined that Nup60p, a nuclear pore basket protein, links the inner core nucleoporin Nup145p to the Mlp proteins. It was further determined that disruption to either Nup145p, Nup60p, Mlp1p or Mlp2p would disrupt telomere clusters (Feuerbach *et al.,* 2002).

However, not long after this study was published, other research efforts began linking the nuclear pore complex to transcription activation rather than repression. The first such report identified several nuclear pore proteins and export factors that could gain

"boundary activities", which prevent the spread of heterochromatin repression caused by Sir proteins. They established that the "boundary activity" of export factors required interaction with the nuclear pore protein Nup2p. It was further reported that tethering Nup2p to chromatin will by itself establish a chromatin boundary activity (i.e. cause repression of chromatin silencing) (Ishii *et al.,* 2002). This conclusion was reinforced by a global analysis which concluded that many nuclear pore components, including the Mlp proteins, associate preferentially with highly transcribed genes. Also it was demonstrated that the *GAL* genes will associate with the nuclear pores upon a media switch from glucose to galactose, which is known to induce activation of these genes (Casolari *et al.,* 2004).

Recently, Laemmli and colleagues extended their conclusions about chromatin boundary activities at the nuclear pores by demonstrating that a variety of gene promoters regularly interact with the NPC (specifically Nup2p). By developing a novel assay, chromatin endogenous cleavage method (ChEC), they measured with high precision (within 100bp) the binding sites for chromatin proteins in the genome. After validating their assay by confirming that *GAL* genes do localize to the nuclear pores (see Casolari above), they determined that while *GAL4* is required for the Nup2 –*GAL* promoter interaction, neither Sus1p nor the SAGA complex are required. Furthermore, since TBP was tested with the ChEC assay and required the SAGA complex for a TBP-*GAL* promoter interaction, it was concluded that the Nup2p-*GAL* promoter interaction does not require TBP or RNA Pol II (Schmid *et al.,* 2006).

The ChEC assay was then performed between Nup2p and the *HXK1* gene, which is also induced by galactose. When repressed with a glucose media, no interaction between Nup2p and the *HXK1* gene was observed, yet upon a switch to galactose media, a strong interaction between Nup2p and the *HXK1* promoter was observed. Additional genes were tested (*CEN6, FRS2, ACT1*) and like the *GAL* genes and *HXK1*, their promoters interacted strongly with Nup2p. The ChEC assay was then applied to a microarray of Chromosome VI where a strong bias for Nup2p interaction with gene promoters was observed. The microarray analysis was then extended to a *sus1*Δ yeast strain and no disruption of the NPC-gene promoter interactions were observed. Using a heat sensitive RNA Pol II yeast strain (*rpb1-1)* also did not interrupt the NPC- gene promoter interactions indicated by the ChEC assay. The collective results of these novel experiments suggested that the interaction between the nuclear pore basket and the promoters of genes is specific and normal. These interactions are not dependent on active transcription, certain transcription components, or SAGA. The interactions between the nuclear pores and gene promoter regions may serve as an initial event in gene activation (Schmid *et al.,* 2006).

In contrast to the CheC results concerning the *SAGA*-independent interaction between *GAL* genes and Nup2p, ChIP assays have demonstrated that a Mlp1p interaction with promoters for *GAL* genes is dependent upon the integrity of SAGA, which also binds to the *GAL2* and *GAL1* promoter regions. Also, components of SAGA interact with both Mlp1p and Mlp2p in an RNA and DNA independent fashion (Luthra *et al.,* 2007). These results agree with other studies linking Mlp proteins to transcription activation (Dieppois *et al.,* 2006; Casolari *et al,* 2005; Casolari *et al.* 2004) and SAGA association with the

nuclear pores (Rodrigues-Navarro *et al.,* 2004; Cabal *et al.* 2006). It remains possible that both Mlp1p and Nup2p interactions occur with the *GAL* promoters and that the Mlp1p interaction is SAGA dependent while the Nup2p interaction is not SAGA dependent. Taken collectively, the data from the Schmid report and Luthra report could indicate separate and parallel mechanisms for *GAL* gene activation at the nuclear pores. Nup2p is a mobile nucleoporin that binds to the nuclear basket protein Nup60p (Dilworth *et al.,* 2001). Recent data suggests that Prp20p, which binds to chromatin, also binds to Nup2p and serves to link chromatin to the NPC (Dilworth *et al.,* 2005). Like Nup2p, the Mlp proteins bind to Nup60p, extend into the nucleus from the pore basket and are not essential, but surprisingly they only associate with a limited subset of nuclear pores and are believed to form a mesh network that controls chromatin location (Strambio-de-Castillia *et al.,* 1999; Fuerbach *et al.,* 2002; Galy *et al.,* 2004).

The migration of the *HXK1* locus to the nuclear pores upon galactose induction was confirmed in a second recent study. The movement of this gene to the nuclear periphery required the promoter region, as well as, the 3'UTR. This localization could also be induced by loss of its repressor, *HXK2,* instead of a shift to galactose media. The telomere binding protein Yku70p, which has been implicated in both activation and repression of chromatin, is not required for activation and relocation of *HXK1* to the nuclear envelope. The viral transcriptional activator VP16 caused a four-fold upregulation to *HXK1* when inserted upstream of the promoter. The activation did not require galactose and did not result in a shift to the nuclear membrane. Therefore, transcription does not require perinuclear anchoring. However, upon a shift to galactose, the VP16-*HXK1* construct did not increase to the normal galactose induced levels nor did the locus relocate to the nuclear envelope. From these results the authors suggest that nuclear pore localization maximizes gene expression. To confirm this possibility *HXK1* was tethered to the nuclear envelope protein Esc1p (discussed above). Once anchored at the periphery, *HXK1* experienced an enhanced repression when supplied glucose, but once shifted to galactose, mRNA levels of *HXK1* doubled beyond their normal galactose induced levels (Taddei *et al.,* 2006).

Still other recent studies have established links between the nuclear pores or nuclear envelope and gene activation. For example, the *INO1* gene is activated upon localization at the nuclear membrane. This upregulation requires the nuclear membrane protein Scs2p (Brickner and Walter, 2004). Also, in a mRNA independent manner, the putative export factor Mex67p and the nuclear pore basket protein Mlp1p are required for positioning active *GAL10* and *HSP104* genes at the nuclear pore (Dieppois *et al.,* 2006). Finally, the *SUC2* locus, which is repressed in glucose and difuse throughout the nucleus, becomes tightly associated with the nuclear rim upon derepression. Proteins necessary for this activation (subunits of Snf1 kinase) are located in the cytoplasm during glucose repression, but move to the perinuclear space upon a switch to derepression conditions (Sarma *et al.,* 2007).

In summary, the most current understanding of yeast molecular biology includes a model where the nuclear pores play important roles in controlling transcription states of many yeast genes. Gene promoters likely interact with several nuclear pore proteins to specifically activate transcription. While transcription can occur in the nuclear interior

and away from the nuclear pores, the normal transcription process may involve the NPC. At minimum, transcription at the nuclear pores would hasten the speed and increase the success of mRNA export. Nuclear exosome decay of nascent mRNAs is a function of time, so from a survival standpoint, rapid mRNA export without a dependency for random diffusion would be more productive. It is therefore possible that promoter sequences of genes have evolved over time to preferentially bind export factors or nucleoporins. The current research also suggests that the loosely associated nuclear pore proteins Nup2p, Mlp1p, and Mlp2p play key roles in modulating chromatin repression states. The Mlp proteins may function as a scaffold system that moves chromatin between active or repressed sub-domains within the nuclear periphery. These proteins are likely key for establishing boundary activities along chromatin so that a specific gene is upregulated but adjacent genes remain repressed (Akhtar and Gasser; 2007; Taddei, 2007; Brown and Silver, 2007).

Recently, a novel mutation and selection technique called global transcriptional machinery engineering (gTME) has generated yeast mutants that experience a dramatic improvement to both ethanol and glucose tolerance. This screen isolated a triple amino acid mutation to gene *SPT15*, which codes for the Tata Binding Protein (TBP). This mutation caused a change in expression patterns for hundreds of genes with the majority being upregulated. Data suggested that the combined upregulation of numerous genes was required for the enhanced tolerances that would be highly desirable for bio-energy production (Alper *et al.*, 2006). Though not addressed in the study, the mass upregulation of hundreds of genes caused by mutation to an individual transcription factor, *SPT15*, is strikingly similar to upregulation expected by tethering the transcription machinery to the nuclear pores. It will be interesting to learn whether the TBP mutation involves the NPC. If it does not, then tethering the enhanced TBP protein or important plasmids to the NPC could further enhance tolerances or production yields of commercial yeasts.

Conclusion

A growing body of evidence demonstrates that transcription, splicing, and pre-mRNA processing (capping, polyadenylation, and mRNP packaging) occur, practically speaking, simultaneously just prior to export. As a result, each step of nuclear expression influences and regulates another. At the same time, a complex of nucleases, called the exosome, degrade transcripts that are left unprotected i.e. without a cap or tail. Indeed, the combined effort of capping and splicing and export factors surrounding a transcript under construction likely protects the nascent transcript from the exosome (Hicks *et al.*, 2006). Delays to mRNA processing or export can result in nuclear retention and decay by the exosome (Hilleren *et al.*, 2001). The impact of splicing and possible nuclear retention of unspliced mRNAs is discussed in Chapter Five. With an exosome acting to continually degrade unprotected transcripts and potentially attack protected transcripts, transcription and mRNP creation is a race against time. Rapid cotranscriptional processing at the nuclear pore serves to minimize wasted cellular resources and energy by efficiently exporting transcripts to the cytoplasm prior to exosome decay (Brown and Silver, 2007; Das *et al.*, 2003).

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Chapter Two

A subset of Mer1p-dependent introns requires Bud13p for splicing activation and nuclear retention

Contribution of Authorship

This chapter was previously published in *RNA* 12:1361-1372. All experiments, figures, figure legends, and Material and Methods selection were conducted or created by Fred Scherrer. Using arguments presented in Fred Scherrer's dissertation proposal, Marc Spingola wrote the Introduction, Results, and Discussion sections of the journal article. Fred Scherrer contributed to editing of the Introduction, Results, and Discussion sections.

Introduction

Precursor messenger RNAs (pre-mRNAs) produced by RNA polymerase II in the yeast *Saccharomyces cerevisiae* undergo several processing events before they are exported to the cytoplasm for translation. One of these processing events, splicing, removes intervening sequences, or introns, from pre-mRNAs to produce mature mRNAs that have uninterrupted translational reading frames. Splicing occurs by two sequential transesterification reactions and utilizes three conserved intronic elements: the 5' splice site sequence, the branchpoint sequence, and the 3' splice site sequence. The reactions are catalyzed by the spliceosome, a macromolecular complex consisting of five small nuclear ribonucleoprotein particles (snRNPs) and several accessory proteins (reviewed in (Staley & Guthrie, 1998; Brow, 2002; Jurica & Moore, 2003; Butcher & Brow, 2005)). In yeast, the splicing process is initiated when the U1 snRNP binds to the 5' splice site region of a pre-mRNA to form a commitment complex (CC) (Seraphin & Rosbash, 1989). This complex is stabilized by base pairing between U1 snRNA and the 5' splice site sequence (Seraphin et al., 1988; Siliciano & Guthrie, 1988) and by several U1 snRNP protein-mRNA interactions (Puig et al., 1999; Zhang & Rosbash, 1999). After the CC has formed, the U2 snRNP binds, and base pairs form between U2 snRNA and the branchpoint sequence of the intron (Parker et al., 1987; Wu & Manley, 1989). The remaining U4, U5, U6 snRNPs bind as a tri-snRNP to the above pre-spliceosome (Cheng & Abelson, 1987), and several conformational changes ensue which lead to the displacement of the U1 and U4 snRNPs and formation of a catalytically active spliceosome (reviewed in (Staley & Guthrie, 1998; Brow, 2002; Butcher & Brow, 2005)).

The above accretion model for spliceosome assembly is based on numerous in vitro studies and was called into question with the isolation of a functional "penta-snRNP" spliceosome holoenzyme from cells (Stevens et al., 2002). However, recent studies measuring spliceosome assembly in vivo support the accretion model (Gornemann et al., 2005; Lacadie & Rosbash, 2005). Regardless of whether the spliceosome binds to pre-mRNA sequentially as individual snRNPs or simultaneously as a holoenzyme, significant RNA and snRNP rearrangements must occur prior to and during both chemical reactions. For example, U6, which also forms base pairs with the 5' splice site (Kandels-Lewis & Seraphin, 1993), cannot pair with the 5' splice site until U1 has been displaced by Prp28p

(Staley & Guthrie, 1999), and U4 must unwind from U6 before U6 can form base pairs with U2 to form the catalytic core of the spliceosome (Hausner et al., 1990; Raghunathan & Guthrie, 1998). This dynamic nature of spliceosome assembly provides ample opportunities for splicing regulators to affect the formation of the spliceosome and alter selection of splice sites.

Mer1p is expressed only during meiosis (Engebrecht et al., 1991), and its expression corresponds to increases in splicing of at least three genes required for meiosis: *MER2/REC107*, *MER3/HFM1*, and *AMA1/SPO70* (Engebrecht et al., 1991; Nakagawa & Ogawa, 1999; Davis et al., 2000). Although evidence suggests that Mer1p activates splicing by affecting the formation or stability of the earliest splicing complexes on pre-mRNAs that contain the Mer1p intronic enhancer element (Nandabalan et al., 1993; Nandabalan & Roeder, 1995; Spingola & Ares, 2000; Spingola et al., 2004), an in vitro demonstration of Mer1p's effects on spliceosome assembly and splicing has been elusive. An alternative hypothesis for Mer1p's function is that its major role is to prevent unspliced enhancer-containing pre-mRNAs from escaping the nucleus or from being degraded before being spliced in the nucleus. Indeed several retention factors have been described and fall into two categories: retention factors that modulate spliceosome activity and retention factors that do not modulate spliceosome activity. The latter category includes Pml1p, a component of the RES complex (Dziembowski et al., 2004), and M1p1p and M1p2p, which line the nuclear pore complex (NPC), prevent pre-mRNAs from exporting to the cytoplasm, downregulate transcription, and may link the NPC to euchromatin (Galy et al., 2004; Casolari et al., 2005; Vinciguerra et al., 2005). While loss of Mlp1p or Pml1p has no effect on splicing, their loss is accompanied by the export and translation of unspliced pre-mRNAs (Dziembowski et al., 2004; Galy et al., 2004). In contrast, the loss of several accessory splicing factors and early-acting snRNP proteins has been shown to have small to moderate reductions on splicing but large increases in the export and translation of unspliced pre-mRNAs. Chief among these proteins are Branchpoint Binding Protein (Bbp1p), and Mud2p, two non-snRNP accessory factors that bind to the commitment complex (Rain & Legrain, 1997; Rutz & Seraphin, 2000). It has been proposed that the essential role of Bbp1p is nuclear retention and not splicing because extracts depleted of Bbp1p (the homolog of mammalian SF1) show no splicing defects with a model pre-mRNA in vitro, temperature-sensitive *bbp1* alleles allow pre-mRNAs to export to the cytoplasm and be translated while only showing a slight reduction in splicing with sensitive splicing reporters that have non-consensus splicing signals, and a *bbp1* allele is synthetic lethal with the deletion of a nonsense-mediated decay gene, *UPF1* (Rutz & Seraphin, 1999, 2000).

Our analysis of pre-mRNA export to the cytoplasm indicates that Mer1p, like many splicing factors that act early during the splicing process (Legrain & Rosbash, 1989), can help retain unspliced pre-mRNA in the nucleus, but that this retention effect cannot be separated or uncoupled from splicing. At a minimum, retention by Mer1p requires a functional 5' splice site, the Mer1p intronic enhancer element, the U1 snRNP protein Nam8p, and the domains of Mer1p that interact with the U1 snRNP and enhancer. Furthermore, *AMA1* pre-mRNA is readily exported to the cytoplasm if Mer1p is not present to activate its splicing, and unlike the *MER2* and *MER3* unspliced pre-mRNAs

that leak to cytoplasm, the *AMA1* pre-mRNA that is exported to the cytoplasm is not degraded by the Nonsense-Mediated Decay process.

Recently a trimeric complex involved in nuclear retention and splicing, the RES complex, was purified from yeast (Dziembowski et al., 2004). Two of its components have been described as splicing and retention factors, Snu17p/Ist3p and Bud13p, and one as a retention factor that does not affect splicing, Pml1p. Snu17p is also a subunit of the U2 snRNP (Wang & Rymond, 2003; Wang et al., 2005). Since Snu17p is required for Mer1p function (Spingola et al., 2004), we tested if the remaining two subunits of the RES complex were critical for Mer1p function. The results show that loss of Bud13p abolishes Mer1p-activated splicing of *AMA1*, impairs Mer1p-activated splicing of *MER2*, and has no effect on Mer1p-activated splicing of *MER3*. Loss of Pml1p had little effect on Mer1p-activated splicing. These results suggest that one function of the RES complex may be to regulate differential splicing during meiosis by modulating Mer1p's activity on specific transcripts. Furthermore, our data support the model that Mer1p activates splicing by stabilizing or promoting the formation of early splicing complexes on pre-mRNA. Our data also support the model proposing that unspliced pre-mRNAs in yeast that are poorly spliced and do not efficiently assemble into spliceosomes are exported to the cytoplasm and not degraded in the nucleus (Hilleren & Parker, 2003).

Methods

Plasmids and Yeast Strains

The construction of many of the plasmids and strains used for splicing analysis in this study were described before (Spingola & Ares, 2000; Spingola et al., 2004). Strains *KH46, BY4741,* or gene deletions in *BY4741* (Invitrogen) were used for isolating RNA. *KH46* is *cup1Δ* and was used for copper sensitivity assays. Strains *AAY334* and *AAY335* (Kebaara et al., 2003) were utilized for the mRNA transcription shutoff experiments and have the genotypes *MATa ura3-Δ-his3-11,15 trp1-1 leu2-3, 112 rpb1-1* and *MATa ura3-Δ-his3-11,15 trp1-1 leu2-3, 112 rpb1-1 upf1-Δ2 (URA3)* respectively. The nonessential splicing gene deletion strains *bud13Δ* and *pml1Δ*, were purchased from Invitrogen, Inc. and are derived from strain *BY4741* (*MAT a his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*). A *bud13Δ::HIS4* strain was produced in the *KH46* background (*cup1Δ*) by the PCR product integration method (Longtine et al., 1998). To distinguish this strain from the *bud13Δ* strain in the *BY4741* background, it is referred to as *KH46-bud13Δ.*

The Splicing Reporter *CUP1* fusion plasmids, pRS316AMA1-CUP1, pRS316MER2-CUP1, and pRS316MER3-CUP1 were described previously (Spingola & Ares, 2000; Spingola et al., 2004). The *AMA1-CUP1* export reporter, pRS316CF7B, was constructed from pRS316AMA1-CUP1 using oligonucleotide site-directed mutagenesis (Kunkel et al., 1991). The synthetic oligonucleotide 5' TTTTCTGGTATA-CGCTTATTTTTTCATTATGAAAAA 3' deletes the G (the – in the sequence above) from the in-frame stop codon in the intron. In addition to deleting the intronic stop codon, the translational frame was altered to ensure that only unspliced mRNA would be in the correct frame for production of reporter protein. Using the mutagenic oligonucleotide 5'TACTAACAAATATTTTCTACAGGGTATTTCTCTGAA, a single

nucleotide was inserted (underlined above) at the beginning of the second exon, which disrupts the reading frame for spliced RNA and creates the correct frame for translation of unspliced RNA. The export reporter pRS316CF7B-G1A was created from pRS316CF7B by making a G to A substitution at the first nucleotide of the 5' splice site using site-directed mutagenesis. Plasmid R1070 (constitutive *MER1* expression) and its parental vector R1130 were gifts from G.S. Roeder and are described in (Engebrecht et al., 1991). Plasmids pGHAMER1 (HA-tagged *MER1*), pGAD (*MER1* splicing Activation Domain) and pGKH (*MER1* RNA-binding KH Domain) were derived from pGAC14 as previously described (Spingola et al., 2004). Plasmid pGAQE was derived by subcloning the constitutive G3PD promoter and *MER1* open reading frame fragment from pGHAMER1 into pRS426 and subsequently performing site-directed mutagenesis to alter the signature KH element GXXG (Siomi et al., 1993) from GAKG to GAQE. Plasmid pGMER1ΔC lacks the carboxy-most terminal peptide (22 residues) adjacent to the KH domain and was constructed by PCR amplifying the gene with primers that amplify all but the last 22 codons of *MER1*. The export reporter plasmid with a nonfunctional *MER1* enhancer element was constructed by oligonucleotide mutagenesis of pRS316CF7b and alters the ATACCCTT enhancer element to CATGGCTT.

The *MER2* export reporter was constructed by oligonucleotide mutagenesis of the *MER2* splicing reporter and removes an intronic stop codon. Using the oligo 5' CATTTACTAACAACTGTAGTACAGgGAAACGTGAAAACCTTAATAAAGG 3' an in-frame stop codon (at the 3' splice site sequence) was altered from TAG to CAG and an additional G nucleotide was inserted in exon two (lower case g) to make the pre-mRNA reading frame open and spliced mRNA out of frame for *CUP1* translation.

The *MER3* export reporter plasmid was created by mutagenesis of the *MER3* splicing reporter using oligos 5' CCAAATAGTAGTAACGAAGCTT**CAACACCCTTATCAGTTTACACC, where ** represents the deletion of AG, and 5' GGTTTTTCTGGAC#AGAATTTCAGAGGACTTACAGAaTATTGACTTTAACG where # represents the deletion of a T. Additionally, the 3' splice site (an in-frame stop) was altered from TAG to CAG, and an A (lower case) was inserted into exon two to make the unspliced reading frame open and spliced mRNA in an incorrect frame for production of Cup1p.

LacZ export and splicing reporters were produced by amplifying the *LacZ* gene from a plasmid by PCR with Vent DNA polymerase and primers containing Kpn I sites at the ends. After digestion with Kpn I, the *LacZ* PCR product was ligated into the *AMA1-CUP1* reporters in which the *CUP1* fragment had been removed by Kpn I digestion.

RNA, splicing assays, and export assays

5 ml overnight cultures were centrifuged, the pellets were washed once with 1 ml H_2O and resuspended in 150 μl of LET (25mM Tris pH 8.0, 100mM LiCl, 20mM EDTA) and 150 μl phenol equilibrated with LET. Glass beads (Sigma) were added and vortexed vigorously for 5 mins. After addition of 250 μl H_2O and 250 μl phenol/$CHCl_3$ (1:1), tubes were again vortexed vigorously. Following centrifugation, the aqueous phase was

transferred to a new tube containing 450 μl phenol heated to 65°C and repeatedly vortexed and incubated at 65°C for five one-minute intervals. The aqueous phase was re-extracted with 450 μl phenol/$CHCl_3$ and 250 μl H_2O. The aqueous phase was extracted a final time with 450 μl $CHCl_3$ and ETOH precipitated.

Primer extension analysis was described before (Spingola et al., 2004) and performed on no fewer than two independent transformants. Primer extension products representing spliced and unspliced RNAs were quantified by phosphorimaging. The formula (*S/(S + U) X 100),* where *S* is spliced product and *U* is unspliced pre-mRNA, was used to calculate splicing efficiency. Primers were designed to anneal to the second exon.

Splicing and mRNA export were also assessed by growth of yeast containing *CUP1* fusion plasmids and various *MER1* or control vectors by streaking transformants on selective media containing 150 μm cupric sulfate and incubating at 30°C for 3–5 days. Alternatively, four microliters and four microliters of a ten-fold dilution of cultures grown in selective media to an optical density of 1.0 at 600 nm were spotted on plates containing 50-800 μm cupric sulfate.

β-galactosidase assays for the *lacZ* reporters were performed in duplicate on at least two independent transformants. Cells were grown in selective media to an optical density of 1.0 at 600 nm prior to assaying and harvested prior to reaching stationary phase. 1 ml of cells was centrifuged, and the pellets were resuspended in 8OO μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). 15 μl of 0.1% SDS and 30 μl of $CHCl_3$ were added to each sample, which was then vortexed vigorously for 3 minutes. 200 μl ONPG (o-nitrophenyl-β-D-galactopyranoside) was then added to each sample, and the samples were quickly mixed and incubated at 37° C for 5 min. The reactions were stopped by adding 500 μl of 1.0 M Na_2CO_3 and centrifuged briefly to pellet the cell debris and separate the chloroform from the aqueous supernatant. The optical density of the supernatant was measured at 420 nm.

RNA polymerase II inactivation and RNA stability assays

Transcription arrest assays were performed as in (Parker et al., 1991) following incubation at the restrictive temperature with the exceptions that RNA was isolated as above and analyzed by primer extension to more clearly distinguish between spliced and unspliced forms. Decay rate constants (*k*) were calculated curve fitting using an exponential function, and half-life values were calculated by ln2/*k*.

Results

The Bud13p subunit of the RES complex has transcript-specific effects on Mer1p-activated splicing

Snu17p is required for Mer1p-activated splicing of *AMA1* (Spingola et al., 2004) and is a subunit of the U2 snRNP (Wang et al., 2005) and the RES complex, which also includes Bud13p, and Pml1p (Dziembowski et al., 2004). Bud13p and Snu17p are thought to be splicing factors while Pml1p has been characterized as a retention factor. Since Bud13p and Pml1p form a complex with a protein that is required for Mer1p-activated splicing,

we determined if these subunits of the RES complex are also important for Mer1p function. Strains deleted of either of the two remaining genes were obtained and transformed with a *MER1* expression plasmid and splicing reporter plasmids. RNA was isolated from these cells and analyzed for Mer1p-activated splicing by primer extension. The results (Figure 1) indicate that Bud13p, like Snu17p, is critical for Mer1p-activated splicing of *AMA1* reporter pre-mRNA. Furthermore, loss of Bud13p causes a reduction in the constitutive splicing that occurs without Mer1p for *AMA1* mRNA (from 31% spliced to 14% spliced, Figure 1 and Table 1). The third component of the RES complex, Pml1p, is not required for Mer1p to activate *AMA1* splicing, but its loss slightly reduced the *AMA1* splicing levels.

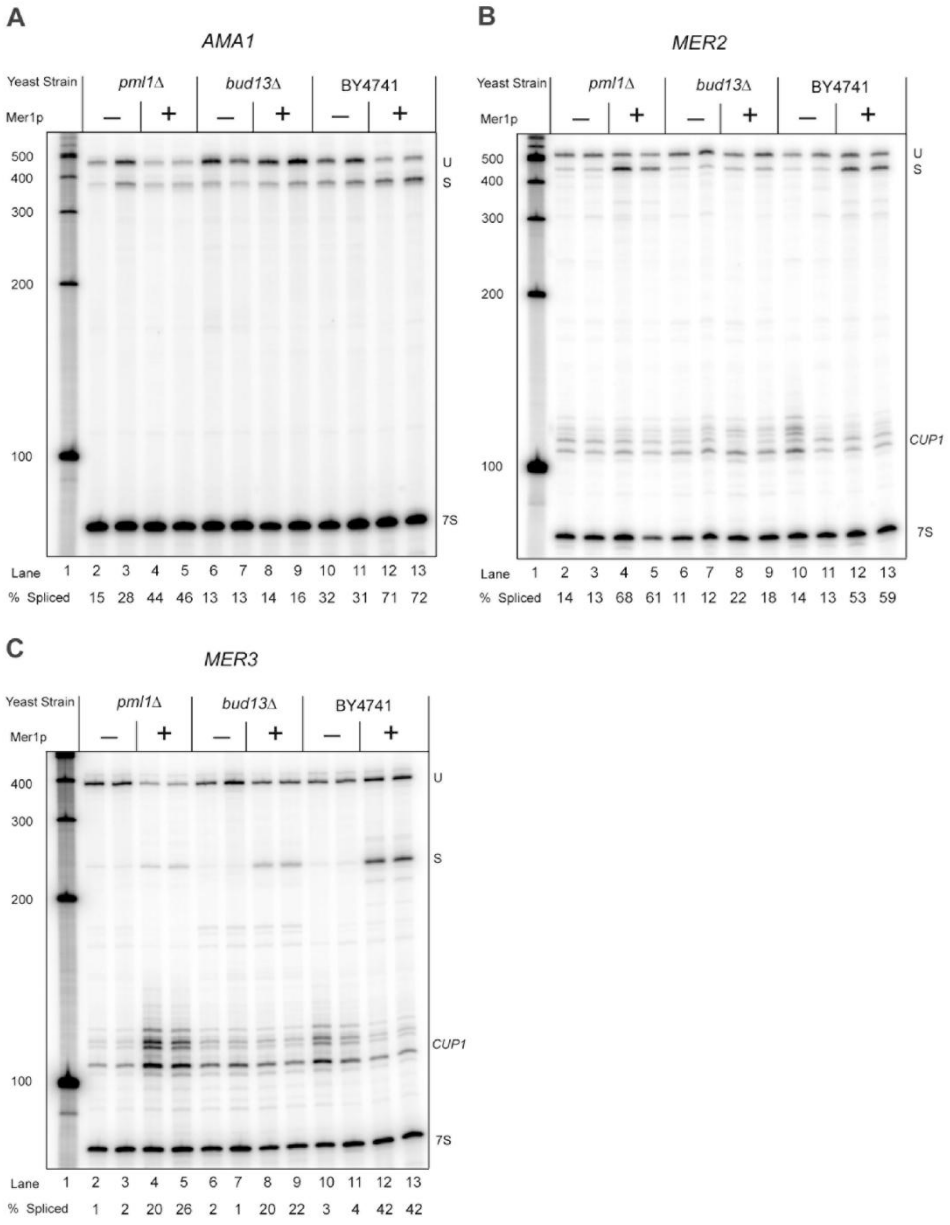

Figure 1. Primer extension analysis of *AMA1*, *MER2*, and *MER3* splicing in wild-type or RES deletion strains with or without constitutive expression of *MER1*. U represents cDNAs from unspliced RNA; S represents cDNA from spliced mRNA. In panel A, a primer complementary to *AMA1* exon 2 was used. In panels B and C, a *CUP1* primer is used, which also primes reverse transcription on endogenous *CUP1* RNA. Splicing efficiencies are reported below each lane using the formula S/(S+U)*100. Primer extension of a polymerase III transcript, 7S RNA, was performed as a loading control.

We extended our splicing studies with the RES deletion strains to *MER2* and *MER3* splicing reporters and surprisingly found dramatically different requirements for each pre-mRNA. Mer1p could not activate splicing of *MER2* to the wild-type level when Bud13p is deleted. When Bud13p is present, there is approximately a four-fold activation of splicing by Mer1p, but only a two-fold activation when Bud13p is deleted. In contrast, loss of Bud13p did not at all hinder the ability of Mer1p to activate the splicing of *MER3* (see Figure 1 and Table 1). Thus, Bud13p is essential for the Mer1p-activated splicing of *AMA1*, helpful but not essential for Mer1p-activated splicing of *MER2*, and not necessary or helpful for the Mer1p-activated splicing of *MER3*. We conclude that Bud13p modulates the activity of Mer1p on certain transcripts.

Table 1. Splicing efficiencies for Mer1p-dependent introns in RES deletion strains

	RNA spliced (percent)		
Strain	*AMA1*	*AMA1* + Mer1p	Splicing activation
BY4741	30.5 +/- 2.9	69.7 +/- 3.3	2.3 fold
bud13Δ	14.2 +/- 1.8	14.9 +/- 1.3	~1
pml1Δ	21.3 +/- 5.5	47.7 +/- 2.6	2.2
snu17Δ *	29.7 +/- 4.0	21.7 +/- 2.7	<1
	MER2	*MER2* + Mer1p	
BY4741	14.0 +/- 1.3	56.6 +/- 3.2	4.0 fold
bud13Δ	12.1 +/- 1.4	21.2 +/- 1.9	1.8
pml1Δ	13.6 +/- 2.0	62.1 +/- 5.9	4.6
snu17Δ *	22.6 +/- 2.3	30.9 +/- 3.0	1.4
	MER3	*MER3* + Mer1p	
BY4741	2.9 +/- 1.0	44.2 +/- 1.8	15.2 fold
bud13Δ	1.9 +/- 0.4	25.4 +/- 3.3	13.3
pml1Δ	1.9 +/- 0.6	25.0 +/- 3.0	13.2
snu17Δ *	4.3 +/- 1.2	27.1 +/- 2.8	6.3

The in vivo splicing efficiencies (percent spliced) and standard deviations for splicing reporter mRNAs with (+ Mer1p) and without constitutive expression of *MER1* are averages of approximately 5-10 primer extension reactions from at least three independent transformants. The splicing activation level (fold increase) is determined by dividing the percent spliced + Mer1p by percent spliced without Mer1p. The *snu17*Δ data (*) are from Spingola et al., 2004.

Mer1p prevents pre-mRNAs containing the intronic splicing enhancer from exporting to the cytoplasm

Several factors first isolated as splicing factors have been shown to prevent pre-mRNA "leakage" to the cytoplasm (Legrain & Rosbash, 1989; Rain & Legrain, 1997; Rutz &

Seraphin, 2000; Dziembowski et al., 2004). Null or conditional alleles of these genes often had minimal affects on splicing but allowed pre-mRNAs to export out of the nucleus into the cytoplasm, suggesting that while these factors may enhance splicing, they have critical roles in retaining pre-mRNAs in the nucleus. Mer1p, with the ability to bind pre-mRNA containing a Mer1p enhancer element (Spingola & Ares, 2000), could potentially block export of pre-mRNAs and retain them in the nucleus for splicing. To address this possibility, we tested if Mer1p affects export of enhancer-containing pre-mRNAs by constructing a reporter gene (*CUP1*) that is fused to a portion of the Mer1p-activatable genes in two different translational frames (see Figure 2). For example the *AMA1-CUP1 splicing* reporter only produces reporter protein, which allows cells to grow in the presence of copper, if the intron is removed by splicing. Unless *MER1* is expressed, the level of spliced reporter mRNA is insufficient to support growth on copper (Spingola & Ares, 2000). The *AMA1-CUP1 export* reporter only produces reporter protein if the intron is *not* removed, and the pre-mRNA is exported to the cytoplasm and translated. Analogous *MER2-CUP1* and *MER3-CUP1* splicing and export reporters were also constructed and tested. When the export reporter plasmids are expressed in *cup1Δ* yeast, cells constitutively expressing *MER1* do not survive on media containing copper (Figure 3A). In contrast, strains that do not express *MER1* grow on media containing copper. These results indicate that (1) unspliced reporter pre-mRNAs are exported to the cytoplasm and translated and (2) that Mer1p inhibits this process, either by facilitating the conversion of pre-mRNA into mRNA or by physically preventing pre-mRNA from exporting to the cytoplasm.

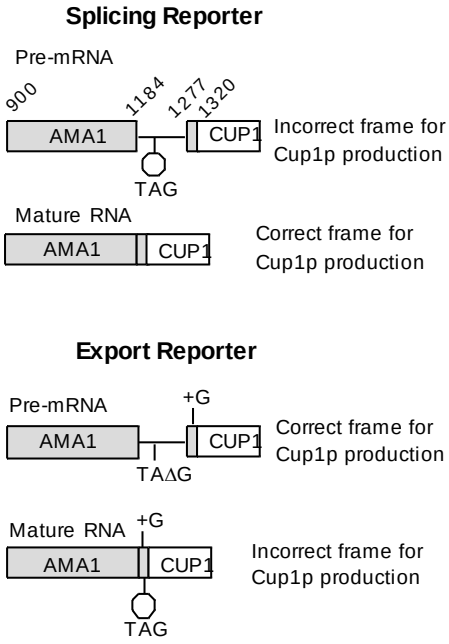

Figure 2. Design of splicing reporter and export reporter plasmids. The numbers indicate the nucleotides of *AMA1* (nt 900-1320) fused to *CUP1* and mark the first nucleotide of the intron (nt 1184) and the first nucleotide of exon 2 (nt 1277). The octagonal stop sign indicates the location of premature stop codons in the constructs. Analogous plasmids were constructed for *MER2* and *MER3* and included the entire exon 1, intron, and approximately 25 nt of exon 2.

Analysis of *AMA1-CUP1* export reporter RNA by primer extension indicates that Mer1p does not affect the abundance of RNA (spliced plus unspliced); neither an increase nor decrease in total reporter RNA is apparent (Figure 3B). Thus it is unlikely that Mer1p causes a down-regulation of transcription of the export reporter that is ultimately responsible for the copper-sensitive phenotype. Rather, an increase in spliced product is measured with a concomitant decrease in unspliced RNA. This suggests that the major reason for a copper-sensitive phenotype when Mer1p is expressed with the export reporter is the conversion of pre-mRNA into mRNA by splicing.

We extended our analysis of splicing and export reporters to *MER2* and *MER3*. The growth patterns on media containing copper largely correlate to their splicing efficiencies. Cells with *MER2* and *MER3* export reporters grew readily on media containing copper, and the amount of growth was reduced by the expression of Mer1p (Figure 3C). Furthermore in the *KH46-bud13Δ* strain, Mer1p did not change the level of growth for either the *AMA1* splicing or export reporter, and Mer1p led to only subtle changes in growth for the *MER2* reporters. In the *KH46-bud13Δ* strain, Mer1p had the same effect on *MER3* as in the wild-type strain: it inhibited growth for cells containing the *MER3* export reporter and stimulated growth for cells containing the splicing reporter. We conclude that Mer1p inhibits the export of unspliced *AMA1*, *MER2*, and *MER3* pre-mRNAs, and that nuclear retention of *AMA1* by Mer1p, like splicing activation, requires Bud13p.

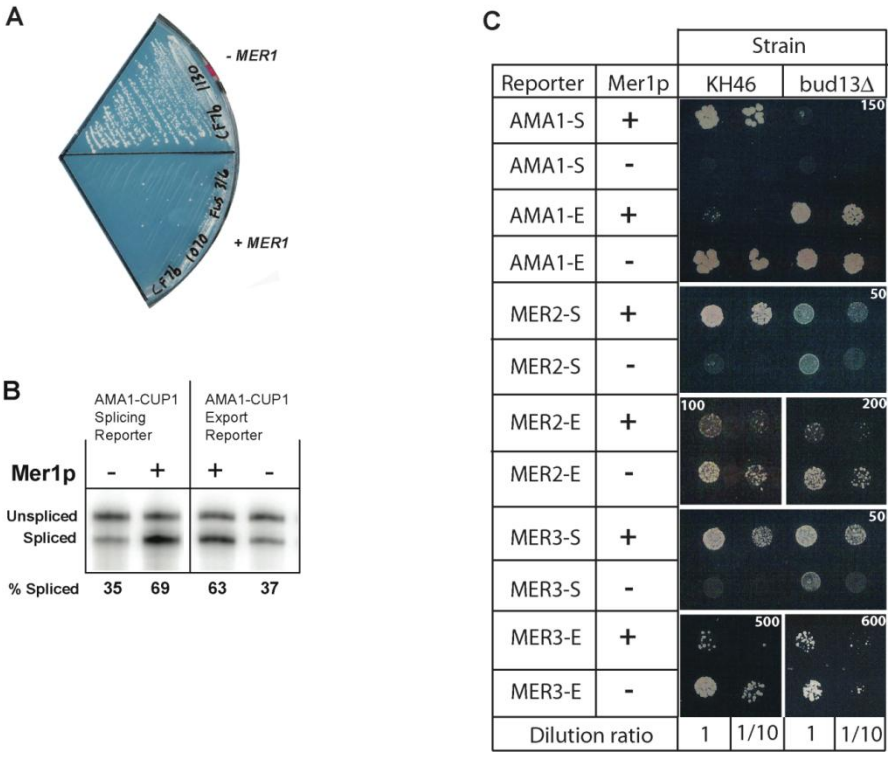

Figure 3. Growth/export assays and splicing assays. (A) Growth of *KH46* yeast (*cup1*Δ) containing the *AMA1–CUP1* export reporter plasmid and either a *MER1*

expression vector or a control vector on media containing 150 µm copper. (B) Primer extension analysis of export and splicing reporter RNAs isolated from *KH46* yeast. Splicing efficiencies are reported below each lane using the formula S/(S+U)*100. (C) Growth at 30°C of *AMA1-*, *MER2-*, and *MER3-CUP1* export (-E) and splicing (-S) reporters on copper in strains *KH46* (wild-type) and *KH46-bud13*Δ with (+) and without (-) *MER1* expression. A variety of copper concentrations was used to best demonstrate differences due to Mer1p. The micromolar concentration of copper is printed on each panel.

Past research has suggested that the primary role for some splicing factors, in particular, Bbp1p, may actually be to retain unspliced pre-mRNA in the nucleus (Rutz & Seraphin, 2000). By measuring the activity of export reporters, unspliced pre-mRNAs are exported to the cytoplasm and translated in strains with temperature-sensitive *bbp1* alleles or deletions of Mud2p, Snu17p, or Bud13p (Rain & Legrain, 1997; Rutz & Seraphin, 2000; Dziembowski et al., 2004). As an additional test to determine if Mer1p might also have a role in retaining pre-mRNAs in the nucleus, we measured β-galactosidase enzyme activity produced from *AMA1* export and splicing reporters that have the *lacZ* gene in place of *CUP1* in a variety of strains including *BY4741* (wild-type), *snu17Δ*, *KH46-bud13Δ*, *pml1Δ*, *mud2Δ*, and *KH46-nam8Δ*. In the wild-type strain, Mer1p has similar effects on both reporters; Mer1p reduces the amount of o-nitrophenol produced by β-galactosidase from the export reporter by about two-fold and increases by about two and half-fold with the splicing reporter (See Figure 4). In *snu17Δ*, *bud13Δ*, and *nam8Δ*, very little β-galactosidase is produced from the splicing reporter, as indicated by the low levels of o-nitrophenol produced, and the levels do not change with *MER1* expression, consistent with the observation that these proteins are needed for Mer1p-activated splicing of *AMA1*. With the export reporter, much more β-galactosidase is produced in *snu17Δ*, *bud13Δ*, and *nam8Δ*, and the levels of o-nitrophenol produced approach that of the wild-type strain. Again, Mer1p has little effect on the amount of β-galactosidase produced from the export reporter in these strains. There is a small difference in the amount β-galactosidase activity in the *snu17Δ* strain when Mer1p is produced. However, the large standard deviations of these samples imply that these differences are not significant, and moreover, this difference is not as large as the differences seen in strains that support Mer1p-activated splicing (*BY4741*, *mud2Δ*, and *pml1Δ*). These results are consistent with the conclusion that *AMA1* pre-mRNAs are best retained in the nucleus by Mer1p only if splicing activation can occur. The loss of Nam8p, Snu17p, or Bud13p, has only a minimal effect, if any, on the basal level of *AMA1* splicing without Mer1p. Consistent with a minimal effect on splicing, the loss of these factors has only a minimal effect on export as well.

The *AMA1* reporter pre-mRNA, which is poorly spliced (~30% is spliced), seems to efficiently leak to the cytoplasm. In the absence of Mer1p, the β-galactosidase activity from the approximately 30% spliced mRNA from the splicing reporter is nearly equal to the signal generated from the approximately 30% unspliced pre-mRNA from the export reporter in the presence of Mer1p; this suggests that most of the unspliced *AMA1* pre-mRNA is leaking to the cytoplasm and being translated. In the *pml1Δ* strain, relative to wild-type, there is a slight reduction in β-galactosidase activity with the splicing reporter and a small increase in activity for the export reporter, consistent with a role for Pml1p in

export and not in splicing. The deletion of Mud2p severely inhibits the activity from the splicing reporter when Mer1p is not produced, but Mer1p restores β-galactosidase levels to about 60% of the wild-type. Previously we have shown that the deletion of Mud2p severely reduces the splicing of *AMA1*, but that Mer1p can activate splicing without Mud2p (Spingola & Ares, 2000). With the export reporter, loss of Mud2p increases the levels of β-galactosidase more than the deletion of the factors required for Mer1p-activated splicing (Snu17p, Bud13p, and Nam8p) and to levels greater than the wild-type. This observation is consistent with Mud2p playing a role in both splicing and nuclear retention.

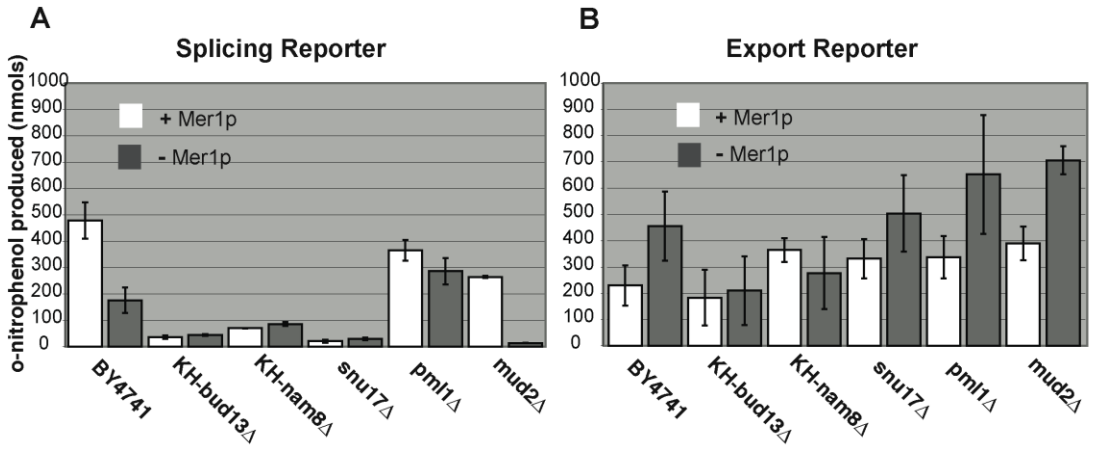

Figure 4. Splicing and pre-mRNA export analysis with the *AMA1-LacZ* reporter plasmids. The nanomoles of o-nitrophenol produced by β-galactosidase cleavage of o-nitrophenyl-β-D-galactopyranoside are averages of duplicates on at least two independent transformants. Strains used include the wild-type (*BY4741*), *KH46-nam8Δ, KH46-bud13Δ, snu17Δ, mud2Δ,* and *pml1Δ*.

Nuclear retention by Mer1p cannot be uncoupled from splicing activation

If Mer1p has a role in retaining pre-mRNAs in the nucleus that is distinct from its role in activating splicing, it may be possible to uncouple the two activities. We attempted this with (1) mutant alleles of *MER1* that cannot activate splicing but still contain the RNA binding KH domain (KH in Figure 5A), (2) with strains of yeast that do not support Mer1p-activated splicing (*nam8Δ*), and (3) with mutant introns that cannot splice because the 5' splice site sequence has been altered from GUACGU to AUACGU (a G1A mutation). In each case, splicing activation could not be uncoupled from nuclear retention, and cells containing the *CUP1* export reporter grew on media containing copper. For example, the KH domain fragment of Mer1p contains the structural motif (Siomi et al., 1993) that binds to RNA, but it does not activate splicing (Spingola & Ares, 2000). If binding to the intronic enhancer were sufficient to elicit nuclear retention, perhaps by preventing export factors from binding, then the KH fragment should cause retention, and the yeast will not grow on copper. However, the KH fragment does not elicit retention, and yeast continue to export and translate the pre-mRNA, which allows growth on media containing copper (Figure 5A). Primer extension analysis verifies that the KH fragment does not activate splicing (Figure 5D, lane 4). Secondly, Mer1p requires a nonessential U1 snRNP protein, Nam8p, to activate splicing (Figure 5D, lanes

8-9). If Mer1p has a role in retention that is independent of splicing, then it should not require a splicing factor for retention. In *nam8Δ* cells, Mer1p does not activate splicing (Spingola & Ares, 2000; and Figure 5D), however cells containing the export reporter grow on copper, indicating that Mer1p also fails to retain pre-mRNA and demonstrating that Nam8p is necessary for this retention effect (Figure 5B). Thirdly, we also tested if a cis-acting mutation to the 5' splice site (G1A) that abolishes splicing would uncouple splicing from nuclear retention. Once again, cells with the export reporter grew on copper, thus Mer1p did not retain the pre-mRNA in the nucleus (Figure 5C). Primer extension results verify that Mer1p does not activate the splicing of the G1A intron (Figure 5D, 10-11).

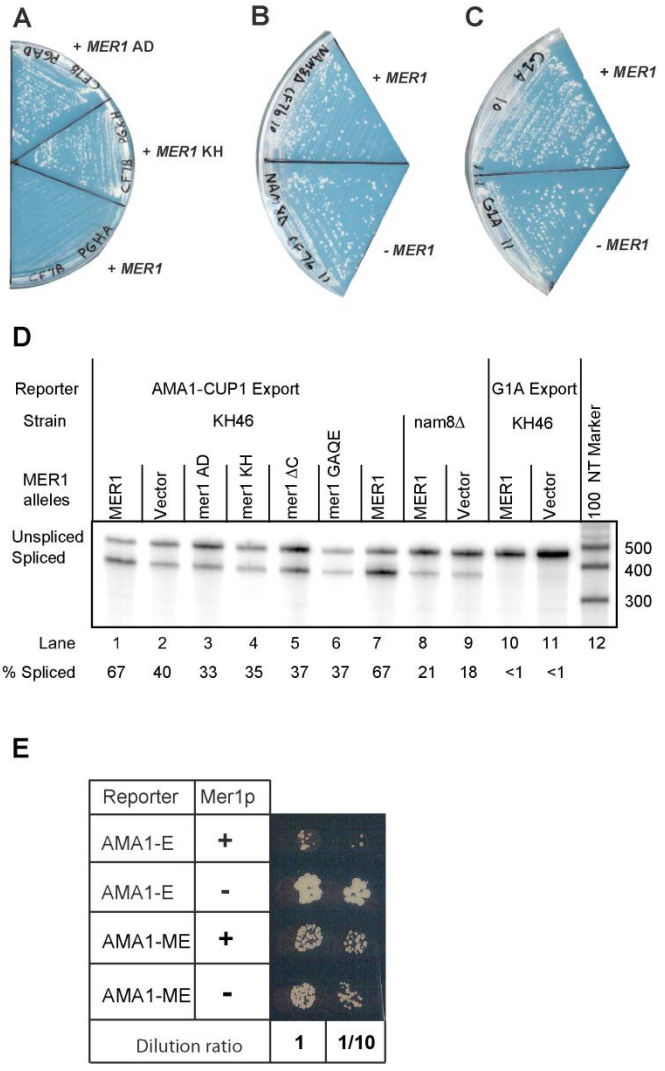

Figure 5. Growth and export assays for (A) yeast containing the *AMA1-CUP1* export reporter and *MER1* alleles that do not activate splicing, including the activation domain (*MER1* AD) or KH domain (*MER1* KH) fragments, (B) *nam8*Δ yeast carrying the *AMA1-CUP1* export reporter, and (C) yeast carrying the *AMA1-CUP1* export reporter with a G1A mutation in the intron, which abolishes its splicing. Media contain 150 µm copper, and cells were grown for 3 days at 30°C. (D) Primer extension assay for splicing of RNAs from cells containing

various export reporters and *MER1* alleles described in the text. Splicing efficiencies are reported below each lane using the formula S/(S+U)*100. (E) Growth of yeast with and without Mer1p on 100 μM copper for strains containing the *AMA1* export reporter or export reporter variant containing mutations to the enhancer element (ME), which abolish Mer1p-activated splicing.

Included in Figure 5D are *AMA1* splicing data for miscellaneous *MER1* alleles that do not activate splicing including the activation domain fragment of *MER1* (AD), a C-terminal deletion of *MER1* (ΔC) that lacks a short peptide adjacent to the KH domain, and an allele (GAQE) with mis-sense mutations in the codons for a highly conserved GXXG peptide element of the KH domain that contacts RNA (Lewis et al., 2000). Cells carrying the above alleles and the export reporter grow readily on copper (Figure 5A, and some not shown), and these alleles cannot facilitate nuclear retention of the *AMA1* export reporter pre-mRNAs. Retention by Mer1p also relies on a functional enhancer element in the pre-mRNA. When the enhancer sequence is altered to one that does not support Mer1p-activated splicing (Figure 5E), Mer1p can no longer retain the pre-mRNA in the nucleus. Nuclear retention of pre-mRNA by Mer1p requires at a minimum a functional 5' splice site, the Mer1p enhancer element, the domains of Mer1p that interact with the U1 snRNP and bind to the enhancer, and an integral component of the U1 snRNP, Nam8p. We conclude that the ability of Mer1p to retain unspliced *AMA1* pre-mRNA in the nucleus is due solely to its ability to activate splicing.

Exported *AMA1* pre-mRNA is not subjected to Nonsense-Mediated Decay

Unspliced pre-mRNAs that are exported to the cytoplasm can be degraded rapidly before ribosomes can initiate multiple rounds of translation on them (Maquat, 2004). This quality control system prevents unspliced pre-mRNAs from being translated into truncated proteins that may be lethal or harmful to the organism, besides being energetically wasteful. Yet *AMA1* export reporter pre-mRNA is exported to the cytoplasm and readily translated in the absence of Mer1p (See Figure 3A). One possible explanation for the translation of unspliced *AMA1* pre-mRNA is that wild-type *AMA1* pre-mRNA is degraded before translation in the cytoplasm by NMD whilst the *AMA1* export reporter is not. In fact, an in-frame premature stop codon had to be deleted from the intron of the export reporter to make its pre-mRNA reading frame open (see Methods). It is possible that by abolishing this stop codon the RNA is rendered resistant to NMD, and thus can be translated. To test this possibility, the half-lives of *AMA1-CUP1* splicing reporter RNA and full length *AMA1* RNA, which both contain the intronic stop codon, were measured in isogenic strains of yeast that differ only by the deletion of the *UPF1* gene (Kebaara et al., 2003), which is critical to NMD. These yeast also contain a temperature-sensitive lesion in a polymerase II subunit (*rpb1-1*) that allows for the inactivation of polymerase II transcription by increasing the temperature to 37° C. RNA was extracted from yeast after shifting to the restrictive temperature and measured by primer extension (Figure 6A). A significant reduction in the half-life of the *AMA1-CUP1* splicing reporter pre-mRNA is not apparent when *UPF1* is deleted. Instead, the RNA has a slightly longer half-life in the presence of Upf1p: 37 mins in the *UPF1* strain and 26 mins in the *upf1Δ* strain (Figure 6B). Since the splicing reporter construct only contains only a small portion of exon 2 of *AMA1*, it was possible that a downstream element important for NMD (Zhang et al., 1995) was not included. A full-length *AMA1* clone

was used to address this possibility, and less than a two-fold difference in pre-mRNA stability was observed when Upf1p was deleted (Figure 6C). The half-life for full-length *AMA1* pre-mRNA was approximately 42 mins in *upf1Δ* cells and 35 mins in *UPF1*. The 35 min $T_{1/2}$ is very close to a previously reported $T_{1/2}$ of 31 mins from a genome-wide microarray measurement of RNA decay (Wang et al., 2002). We conclude that although *AMA1* pre-mRNA contains a premature stop codon in its intron, it is not subjected to NMD.

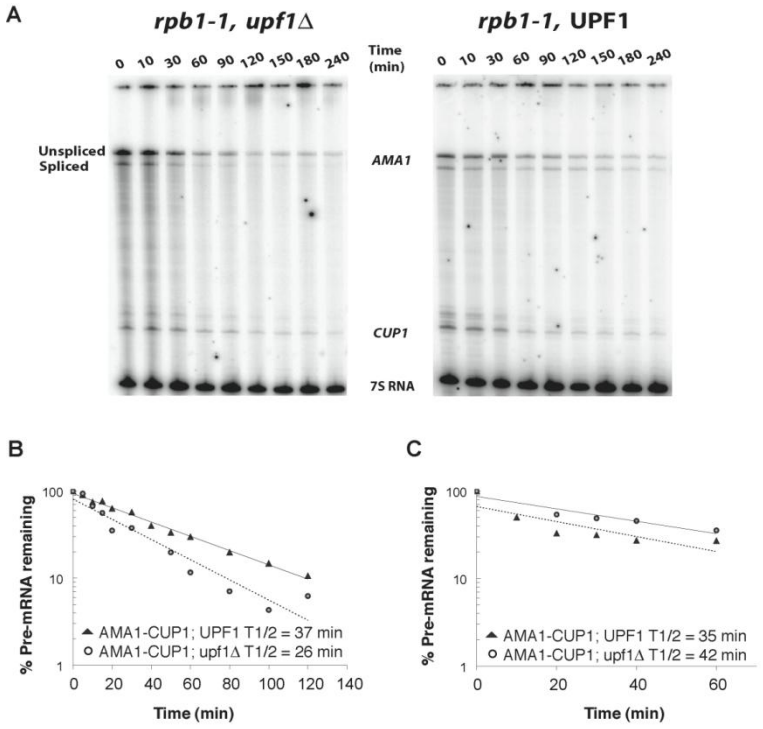

Figure 6. (A) Primer extension assay of pre-mRNA stability following the inactivation of RNA polymerase II in isogenic strains containing *UPF1* or *upf1*Δ. Bands corresponding to unspliced pre-mRNA from the splicing reporter were quantified and normalized to a polymerase III transcript, 7S RNA, and plotted as the percent RNA remaining from time zero in (B) from five separate experiments. (C) A plot for the decay of full-length *AMA1* RNA after inactivation of RNA polymerase II in isogenic strains containing or lacking *UPF1*. The data are averages from two independent trials.

Discussion

Mer1p is a splicing regulator that prevents export of pre-mRNAs by facilitating their splicing

It has been proposed that several factors first characterized as splicing factors, in particular, Bbp1p and Mud2p, may have critical roles in retaining pre-mRNAs in the nucleus that cannot be attributed to their roles in splicing (Rain & Legrain, 1997; Rutz & Seraphin, 2000; Dziembowski et al., 2004). The deletion or inactivation of these genes has little effect on the splicing of various reporters but induces the export of the same reporter pre-mRNA. If Mer1p also has a role in preventing export of enhancer-

containing pre-mRNAs, then the observed increases in splicing of these pre-mRNAs attributed to Mer1p could be the indirect result of nuclear retention. By retaining the pre-mRNA in the nucleus, the pre-mRNAs would have more opportunities to interact with snRNPs, which might account for the increase in splicing when Mer1p is expressed. We addressed whether Mer1p might also have a role in retaining unspliced pre-mRNAs in the nucleus by measuring the effect of Mer1p on splicing reporters and export reporters. We found that unspliced *AMA1* reporter pre-mRNA is exported to the cytoplasm and translated, and that Mer1p reduces the amount of pre-mRNA that is exported and translated. Thus, in the broadest sense Mer1p is a retention factor.

To further support the above claim, we attempted to uncouple splicing from nuclear retention with a mutant allele of *MER1* that does not activate splicing, and with cis- and trans-acting mutants that disrupt splicing. In none of the cases above were we able to uncouple splicing activation from nuclear retention. The KH domain fragment of Mer1p lacks a splicing activation domain but contains the RNA binding domain. Presumably, the KH domain could bind to enhancer-containing pre-mRNA and block the binding of export factors, thus causing retention. Since the KH domain fragment cannot activate splicing but can bind pre-mRNA, it could possibly uncouple retention from splicing activation. However, the KH domain failed to retain unspliced *AMA1* export reporter pre-mRNA. Mer1p-mediated retention also failed to occur in cells missing a nonessential snRNP splicing factor protein, Nam8p, which is required for Mer1p function. Lastly, Mer1p-mediated retention did not occur if the 5' splice site sequence is altered to one that abolishes splicing. Any nuclear retention by Mer1p required a functional 5' splice site, a functional Mer1p intronic splicing enhancer element, the domains of Mer1p that interact with snRNPs and the enhancer, and the U1 snRNP protein Nam8p. Although we attempted only a few means of uncoupling nuclear retention from splicing, and by no means have exhausted a search for alleles that could uncouple splicing from retention, the results strongly suggest that Mer1p's ability to retain pre-mRNA in the nucleus is functionally linked to activating splicing and that Mer1p does not increase splicing indirectly by preventing export of pre-mRNA.

AMA1 pre-mRNA is not a substrate for Nonsense-Mediated Decay

Several quality control mechanisms that degrade aberrantly spliced and unspliced pre-mRNAs have been described that function in the nucleus or in the cytoplasm (Bousquet-Antonelli et al., 2000; Das et al., 2003; Hilleren & Parker, 2003; Conti & Izaurralde, 2005). These quality control systems would seem to be critical in preventing aberrant or unspliced pre-mRNAs from being translated into proteins that are truncated and could have deleterious effects on the organism. Surprisingly, unspliced *AMA1* pre-mRNA seems to evade these quality control mechanisms and is translated. *AMA1* pre-mRNA contains a premature stop codon in its intron, but no differences in its degradation rate were measured when NMD was functional or nonfunctional. In contrast, both *MER2* and *MER3* pre-mRNAs are substrates for NMD (He et al., 1993; He et al., 2003), and when NMD is disabled, their pre-mRNAs accumulate 10-fold and 5-fold respectively (He et al., 2003). Two important requirements for NMD in yeast are the position of the premature stop codon and the presence of an element downstream of the premature stop codon (Hagan et al., 1995). In the *AMA1-CUP1* reporter pre-mRNA and the full length *AMA1*

pre-mRNA the premature stop codons are found at nucleotide 330 in a 612 nt transcript and at nucleotide 1230 in a 2283 nt transcript respectively (positions are relative to the annotated start and stop codons). Hence the first 54% of the *AMA1* pre-mRNA could be translated before the premature stop codon is encountered. In contrast, only the first 38% of *MER2* and 2% of *MER3* pre-mRNAs would be translated before the premature stop codon is encountered (stop codons at nt 396 of 1025 total and at 75 of 3716 total, respectively). With other model transcripts, a premature stop codon found in the last third of the transcript to does not elicit NMD, and the NMD response diminishes as a larger fraction of the transcript is translated prior to encountering the stop codon (Hagan et al., 1995). Although the position of the *AMA1* stop codon is not in carboxy terminal third of the protein coding region, a larger fraction and many more codons of *AMA1* pre-mRNA would be translated before the stop codon is encountered relative to *MER2*, and this may circumvent any NMD. Moreover, *MER2* and *MER3* may have strong NMD downstream elements and *AMA1* may not, although sequence analysis indicates that none of the three genes has a perfect match to the consensus.

Hilleren and Parker have proposed that "the vast majority of pre-mRNAs that are unable to assemble into spliceosomes degrade by the cytoplasmic mRNA turnover enzymes" (Hilleren & Parker, 2003). Our results for *AMA1* pre-mRNA, which is poorly spliced in the absence of Mer1p, support Hilleren and Parker's model that pre-mRNAs that do not assemble into spliceosomes and do not undergo the first step of splicing are exported to the cytoplasm and not degraded in the nucleus. Other pre-mRNAs that undergo regulated splicing are also degraded in the cytoplasm. For example, the splicing of the *RPL30* pre-mRNA can be inhibited when concentrations of Rpl30p are higher than needed for ribosome assembly. The U1 snRNP binds to the *RPL30* pre-mRNA, but U2 snRNP is blocked from binding if Rpl30p is bound to the pre-mRNA (Vilardell & Warner, 1994). Eventually, U1 snRNP dissociates, the Rpl30p-bound pre-mRNA is exported to the cytoplasm, and upon dissociation of Rpl30p the pre-mRNA is subjected to NMD (Vilardell et al., 2000). The remaining Mer1p-regulated pre-mRNAs, *MER2* and *MER3*, suffer a similar fate in the cytoplasm. In the absence of Mer1p their pre-mRNAs are very poorly spliced (Engebrecht et al., 1991; Nakagawa & Ogawa, 1999), and their unspliced pre-mRNAs are exported to the cytoplasm and subjected to NMD (He et al., 1993; He et al., 2003).

A role for the RES complex in Mer1p-regulated splicing and meiosis

Snu17p has been described as a U2 snRNP protein (Wang et al., 2005) that is necessary for Mer1p-activated splicing and as a subunit of the RES complex, which also includes Bud13p and Pml1p (Dziembowski et al., 2004). Bud13p, a splicing factor, is also essential for Mer1p activity on specific transcripts but Pml1p, a retention factor, is not. Although the basis for the requirement of Bud13p and Snu17p in the Mer1p-activated splicing has yet to be determined, our data imply that the RES complex, or two of its subunits, are critical to regulating splicing of a subset of Mer1p-regulated transcripts during meiosis. Based on the observations that loss of Bud13p has the same transcript-specific effects on Mer1p-activated splicing as loss of Snu17p, it is possible that only one of these subunits is needed, but that the loss of the other affects the stability or expression of the required factor. Purifications of the SF3b particle of U2 by the TAP tag method

demonstrate that if the tag is on U2 snRNP protein Cus1p, Snu17p co-purifies with U2 but only a minor trace of a peptide the size of Bud13p co-purifies with U2 (Wang et al., 2005). This suggests that at least a fraction of Snu17p is stable when it is not associated with Bud13p. However, additional experimentation is needed to determine if loss of Snu17p affects the stability of Bud13p or vice versa.

Further experimentation is needed to determine the cis-acting features that make some Mer1p-regulated introns require the Bud13p and Snu17p. Of note, *AMA1* is most obviously different from *MER2* and *MER3* by 5' exon size. *AMA1* has a very large 5' exon (1183 nt from the start codon) whereas *MER2* and *MER3* have much shorter 5' exons (317 nt and 58 nt respectively). Large 5' exons have been shown to reduce splicing efficiency, perhaps by destabilizing interactions between the cap-binding complex (CBC) and the commitment complex (CC) (Lewis et al., 1996a; Lewis et al., 1996b; Spingola & Ares, 2000). A stable CBC-CC interaction may occur with the shorter 5' exon of *MER3*, and to a lesser extent with *MER2*, but not with *AMA1*. We are currently testing if the RES complex may stabilize commitment complexes formed on mRNAs with large 5' exons or whether Bud13p and Snu17p stabilize the binding of U2 to commitment complexes formed on pre-mRNAs with large 5' exons.

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Chapter Three

Mer1p and U1 snRNP Protein Interactions

Introduction

Pre-mRNA splicing is a nuclear event that starts with a stepwise assembly of the 5 snRNPs around a nascent transcript. While ChIP assays indicate that both cotranscriptional and posttranscriptional splicing occurs in yeast, in either case the first round of the assembly process begins with the U1 snRNP binding to the 5' splice site (Tardiff *et al.,* 2006). The yeast 5' splice site is highly conserved and the first six nucleotides of an intron play a vital role by binding directly to the 5' end of the U1 snRNA to promote spliceosome assembly (Seraphin *et al.,* 1988). Later in the splicing reaction these same intron nucleotides form base pairs with the U6 snRNA and help catalyze the first transesterification reaction (Ares and Weiser, 1995). An early study of splicing kinetics concluded that splicing catalysis is not the rate-limiting step of the splicing process. Instead, the 5'splice site recognition and the transition to an assembled spliceosome are the largest hurdles to the splicing reaction (Pikielny and Rosbash, 1985). As a result, this suggests that the proper understanding of early spliceosome assembly remains key to appreciation of the entire splicing process.

The important role of the U1 snRNA in yeast splice site selection was not realized during the first several years of splicing research. Though a metazoan U1 snRNA was identified, no obvious homolog in yeast was initially recognized. Early research proposed that the highly conserved branchpoint sequence, UACUAAC, formed base pairs with the 5'splice site and essentially functioned as the metazoan U1 snRNA (Pikielny *et al.,* 1983). However, in the following years, the proper role of the branchpoint sequence was discovered (i.e. the contribution of a 2' OH group for the initial intron excision) and a yeast RNA, snR19, was recognized as the homolog for the metazoan U1 snRNA. Interestingly, these RNAs are quite different in size, sequence, and probable secondary structure. For example, the yeast U1 snRNA is 568 nucleotides in length, whereas the metazoan snRNA is only 165 nucleotides in length (Seraphin *et al.,* 1988; Kretzner *et al.,* 1990).

For yeast, the consensus 5' splice sequence is GUAPyGU and five of these six nucleotides form Watson and Crick pairing with the U1 snRNA (Parker and Guthrie, 1985; Lesser and Guthrie, 1993). Mutations to this conserved 5' splice site can lead to a block in splicing by preventing U1 snRNA binding and subsequent splicing complex formation. For example, a G5A 5'splice site alteration will inhibit splicing and cause an accumulation of pre-mRNA. Furthermore, this mutation causes an intermediate lariat accumulation and likely interferes with the U6 / 5'splice site binding during the splicing reaction. Extensive analysis of this mutation indicated that this "frozen" or "dead end" lariat was slightly larger than the comparable lariat from wild-type transcript. Though U1 snRNA binds to the mutant splice site, the actual U6 snRNA-mediated cleavage occurs several bases upstream of the mutant splice site. This perturbation was observed in three

different reporter transcripts and indicates that a second recognition of the 5' splice site occurs (by U6 snRNA) after U1 snRNA binding (Parker and Guthrie, 1985; Fouser and Friesen, 1986; Seraphin *et al.*,1988).

Another 5' splice site mutation, G1A, will completely block splicing. *In vitro* splicing reactions demonstrate a delayed and decreased, but persistent, 3' exon-intron lariat accumulation and no spliced product. This suggests that U1 snRNA binding and commitment complex formation are inhibited and while the first transesterification reaction occurs to limited degree, exon ligation does not occur (Newman *et al.*, 1985). Like the G1A 5'splice site mutation, a G1C mutation also completely blocks splicing. However, in this case, splicing gels do not indicate accumulation of the intermediate lariat, which suggests the U1 snRNA binding and subsequent spliceosome assembly is inhibited completely (Fouser and Friesen, 1986). In humans, the 5'splice site is less conserved. Mutations to the consensus GUPuAGU sequence allow the selection of nearby cryptic or alternative 5' splice sites. Utilization of cryptic 5'splice sites in yeast is much less common (Parker and Guthrie, 1985).

Achieving a detailed understanding of splice site selection and the spliceosome assembly pathway was primarily facilitated by the development of a yeast *in vitro* splicing system. Though the first protocols failed to indentify a splicing complex containing the U1 snRNA, it was firmly established that the U2 snRNA bound to the branchpoint sequence and formed a pre-spliceosome complex. Native gel time-course assays demonstrated that pre-spliceosome complexes developed prior to and migrated faster than bands representing mature and active spliceosomes. By utilizing a U2 snRNA-depleted splicing extract, the Rosbash group developed a chase experiment that suggested a splicing complex formed prior to the U2 snRNA pre-spliceosome complex. While visualization of this "commitment complex" could not at first be achieved on native gels, its function was in evidence because splicing complexes quickly reappeared on native gels when active U2 snRNA was added to the splicing reaction. This occurred despite the treatment or chase with excess cold substrate. It was concluded that an initial complex of hot substrate and unknown splicing factors formed in the absence of active U2 snRNA that "committed" the substrate to the splicing reaction. Under normal splicing extract conditions, excess cold substrate prevented visualization of splicing complexes, but commitment complex formation during a pre-incubation period would prevent such competition by cold substrate addition. Additional experiments revealed that while ATP is required for the U2 pre-spliceosome complex formation, it is not required for commitment complex formation. Interestingly, it was also determined that stable commitment complex formation requires an intact branchpoint sequence. This implied that a splicing factor interacts with the branchpoint sequence prior to the U2 snRNA and branchpoint interaction in the pre-spliceosome complex (Legrain *et al.* 1988).

Soon after discovery of the yeast commitment complex, improvements to *in vitro* splicing assays allowed for visualization of this complex and it was determined to contain the U1 snRNA. Splicing extracts devoid of U1 snRNA failed to form either commitment complexes or pre-spliceosome complexes during *in vitro* splicing assays. Yet when the U1 depleted extract was combined with a U2 depleted splicing extract, a pre-spliceosome

complex appeared on native gels (Seraphin and Rosbash, 1989). With this identification of the U1 snRNA-based commitment complex, the various steps to the yeast spliceosome assembly were largely recognized. First U1 snRNA binds to the 5' splice site and forms the commitment complex. Second, addition of the U2 snRNA to the branchpoint sequence creates the pre-spliceosome. Finally, the tri-snRNP (U4, U5 and U6) replaces U1 at the 5' splice site and U4 is displaced as U6 and U2 bind together to create an active assembled spliceosome (see Figure 1) (Staley and Guthrie, 1998; Ares and Weiser, 1995).

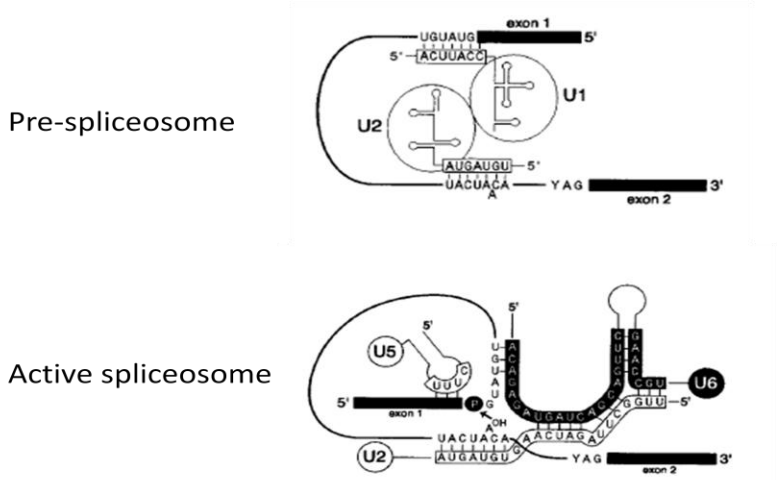

Figure 1. Model of yeast splicing complexes. (Adapted from Nagai *et al.,* 2001).

With many of the basic questions of splicing answered by the early 1990s some of the new efforts in splicing research turned to identification of the numerous proteins that associate with the 5 snRNAs required for splicing. Because the splicing process in yeast and humans turned out to be surprisingly similar, the identification of obvious homologs sped up this process. For example, in both yeast and humans, a core set of 7 Sm proteins forms a doughnut-like complex around the Sm binding site sequence (AUUUUUG) found on all splicing snRNAs except U6. These proteins play a key role in the biogenesis of a snRNA into a functional snRNP (Ares and Weiser, 1995; Yong *et al.* 2004; Stark *et al.* 2001; Kambach *et al.*, 1999).

After transcription by RNA polymerase II, the splicing snRNAs (not including U6) are exported to the cytoplasm. Here, the Sm proteins assemble around a snRNA; the 5' end of the snRNA is trimmed and a trimethyl guanosine cap is then added to this new end. Nuclear import of the snRNP is dependent on the Sm protein core binding to the snRNA. Numerous snRNP-specific proteins also bind to the maturing snRNP either in the cytoplasm or upon return to the nucleus (Mattaj and Roberts, 1985; Jarmolowski and Mattaj, 1993; Jones and Guthrie, 1990; Yong *et al.* 2004). For the U6 snRNA, which does not export to the cytoplasm and is transcribed by RNA pol III, a set of proteins similar to the Sm proteins was recently recognized. Aptly named the Lsm (like Sm) proteins, these 7 proteins, Lsm2-Lsm8, co-precipitate with U6 snRNA and fulfill a vital role since the *LSM2-4* and *LSM8* genes are essential (Salgado-Garrido, 1999).

Though common to the splicing snRNAs, a function for the core Sm proteins besides cap modification and snRNP nuclear importation was difficult to ascertain (Salgado-Garrido, 1999). Yet recently, an additional function for the Sm core was identified within the yeast commitment complex. By creating viable yeast strains containing Sm proteins devoid of their positively charged C-terminal tails, the Rosbash lab demonstrated through pre-mRNA cross-linking studies that the tail-less SmD1, SmD3, and SmB proteins lose their abilities to bind pre-mRNA. Furthermore, it was concluded that this loss of pre-mRNA contact by the core Sm proteins impaired splicing efficiencies and commitment complex stability. Synthetic lethality between Sm mutants and growth rate defects were also documented (Zhang *et al.*, 2001).

In addition to the core Sm proteins, many snRNP-specific proteins have been identified, as well as, accessory splicing factors that act independently of snRNAs. In 1998, it was estimated the spliceosome consisted of more than 50 proteins (Staley and Guthrie, 1998). Today with better protein purification methods and additional research, over 82 unique proteins are believed to contribute to the yeast splicing reaction. Table 1 provides a comprehensive listing of proteins participating in yeast splice site selection and pre-spliceosome formation. Of course, many additional splicing factors and proteins associated with the tri-snRNP (U4, U5, and U6) also comprise the fully assembled spliceosome.

Several of the non-snRNP splicing factors listed in Table 1 play key roles early in the spliceosome assembly pathway. Of particular interest are Mud2p, Sub2p, and BBP. Continued analysis of yeast commitment complex formation revealed that, in fact, two commitment complexes of varying size (CC1 and CC2) could form during *in vitro* splicing reactions in the absence of U2 snRNA. The smaller complex, CC1, required an intact 5' splice site, but not a branchpoint sequence. However, the larger complex, CC2, did require an intact branchpoint sequence in addition to U1 snRNA and the 5' splice site. These distinctions between CC1 and CC2 suggested that an unrealized splicing factor (factor X) bound to the branchpoint prior to U2 snRNA binding (Seraphin and Rosbash, 1991). A synthetic lethal screen with mutant U1 snRNA identified several candidate genes. One candidate, *MUD2*, could be mutated to prevent proper CC2 formation. While Mud2p will not co-precipitate with the U1 snRNP, it will precipitate with U1 snRNP in the presence of pre-mRNA containing an intact branchpoint sequence. Mud2p will also UV cross-link to wild-type pre-mRNA, but not pre-mRNA with a mutant branchpoint. Collectively, these observations pointed to Mud2p as the missing component of CC2 (Abovich *et al.*, 1994).

An additional role for Mud2p during spliceosome assembly was proposed when a two-hybrid interaction between Prp11p, a U2 snRNP component, and Mud2p was discovered along with a synthetic lethal relationship between *PRP11* and *MUD2* (Abovich *et al.*, 1994). Besides identification as the unknown "factor X" of the CC2 *in vitro* complex, Mud2p could serve to attract the U2 snRNA to the pre-mRNA branchpoint sequence via its interaction with Prp11p (Abovich *et al.*, 1994). It was later discovered that mutations to the nucleotide just upstream of the branchpoint sequence impair Mud2p's ability to

stabilize U2 snRNP addition to the CC2 and lead to an increase in pre-mRNA export (Rain and Legrain, 1997).

Table 1. Yeast Pre-Spliceosome Proteins

Yeast Protein	Aliases	Type	Essential	Size (kDa)	Citation	Metazoan Homolog
SmD1		snRNP core	yes	18	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	SmD1
SmD2		snRNP core	yes	15	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	SmD2
SmD3		snRNP core	yes	10	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	SmD3
SmB		snRNP core	yes	28	Gottschalk *et al.*, 1998	SmB/B'
SmE		snRNP core	yes	12	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	SmE
SmF		snRNP core	yes	11	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	SmF
SmG		snRNP core	yes	9	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	SmG
Snp1p		U1 snRNP-specific	some strains	34	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	U1-70K
Mud1p		U1 snRNP-specific	no	37	Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	U1-A
U1-C	Yhc1p	U1 snRNP-specific	yes	31	Tang *et al.*, 1997; Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	U1-C
Prp39p		U1 snRNP-yeast specific	yes	69	Lockhart and Rymond, 1994; Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	none
Prp40p		U1 snRNP-yeast specific	yes	69	Kao and Siliciano, 1996; Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998	none
Snu71p		U1 snRNP-yeast specific	yes	77	Gottschalk *et al.*, 1998	none
Snu56p		U1 snRNP-yeast specific	yes	52	Gottschalk *et al.*, 1998; McLean and Rymond, 1998	none
Nam8p	Mre2p	U1 snRNP-yeast specific	no	57	Ekwall *et al.*, 1992; Gottschalk *et al.*, 1998; Puig *et al.*, 1999	TIA-1
Snu65p	Prp42p	U1 snRNP-yeast specific	yes	57	Gottschalk *et al.*, 1998	none
Luc7p		U1 snRNP-yeast specific	yes	32	Fortes *et al.*, 1999	none
Npl3p		Pre-mRNA binding	yes	55	Gottschalk *et al.*, 1998	none
Cbp20p	Mud13p	Cap binding	no	24	Fortes *et al.*, 1999	CBP20
Cbp80p		Cap binding	no	100	Fortes *et al.*, 1999	CBP80
Snu17p		RES complex	no	17	Gottschalk *et al.*, 2001; Wang and Rymond, 2003; Dziembowski *et al.*, 2004	CGI-79
Bud13p		RES complex	no	34	Dziembowski *et al.*, 2004	MGC13125
Pml1p		RES complex	no	25	Dziembowski *et al.*, 2004	Snip
Mud2p		Accessory Splicing Factor	no	60	Abovich *et al.*, 1994; Rain and Legrain, 1997; Kistler and Guthrie, 2001	U2AF65
Bbp1p	Msl5p	Accessory Splicing Factor	yes	53	Abovich and Rosbash, 1997; Bergland *et al.*, 1997	SF1
Sub2p	yUAP	Accessory Splicing Factor/DExD/H box	yes	50	Zhang and Green, 2001; Kistler and Guthrie, 2001; Libri *et al.*, 2001	UAP56
Prp5p		Accessory Splicing Factor/DExD/H box	yes	96	Ruby *et al.*, 1993; Wells and Ares, 1994; Will *et al.*, 2002	hPrp5p
Lea1p		U2 snRNP	no	27	Caspary *et al.* 1999	U2A'
Msl1p	Yib9p	U2 snRNP	no	13	Tang *et al.*, 1996; Caspary et al. 1999	U2B''
Cus2p		U2 snRNP	no	32	Yan *et al.*, 1998; Perriman and Ares, 2000	Tat-SF1
Prp9p		U2 snRNP SF3a subunit	yes	66	Ruby *et al.*, 1993; Wells and Ares, 1994	SF3a60
Prp11p		U2 snRNP SF3a subunit	yes	29	Ruby *et al.*, 1993; Wells and Ares, 1994	SF3a66
Prp21p		U2 snRNP SF3a subunit	yes	31	Ruby *et al.*, 1993; Wells and Ares, 1994	SF3a120
Rse1p		U2 snRNP SF3b subunit	yes	148	Gottschalk *et al.*,1999; Caspary et al. 1999; Wang and Rymond, 2003	SF3b/SAP130
Hsh49p		U2 snRNP SF3b subunit	yes	28/22	Gottschalk *et al.*,1999; Pauling et al., 2000; Wang and Rymond, 2003	SF3b/SAP49
Cus1p		U2 snRNP SF3b subunit	yes	~66	Pauling *et al.*, 2000; Wang and Rymond, 2003	SF3b/SAP145
Hsh155p		U2 snRNP SF3b subunit	yes	~110	Pauling *et al.*, 2000; Wang and Rymond, 2003	SF3b/SAP155
Rds3p		U2 snRNP SF3b subunit	yes	12	Wang and Rymond, 2003	SF3b14/hRds3p
Ysf3p		U2 snRNP SF3b subunit	yes	10	Dziembowski *et al.*, 2004	SF3b10

While Mud2p is a component of the CC2 complex, it is absent in the pre-spliceosome complex (Rutz and Seraphin, 1999). This observation refined the Mud2p bridging activity proposed earlier by Abovich to now suggest that: 1. Mud2p brings the 5' splice

site and branchpoint sequence together in close proximity by interactions with the U1 snRNP and the branchpoint sequence; 2. Mud2p attracts the U2 snRNA to the branchpoint by interaction with Prp11p; 3. Mud2p disassociates from the rearranging complex as U2 snRNA binds the branchpoint sequence (Abovich *et al.*, 1994; Rutz and Seraphin, 1999).

Because U2 snRNP addition to the commitment complex during the transition to the pre-spliceosome complex requires ATP, the disassociation of Mud2p may be catalyzed by an ATPase DExD/H box protein. One such protein, Prp5p, associates with the U2 snRNP and has been identified as the putative factor responsible for U2 snRNA rearrangement as it binds the branchpoint sequence (Ruby *et al.*, 1993; Wells and Ares, 1994; Dayyeh *et al.*, 2002). Yet a second DExD/H protein, Sub2p, was recently recognized to more likely catalyze the removal of Mud2p. Sub2p is an essential ATPase that is required for *in vivo* and *in vitro* splicing (Kistler and Guthrie, 2001; Zhang and Green, 2001; Libri *et al.*, 2001). Surprisingly, deletion of *MUD2* alleviates the need for the essential *SUB2*. Thus, Sub2p may serve to remove Mud2p while Prp5p acts to rearrange U2 snRNA (Kistler and Guthrie, 2001).

Though the revelation of a second APTase required for yeast pre-spliceosome formation mirrored discoveries in metazoan splicing, some data concerning Sub2p's activities during splicing remain puzzling. In particular, the requirement for this ATPase during CC2 formation is difficult to grasp since this complex forms without ATP (Seraphin and Rosbash, 1989). Yet convincing evidence from three labs in simultaneous publications demonstrates both a Sub2p requirement for CC2 formation and a Sub2p-ATPase-dependent role after U2 snRNP addition (Kistler and Guthrie, 2001; Zhang and Green, 2001; Libri *et al.*, 2001). Ultimately, the exact roles for Sub2p may be tough to delineate because it is a ubiquitous, versatile and important protein. In addition to splicing, Sub2p plays an essential role in nuclear export of both intron and intronless mRNA. As part of the TREX complex, Sub2p loads onto nascent mRNAs during transcription. Then it attracts the export factor Yra1p to the mRNP by binding to it. Finally, Sub2p disassociates from Yra1p and the mRNP and remains in the nucleus as the putative export receptor Mex67p binds to Yra1p and escorts the mRNP to the cytoplasm (Straber and Hurt, 2001; Straber *et al.*, 2002; Lei and Silver, 2002).

Because the CC2 migrates more slowly on native gels than CC1, it is more massive. After identifying the "factor X" of CC2 as Mud2p, the Rosbash group suggested an additional, but unknown, component to CC2 was probable. Several years later, the Branch Point Bridging protein (BBP) was identified as this unknown CC2 component using a genetic screen. This screen revealed synthetic lethality between *MUD2* and *MSL5* (gene coding for BBP). Subsequent analysis showed that BBP and Mud2p, as well as, BBP and Prp40p (a U1 snRNP-specific protein) interact via the yeast two-hybrid system. Additionally, BBP is an essential protein whose conditional depletion will completely prevent CC2 formation. A model was proposed where BBP through its interaction with Mud2p and Prp40p served to bring the branchpoint sequence in close contact with the 5' splice site (Abovich and Rosbash, 1997). Experiments that subjected commitment complexes to UV cross-linking followed by RNase T1 digestion and co-

immunoprecipitation demonstrated that BBP binding to pre-mRNA requires an intact branchpoint sequence. The yeast BBP contains three RNA binding motifs. A KH domain binds directly to the branchpoint sequence, but stable binding also requires the two additional Zn knuckle domains to provide non-specific binding to the pre-mRNA phosphate backbone (Bergland *et al.*, 1997; Bergland *et al.*, 1998).

Like Mud2p, BBP likely acts to stabilize the commitment complex and attract the U2 snRNP. As the U2 snRNP binds to the branchpoint sequence, BBP is displaced from the complex (Rutz and Seraphin, 1999). Thus, at least four similarities between Mud2p and BBP are apparent: 1. both are required for CC2 formation; 2. neither takes part in CC1 formation; 3. Mud2p and BBP interact via the yeast two-hybrid; 4. both are displaced from the nascent spliceosome as the ATP-dependent U2 snRNP addition takes place (Abovich and Rosbash, 1997; Rutz and Seraphin, 1999). Because of these similarities, it was proposed that Sub2p may displace both Mud2p and BBP to create a competent pre-spliceosome complex (Kislter and Guthrie, 2001). See Figure 2 for a graphic summary of initial steps during spliceosome assembly.

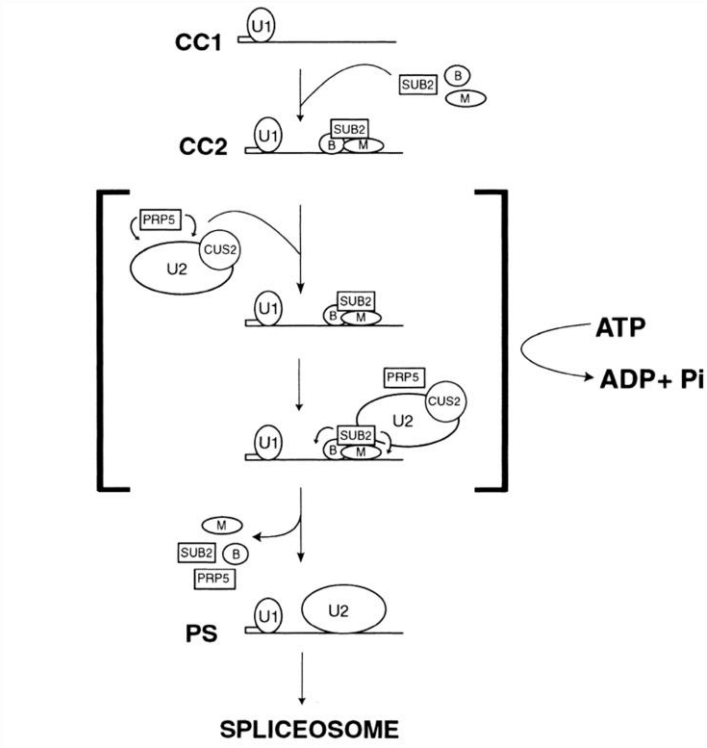

Figure 2. Graphic summary of *in vitro* splicing complexes where circled B is BBP, circled M is Mud2p, PS is pre-spliceosome. (Adapted from Kislter and Guthrie, 2001).

Besides the core Sm proteins and the accessory splicing factors described above, the snRNP-specific proteins of U1 and U2 also play key functional roles during the commitment complex and pre-spliceosome complex assembly process (Wells and Ares, 1994; Mc Lean and Rymond, 1998). Several detailed analyses of these two snRNP's protein components have been completed (Neubauer *et al.*, 1997; Gottschalk *et al.*, 1998;

Wang and Rymond, 2003). Perhaps because the yeast U1 and U2 snRNAs are significantly larger than their human homologues, 3.4 X and 6.3 X respectively, several yeast proteins have no apparent human homologues (see Table 1). Accordingly, some yeast pre-spliceosome proteins are not essential and it was demonstrated that a very large proportion (82%) of the yeast U2 snRNA or (60%) of the yeast U1 snRNA can be deleted without serious defects (Igel and Ares, 1988; Liao *et al.,* 1990; Siliciano *et al.,* 1991).

Exploring Mer1p Function During Pre-Spliceosome Assembly

While convincing evidence suggests Mer1p functions as a splicing factor to stabilize early commitment complex formation on the transcripts *AMA1, MER2,* and *MER3*, the extent of interaction between Mer1p and the U1 snRNP remains uncertain. The KH domain of Mer1p does bind the enhancer sequence on transcripts it regulates, but the function of the Mer1p activation domain has not been firmly established (Spingola and Ares, 2000; Spingola *et al*., 2004). Previous efforts probing activities of this domain were accomplished by performing a two-hybrid screen between Mer1p and many of the proteins participating in the pre-spliceosome complex. Interactions between Mer1p and several proteins were reported, but some interactions were weak and possibly resulted from secondary interactions. The two most significant interactions were Prp11p, a U2 snRNP-specific protein and Snu71p, a U1 snRNP-specific protein. While these interactions certainly support the proposed function of Mer1p as a splicing factor by linking it to the pre-spliceosome complex, this initial screen was not exhaustive (Spingola *et al.,* 2004). To continue with the determination of activation domain activities for Mer1p, I have extended the two-hybrid testing to include the remaining U1 snRNP proteins required for pre-spliceosome formation. A strong two-hybrid interaction occurred between Mer1p and Prp39p. When Mer1p was truncated, the Mer1p activation domain also reacted with Prp39p. These results indicate a modified model for Mer1p function. Furthermore, this two-hybrid screen was also used to probe protein-protein interactions within the yeast U1 snRNP. This data combined with previous documentation of U1 snRNP proteins creates a model for the U1 snRNP structure.

Methods

Plasmids and Yeast Strains

Construction of the bait plasmids containing full-length *MER1* (pBTM-MER1), the *MER1* activation domain (pBTM-MER1AD), and the *MER1* KH domain (pBTM-MER1KH) were described previously (Spingola *et al.,* 2004). These plasmids contain the *TRP1* and *amp*^{r} markers and the *lexA* DNA binding domain located just upstream of a multi-cloning site. The parent prey plasmid (pACT2) contains *LEU2* and *amp*^{r} markers and features a *GAL4* activation domain located just upstream of a multi-cloning site. Full-length yeast splicing factors and various truncations of *PRP39* were PCR amplified from yeast genomic DNA using high fidelity Vent DNA polymerase and ligated into pACT2 using standard techniques. The primers utilized are listed in Table 2. Cloning success was confirmed by restriction digestion. Prey and bait plasmids were transformed in yeast strain L40 (*MATa, his3Δ200, trp1-901, leu2-3, 112, ade2, LYS2*::(*lexA*op)$_4$-*HIS3, URA3::(lexA*op)$_8$-*lacZ, Gal4*). Interaction between prey and bait fusion proteins

brings the *GAL4* activation domain in close proximity to the *lexA* operator sites and genes coding for histidine or β-galactosidase are transcribed.

Table 2. Primers used for yeast two-hybrid constructs

Yeast protein	Primer upstream (sense)	Restriction site	Primer downstream (anti-sense)	Restriction site
Sm G	CGGGATCCGA**ATG**GTTTCTACCCCTGAACTGAAG	BamHI	GCGCTCGAG**TTA**TATGGCATCTAGAGCCTCTAG	Xho I
Sm F	CGGGATCCGA**ATG**AGCGAGAGCAGTGATATCAG	BamHI	GCGCTCGAG**TTA**GTTCGGCAGCTCCCTGATGT	Xho I
Sm E	CGGGATCCGA**ATG**TCGAACAAAGTTAAAACCAAGG	BamHI	GCGCTCGAG**TCA**GTCCGCTGATGTTATCAATGT	Xho I
Sm D1	CGGGATCCGA**ATG**AAGTTGGTTAACTTTTTAAAAAAGC	BamHI	GCGCTCGAG**TCA**TAGACCTCTTCTTGGCCTTTTA	Xho I
Sm D2	CGGGATCCGA**ATG**TCGTATGTTTGATCTTAACCATT	BamHI	GCGCTCGAG**TTA**CTCAACAGGGGTTTTTAACACA	Xho I
Sm D3	CGGGATCCGA**ATG**ACTATGAATGGAATACCAGTGA	BamHI	GCGCTCGAG**TCA**CCTTCTCTTAGGTCCTCTTATT	Xho I
Sm B	CGGGATCCGA**ATG**AGCAAAATACAGGTGGCACATA	BamHI	GCGCTCGAG**TTA**TTTTCTTTTAAAACCTGGTGGGG	Xho I
Snp1	CGCCCGGGA**ATG**AATTATAATCTATCCAAGTATCCA	Sma I	GCCGAGCTC**TCA**ATAGTCGGGCGCTTCTTTGG	Sac I
Mud1	CGGGATCCGA**ATG**TCAGCGTATGTATATACCTTGT	BamHI	GCGCTCGAG**CTA**CTTAGCAAATCCTATGGTAACG	Xho I
Snu65	CGCC**ATG**GATAAATATACTGCTTTGATTCACG	Nco I	GCCGAGCTC**CTA**AGGTTCTTCAGTAAAC	Sac I
Snu65 (Bait)	TACGGATCCGT**ATG**GATAAATATACTGCTTTGATTCACG	BamHI	TCCGTCGAC**CTA**AGGTTCTTCAGTAAAC	Sal1
Prp39	CGCCCGGGA**ATG**CCAGATGAAACAAATTTTACAATA	Sma I	GCGCTCGAG**TCA**TTTACCTTCCTTAAGAAATTCTTC	Xho I
Prp40	CGGGATCCGA**ATG**TCTATTTGGAAGGAAGC	BamHI	GCGCTCGAG**TCA**ATAHTCCAATTCCAC	Xho I
Cbp20	CGGGATCCGA**ATG**TCCCTGGAAGAATTTGACGAA	BamHI	GCGCTCGAG**CTA**CTGAGGTACGTAGTTATCATCT	Xho I
Bud13	CGCC**ATG**GCATTGCATCAGTATTTATCAG	Nco I	GCCGAGCTC**TCA**ATAATCCTCCTGTAGGGTGTA	Sac I
Pml1	CGGGATCCGA**ATG**TTTCACAGACGCAAAAGACCTT	BamHI	GCGCTCGAG**TTA**TACATTCATGAAGATGAGTTCGT	Xho I
Prp39 (1-50 aa)	CGCCCGGGA**ATG**CCAGATGAAACAAATTTTACAATA	Sma I	GCGCTCGAG**TTA**TTGGGTCAACGAAGAAATATCTGACC	Xho I
Prp39 (1-504 aa)	CGCCCGGGA**ATG**CCAGATGAAACAAATTTTACAATA	Sma I	GCGCTCGAG**TCA**TACTGTAGTCGGCAAGTATATTTCTAC	Xho I
Prp39 (51-504 aa)	CGCCCGGGA**ATG**GTAGATGTTATAGAGCAAACAG	Sma I	GCGCTCGAG**TCA**TACTGTAGTCGGCAAGTATATTTCTAC	Xho I
Prp39 (51-629 aa)	CGCCCGGGA**ATG**GTAGATGTTATAGAGCAAACAG	Sma I	GCGCTCGAG**TCA**TTTACCTTCCTTAAGAAATTCTTC	Xho I
Prp39 (505-629 aa)	CGCCCGGGA**ATG**AATGATATTTTGACGGATTATAAG	Sma I	GCGCTCGAG**TCA**TTTACCTTCCTTAAGAAATTCTTC	Xho I

β-Galactosidase Assay

Transformed L40 yeast strains were streaked on selective plates and incubated at 30 degrees for 3-4 days. These fresh yeast colonies were transferred to Whatman #5 filter disks and flash frozen in liquid nitrogen. The yeast/filter disk was thawed and then placed into a disposable petri dish containing two filter disks soaked with 2.5 ml Z Buffer (60 mM Na_2HPO_4 (anhydrous), 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH adjusted to 7.0), 5.4 ul β-mercaptoethanol, and 20 ul x-gal solution (10% x-gal in NN-Dimethyl-formamide . The disks were pressed together without disturbing the yeast. The dishes were covered and left in the dark at room temperature overnight. The previously described prey plasmid, pACT2-NAM8 served as a negative control (Spingola *et al.*, 2004).

Two-Hybrid Screen of U1 snRNP Protein-Protein Interactions

The protein components of the yeast U1 snRNP are well defined (Gottschalk *et al.*, 1998), but a fine structure for the particle is not yet determined. In humans, however, a three-dimension structure of the U1 snRNP was determined to a 10 angstrom resolution by creating an electron cryomicroscopy map applied to a U1 snRNP model formed from data including: RNA-protein interactions, protein-protein interactions, immunoelectron microscopy, and X-ray crystallography of individual proteins and the Sm core (Stark *et al.*, 2001). Though many similarities exist between the yeast and human snRNAs and Sm protein core, the existence of five novel yeast U1 snRNP proteins suggests the proposed

human U1 snRNP fine structure cannot be applied to yeast until more data is collected that details protein-protein interactions in the yeast U1 snRNP.

To begin this task, I modified the Mer1p-specific two-hybrid screen to expand the analysis to include all U1-snRNP proteins and several other early splicing factors. This larger screen probed 460 possible interactions. The plasmids described above were utilized, as well as, prey plasmids for genes *NAM8, SNU71, SNU56, LUC7, YHC1, BBP*, and *MUD2* that were previously described (Spingola *et al.*, 2004). A *MER1* prey plasmid was constructed by PCR amplification from genomic DNA. This fragment was cloned into pACT2. 22 bait plasmids were constructed by sub-cloning prey plasmid fragments into pBTM116 or by PCR amplification from genomic DNA.

Prey plasmids were transformed into strain L40 and bait plasmids were transformed into strain KH46 (*MATα, ura3-52, leu2-3, 112, trp1-1, lys2, his3-1, ade2-101, cup1Δ::ura-3-52*). The haploid strains were individually crossed and diploids were selected on restrictive media plates. The 460 diploid strains were tested with the β-galactosidase assay described above.

Results

Mer1 Interactions with U1 snRNP Proteins

To extend the two-hybrid screen between Mer1p and the remaining untested pre-spliceosome proteins, I amplified 15 genes by PCR using genomic DNA and Vent DNA polymerase. These full-length genes were cloned into the prey plasmid (pACT2), which codes for the *GAL4* activation domain and thereby creates a *GAL4*AD-Splicing Factor fusion. The bait plasmid (pBTM-MER1) contained *MER1* just downstream of the *LexA* DNA binding domain. These plasmids were transformed into the yeast strain L40 that features a *LacZ* reading frame just downstream of multiple repeats of *LexA*. If the hybrid bait and prey proteins interact with each other, they will drive transcription of *LacZ* whose protein product is β-galactosidase. Accumulation of this enzyme is confirmed by a colorimetric assay. Strain L40, in addition to featuring the *LexA-LacZ* sequence also contains a *LexA-HIS3* fusion. Table 3 lists the 15 proteins probed by Mer1p. While 14 proteins produced no interaction with Mer1p, Prp39p did create a strong and reproducible interaction with Mer1p (see Figure 3).

To confirm the Mer1p-Prp39p interaction indicated by the β-galactosidase assay, a L40 yeast strain containing the *MER1* bait plasmid and the *PRP39* prey plasmid was cultured on media plates lacking hisitidine (see Figure 4A). The robust growth confirmed the interaction. The *PRP39* prey plasmid was then transformed into the L40 strain with either the *MER1* activation domain or *MER1* KH domain bait plasmids (see Figure 4B). The results indicate a new function for the *MER1* activation domain to stabilize commitment complex formation via Prp39p for pre-mRNA containing the Mer1p enhancer sequence. This interaction along with previous data (Spingola and Ares, 2000; Spingola *et al.*, 2004) suggest a model where the two domains of Mer1p act in concert to promote splicing efficiencies. The KH domain binds to introns of pre-mRNA in a sequence dependent manner, while the activation domain serves to attract the U1 snRNP and nucleate spliceosome assembly.

Table 3. Two-hybrid interactions between Mer1p and pre-spliceosome proteins

Yeast Protein	Color intensity of β-galactosidase assay
Sm G	-
Sm F	-
Sm E	-
Sm D1	-
Sm D2	-
Sm D3	-
Sm B	-
Snp1	-
Mud1	-
Snu65	-
Prp39	+++
Prp40	-
Cbp20	-
Bud13	-
Pml1	-
Prp39 (1-50 aa)	-
Prp39 (1-504 aa)	++
Prp39 (51-504 aa)	-
Prp39 (51-629 aa)	+++
Prp39 (505-629 aa)	-

Figure 3. Mer1p two-hybrid screen using β-galactosidase assay. Mer1p bait plasmid and splicing factor prey plasmids are transformed in yeast L40 strain. Flash frozen yeast were treated with X-gal solution. Top plates are media plates from which the bottom assay plates were replica plated. Left plates clockwise from top: prey = Prp39p, Nam8p (negative control), SmGp, SmGp, SmGp, SmFp, SmFp, SmFp. Right plates clockwise from top: prey = Prp39p, Nam8p (negative control), Cbp20p, Cbp20p, Cbp20p, SmD_2p, SmD_2p, SmD_2p.

The essential U1 snRNP protein Prp39p (629 aa) shares a high degree of sequence similarity (50%) with the U1 snRNP protein Snu65p (544 aa) (McLean and Rymond, 1998). Interestingly, the two-hybrid results did not indicate an interaction between Mer1p and Snu65p (see Figure 4B). This suggests the Mer1p-Prp39p interaction is mediated by a unique Prp39p domain or peptide sequence. To test this possibility, a series of *PRP39* truncations were prepared and tested against the *MER1* bait plasmid. Though sequence similarities extend throughout the coding regions for the proteins, these similarities are concentrated in a series of 11 tetratricopeptide repeats located in the middle regions of both proteins (McLean and Rymond, 1998). Thus, it was anticipated that either an N-terminal (1-50 aa) or C-terminal (505-629 aa) fragment of Prp39p would interact with Mer1p. However, upon testing, neither terminal fragment interacted with Mer1p, but the (51-629 aa) Prp39p fragment did provide a strong interaction (see Table 3). This Prp39p truncation produces a viable, but splicing impaired strain (Lockhart and Rymond, 1994). Taken together, the Mer1p activation domain interaction with Prp39p likely involves a Prp39p peptide sequence located in the middle of the protein, but not part of the TPR repeat sequences.

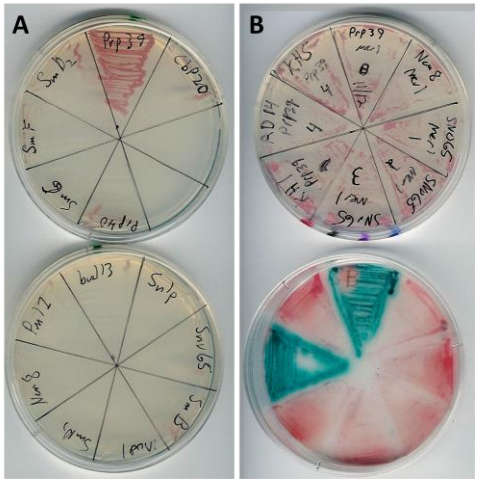

Figure 4. Mer1p two-hybrid screen. Panel 4A is selection on (-His) plates with L40 strain containing bait pBTM-MER1 and various prey pACT2 candidates. Top plate clockwise from top: prey = Prp39p, Cbp20p, empty, empty, Prp40p, SmGp, SmFp, SmD_2p. Bottom plate clockwise from top: prey = Bud13p, Snp1p, Snu65p, SmBp, Mud1p, SmD_1p, Nam8p (negative control), Pml1p. Panel 4B tests domains of Mer1p against Prp39p using β-galactosidase assay. Top plate is the media plate. Bottom plate is assay plate. Both plates clockwise from top: bait-prey = Mer1p Prp39p, Mer1p-Nam8p, Mer1p-Snu65p, Mer1p-Snu65p, Mer1p-Snu65p, Mer1p KH domain-Prp39p, Mer1p activation domain-Prp39p, Mer1p KH domain-Prp39p.

U1snRNP Protein-Protein Interactions

Having identified a new interaction between Mer1p and the U1 snRNP, I probed the yeast U1 snRNP structure by applying the two-hybrid assay. Although a great deal of structural information has been generated for the human U1 snRNP, this data may not be relevant to Mer1p function since critical differences exist between the two protein complexes (Stark *et al.,* 2001). For example, there are five yeast U1 snRNP proteins

(including Prp39p) without human homologs (see Table 1). Because testing all the possible pair-wise protein interactions within the U1 snRNP complex would involve hundreds of transformations, a different strategy was employed to efficiently probe the U1 snRNP and several associated splicing factors.

A collection of U1 snRNP bait plasmids was created by subcloning from the existing prey plasmids (except Snu71p). Then each prey plasmid was separately transformed into the L40 strain and each bait plasmid was separately transformed into the KH46 strain, which has an opposite mating type to L40. The multiple L40 strain variants were crossed with all the KH46 strain variants and these 460 crosses were selected for diploids on restrictive media. After sufficient growth, the β-galactosidase assay was performed on the surviving yeast colonies that contained both prey and bait plasmids. With few exceptions, each possible interaction was tested four times apiece. The interactions are summarized in Table 4. Because the *NAM8* bait plasmid strongly interacted with every prey plasmid tested, it was apparent these were false-positive interactions (data not shown). The *NAM8* prey plasmid, however, generated numerous negative results and therefore its positive interactions can be considered valid.

For the core Sm proteins, I observed three strong and reciprocal interactions. They were: SmE-SmG and SmD_3-SmB and SmD_2-SmE. The first two have been reported previously, but the SmD_2-SmE interaction is novel (see Discussion). Besides interactions among Sm core proteins, I observed three other strong interactions with reciprocals: Bud13p-Pml1p; Prp39p-Snu65p; Mer1p-Prp39p (Mer1p will be discussed below). The Bud13p-Pml1p interaction by two-hybrid analysis is novel, but Bud13p, Pml1p, and Snu17p have been isolated in a trimeric complex (RES) linked to splicing (Dziembowski *et al.*, 2004). Likewise the Prp39p-Snu65p interaction has not been reported previously, but their strong sequence similarity and multiple TPR sequences does not make this interaction a great surprise. In fact, both Prp39p and Snu65p interacted with several U1 snRNP associated proteins (Table 4).

While the Prp39p-Mud2p and Prp39p-BBP interactions were observed previously, no Snu65p two-hybrid interactions have been documented (Fromont-Racine, *et al.*, 1997). Since the TPR sequence is a recognized protein binding domain and because both the essential proteins Snu65p and Prp39p contain multiple copies of TPR, the results reported here suggest new activities for these proteins (McLean and Rymond, 1998). Though I was not successful in creating a Snu71p bait plasmid, the Snu71p prey plasmid did generate two strong one-way interactions against the Luc7p and Prp40p bait plasmids. A Snu71p-Prp40p interaction was reported previously, but the Snu71p-Luc7p interaction is novel (Ito *et al.*, 2001).

Table 4. U1 snRNP protein-protein interactions indicated by two-hybrid assay

	SmF	SmG	SmE	SmD1	SmD2	SmD3	SmB	Mud1	Snu65	Prp39	CBP20	Snu56	U1-C	Pml1	Bud13	Luc7	Prp40	Sn1p	Mer1
SmF	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
SmG	-	-	+++	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
SmE	-	+++	++	-	++	-	-	-	+	-	-	-	-	-	-	-	-	-	-
SmD1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SmD2	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SmD3	-	-	-	-	-	-	++	-	-	+	-	-	-	-	-	-	-	-	-
SmB	-	-	-	-	-	+++	-	-	-	-	-	-	-	+	-	-	-	-	-
Mud1	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-
Snu65	-	-	-	-	-	-	-	-	+++	++	-	-	+++	+	-	-	-	-	-
Prp39	-	-	-	-	-	-	-	-	+++	+++	-	-	-	+	-	-	-	-	+++
CBP20	-	-	-	-	-	-	-	-	++	-	++	-	-	+	-	-	-	-	-
Nam8	-	-	-	-	-	-	-	-	+++	++	-	-	-	+	-	-	-	+	-
BBP	-	-	-	-	-	-	-	-	++	++	-	-	-	++	-	-	-	-	-
Snu56	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-
U1-C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mud2	-	-	-	-	-	-	-	-	++	+++	-	-	-	++	-	-	-	-	-
Pml1	-	-	-	-	-	-	-	-	-	++	-	-	-	++	+++	-	-	-	-
Bud13	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-
Snu71	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+++	+++	-	-
Luc7	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+++	++	-	-
Prp40	-	-	-	-	-	-	-	-	+	+	++	-	-	-	-	-	++	-	-
Sn1p	-	-	-	-	-	-	-	-	-	-	++	-	-	+	-	-	-	-	-
Mer1	-	-	-	-	-	-	-	-	++	+++	-	-	-	+	-	-	-	-	-

Prey plasmids (rows) and bait plasmids (columns) were transformed in strain L40 and KH46 respectively. Strains were mated and the diploids were selected by growth on restrictive plates. Interactions were probed using X-gal as a substrate for β-galactosidase. Minus signs indicate no color change. Plus signs (+), (++), or (+++) represent color change intensity.

Discussion

The two-hybrid system has been utilized extensively to determine probable protein-protein interactions. For example, Camasses and colleagues have tested the pair-wise interactions of the Sm protein core and the Rymond group has probed interactions within the yeast U2 snRNP (Camasses *et al.*, 1998; Wang *et al.*, 2005). In this study, I examined possible Mer1p interactions with core Sm proteins, U1-specific snRNP proteins, and several accessory splicing factors. Furthermore, I tested for protein-protein interactions among the yeast U1 snRNP proteins. While I have documented several novel interactions, other interactions that I report agree with data generated from previous two-hybrid reports and structural studies. For example, the SmE-SmG and SmG-SmE reciprocal interactions reported here agree with Camasses *et al*., (1998) and the identification of a particle consisting of SmE, SmF and SmG (Raker *et al.*, 1996). Likewise, the SmB-SmD_3 and SmD_3-SmB reciprocal interactions reported here agree with previous two-hybrid data, as well as, the isolation and crystal structure of a SmB-SmD_3 particle (Raker *et al.*, 1996; Camasses *et al.*, 1998; Kambach *et al.*, 1999). Interestingly, the SmE-SmD_2, SmD_2-SmE reciprocal interactions reported here have not been previously observed. This data conflicts with Kambach's proposed structure of the Sm core heptameric ring because this ring model separates SmD_2 and SmE with SmF in between. However because this doughnut model was generated using the SmD_3-SmB and SmD_1-SmD_2 crystal structures in combination with reported metazoan Sm core protein two-hybrid interactions, it remains possible the yeast Sm core structure could vary slightly (Kambach *et al.*, 1999). A second ring model based on electron microscope

data generated from the SmE,F,G particle suggests that two SmE,F,G particles form a flat hexamer ring to which the SmD_1-SmD_2 and SmD_3-SmB particle attach (Plessel, *et al.*, 1996). The data presented here does not conflict with this second ring model. In combination with other reports of interactions, structures, and functions, along with this two-hybrid data, I propose a model for U1 snRNP structure; see Figure 5.

Mer1p-Prp39p interaction

In the case of Mer1p regulated pre-mRNA splicing, the model in Figure 5 would vary slightly. Here Mer1p is bound to the pre-mRNA via the KH domain. Mer1p will primarily contact the U1 snRNP by its interaction between the activation domain and Prp39p. It is this link from the essential Prp39p through Mer1p to the enhancer sequences on *AMA1*, *MER2*, and *MER3* transcripts that give the transcripts the extra stability to form productive commitment complexes and increase the splicing efficiencies during meiosis when Mer1p is expressed; see Figure 6. This model suggests that future studies involving the splicing regulator Mer1p could utilize mutant *prp39* alleles to better understand the splicing reaction and possibly amplify the swing in splicing efficiencies observed with and without Mer1p.

Overall, the collective two-hybrid analysis presented here tested hundreds of possible protein-protein interactions. Because the vast majority of these interactions were negative and many of the positive interactions have been independently confirmed, it lends confidence that the novel interactions reported here are bona fide. While all of the proteins discussed here have been previously grouped together by genetic interaction or by affinity capture and other protein purification techniques, this study identifies specific interactions that provide stability to the U1 snRNP, a key participant in the spliceosome assembly process.

Note Added in Proof

After submission of this dissertation to my dissertation committee and the graduate dean, a journal article was published in print that identified a specific requirement for the U1 snRNP protein Snu56p for Mer1p-regulated splicing. This article confirmed a weak two hybrid interaction between Snu56p and Mer1p (Spingola *et al.*, 2004) and identified a strong two-hybrid interaction between Snu56p and Mud2p. These results do not significantly alter the Mer1p-regulated splicing model that I present in Figure 6. Interestingly, this research group demonstrates that Snu56p is not essential to mitiotic splicing whereas it was previously demonstrated as an essential U1 snRNP protein for mitosis. This suggests that Snu56p has a second activity during mitosis in additional to this newly identified role essential for Mer1p-activated splicing during meiosis (Balzer and Henry April, 2008).

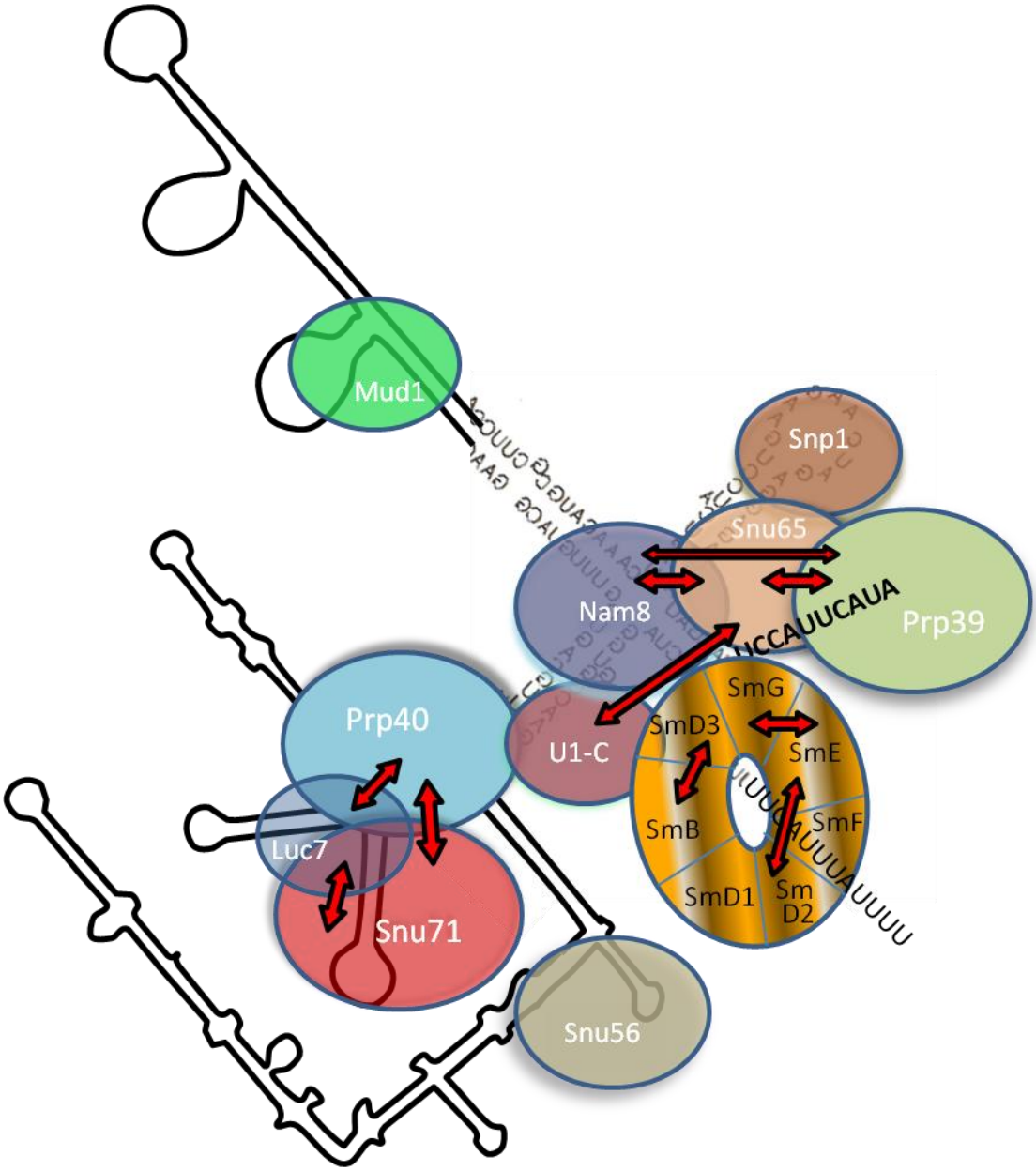

Figure 5. 2D Model of the U1 snRNP. The underlying snRNA structure was proposed by Kretzner *et al.* (1990). The globular proteins are sized proportionally to reported masses; see Table 1. Mud1p and Snp1p are aligned where their human homologs are reported to bind key snRNA stem loops (Stark *et al.* 2001). The structure of the Sm core doughnut was determined by Kambach *et al.* (1999). Singled stranded U1 snRNA threads through the Sm core doughnut hole (McConnell *et al.,* 2003). U1-C and SmB are oriented relative to human homologs (Stark *et al.,* 2001). Two-hybrid interactions are represented by arrows and were reported in this study and previously (Ito *et al.,* 2001; Camasses *et al.,* 1998).

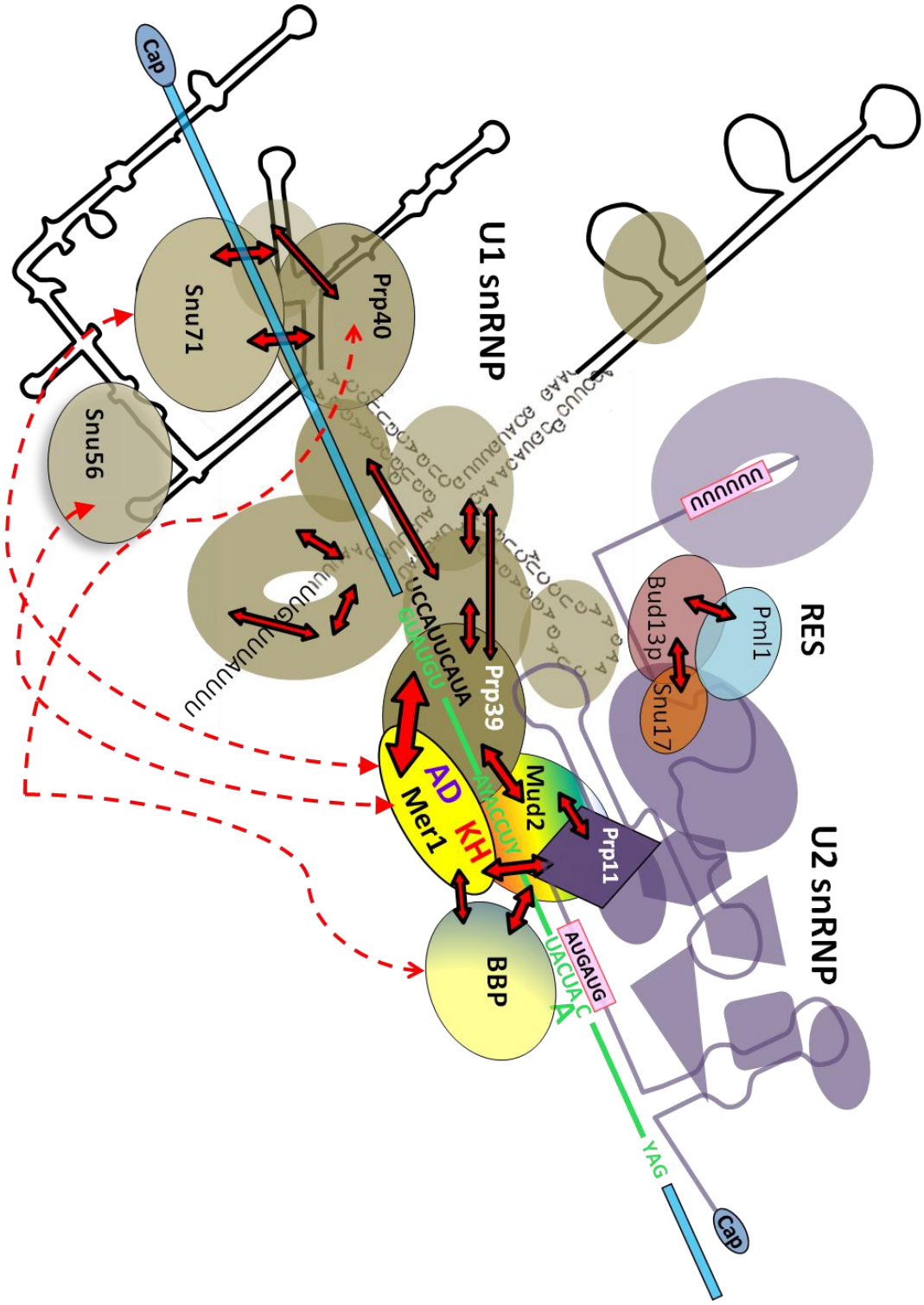

Figure 5. 2D Model of Mer1p interaction with the assembling pre-spliceosome complex. The Mer1p KH domain binds to pre-mRNA containing the enhancer AYACCUY. Mer1p's participation with the pre-spliceosome is mediated by Prp39p and other reported interactions (Spingola *et al.,* 2004). Two hybrid interactions are indicated by red arrows. In a 3D model, the dashed red arrows would fold under this flat representation (Abovich and Rosbash, 1997). As U2 snRNP binds to the branchpoint, Mud2p and BBP will disassociate from the complex.

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Chapter Four

Probing a Meiotic Function for the RES Complex

Introduction

Whether a haploid or a diploid, yeast can quickly reproduce asexually during mitosis by budding. In fact, given a steady supply of sugar and nutrients yeast forego meiotic cell division, which is a necessary step in the gamete production and sexual reproduction of higher eukaryotes. However, in times of starvation, yeast diploids will exit mitosis and enter meiosis where a round of DNA replication followed by two successive chromosomal segregations leads to the production of a tetrad containing four haploid spores. For yeast, meiotic cell division can be considered a defensive reaction to environmental conditions as spores can remain dormant until nutrients are available (Sherman *et al., 1986*). Yet besides this obvious defensive component, meiosis allows yeast to alter their genetic makeup by enhanced levels of recombination. Compared to mitosis, recombination events are 1000 times more common in meiosis. Recombination is facilitated in meiosis by creation of synaptonemal complexes and formation of double-stranded breaks (Ogawa *et al.,* 1995).

Because tetrad formation and enhanced recombination requires many specialized proteins, there are over 150 yeast genes whose products function only during meiosis. A microarray analysis of sporulation concluded that over 1000 mRNAs experience significant changes to expression levels during meiosis with about one-half being upregulated (Chu *et al.,* 1998). Just recently, in a screen of 4323 "non essential" yeast genes, 334 genes were characterized as essential for sporulation (Enyenihi and Saunders, 2003). One way the yeast organism regulates genes specific for meiosis is by expression of several key meiotic transcription factors. For example, in the middle of the meiotic cell cycle, Ndt80p regulates transcription by binding to an upstream sequence called the middle gene sporulation element (MSE) found on genes required for meiotic metaphase. Two other meiotic transcription factors Ume6p and Ime1p regulate genes required for the start of meiosis (Chu *et al.,* 1998). They are arguably the two most important proteins controlling meiosis and both are essential for sporulation. Ume6p directly regulates at least 74 genes containing URS1 activation sites including *NDT80* (Williams *et al.*, 2002). Interestingly, Ume6p serves as a transcriptional repressor during mitosis when Sin3p is bound to it. Yet when Ime1p is upregulated by starvation conditions and other cellular cues, Ime1p serves to destabilize the Ume6p-Sin3p complex, which in turns allows a Ume6p-Ime1p complex to act as a transcription activator (Washburn and Esposito, 2001).

A second way that yeast control meiotic cell cycle initiation and progression is through regulated splicing. Indeed, several studies document changes to splicing patterns during meiosis. Besides the example of Mer1p and its role with *AMA1*, *MER2*, and *MER3* pre-mRNA splicing discussed in Chapter Two, Nam8p is expressed during meiosis and can specifically alter splicing patterns of meiotic transcripts. Because it cross-links to intronic regions of pre-mRNA and is a yeast specific U1 snRNP protein, it likely

functions to stabilize commitment complex formation around inefficiently spliced transcripts (Zhang and Rosbash, 1999; Nakagawa and Ogawa, 1999; Puig *et al.,* 1999). Still other meiotic splicing factors likely remain undetected. Juneau and colleagues recently made this conclusion when they determined that 100% of the meiotic-specific transcripts (containing introns) splice inefficiently during mitosis. Yet during sporulation, the splicing efficiencies of all 13 meiotic transcripts improve; several of these transcripts undergo a dramatic splicing enhancement. Thus, Juneau concludes that splicing regulators, in addition to Mer1p and Nam8p, are almost certainly expressed during meiosis (Juneau *et al.,* 2007).

Data presented in Chapter Two demonstrate that *BUD13* and *SNU17* can be required to observe Mer1p-activated splicing. Since both Bud13p and Snu17p are components of the trimeric RES complex, these findings suggest the RES complex contributes to Mer1p-mediated splicing regulation. This role is consistent with the previous report identifying the RES complex as a participant in the splicing regulation of inefficiently spliced transcripts (Dziembowski *et al., 2004*). However, there is a caveat to this relationship; in wild-type yeast, Mer1p is expressed only during meiosis, while the experiments in Chapter Two that established a link between Mer1p-activated splicing and the RES complex were conducted during mitosis using plasmids to constitutively express Mer1p.

Not only has Northern blot analysis demonstrated that sporulation media is required for *MER1* upregulation, further experimentation revealed that Mer1p expression requires both starvation conditions and expression of the *MAT*a and *MAT*α gene products (Engebrecht and Roeder, 1990). Yet besides these general conditions required for entry into meiosis, the determinants of *MER1* transcription are not known. *MER1* does not contain the MSE or URS1 activation sequences needed for upregulation by Ndt80p or Ume6p (Chu *et al.,* 1998; Williams *et al.,* 2002). None the less, the expression of Mer1p peaks at the onset of Meiosis I (Primig *et al.,* 2000). These increased levels of Mer1p then act to regulate splicing of *AMA1, MER2,* and *MER3* whose gene products play important roles during spore formation (Spingola and Ares, 2000).

Though the previous study of the RES complex did not propose a specific meiotic role, a large scale sporulation study revealed upregulation of both *BUD13* and *SNU17* during meiosis (Dziembowski *et al., 2004;* Primig *et al.,* 2000). Taken together, this suggests the primary activity of the RES complex could involve meiotic splicing. To explore this possible function, I developed a working hypothesis which states: if the RES complex is required for Mer1p-dependent splicing of the *AMA1* transcript, then loss of *BUD13* or *SNU17* during meiosis should produce a phenotype similar to the loss of *MER1* or the loss of *AMA1*. Both deletions impact spore formation; *mer1*Δ strains produce inviable spores and *ama1*Δ strains do not create spores (Engebrecht and Roeder, 1990; Enyenihi and Saunders, 2003). Below I present experiments that test this hypothesis.

Experiments from Chapter Two further suggested the RES complex contribution to Mer1p-activated splicing is transcript specific. Primer extension analysis demonstrated that loss of RES components affected the splicing efficiencies of *AMA1* to a greater degree than observed with *MER3*. A reasonable conclusion from this observation is that

sequence differences between the two transcripts would explain the requirement for the RES complex with one transcript, but not the other. Sequence differences by a variety of means could prevent or require RES complex activity. For example, an RNA secondary structure could form within one transcript that serves to impair spliceosome assembly. In this case, the RES complex could overcome this assembly inhibition by adding commitment complex stability or melting the secondary structure. Supporting this scenario is the finding that RNA hairpins, as short as 6 nucleotides, located at the 5' splice site or the branchpoint sequence can impair splicing levels (Goguel *et al.*, 1993).

Alternatively, an intronic or exonic sequence contained within *AMA1* may attract Bud13p or Snu17p directly. With this binding situation the affinity between a specific transcript sequence and the RES complex would provide extra stability for commitment complex or pre-spliceosome formation in a manner similar to the putative Mer1p activity. Because Snu17p contains an RNA recognition motif (RRM), this direct binding to pre-mRNA is plausible and agrees with co-immunoprecipitation assays that have recovered labeled pre-mRNA using a Snu17p-protA fusion (Gottschalk *et al.*, 2001). The possible contribution of Bud13p and Snul7p to pre-spliceosome formation and stability is further supported by their close association with the U2 snRNP SF3b particle (Wang *et al.*, 2005).

Apart from secondary structure or sequence specific binding there are several other explanations for the RES complex activity favoring *AMA1* over *MER3*. Since both intron length and 3'exon length can affect splicing efficiencies, RES complex function could depend on pre-mRNA spatial requirements for optimal activity (Klinz and Gallwitz, 1985; Nandabalan and Roeder, 1995). Furthermore, it is possible that *MER3* has inhibitory proteins binding to it that block RES function.

To explore which sequence differences between *MER3* and *AMA1* determine the need for the RES complex activity during splicing, I questioned whether the RES activity was based on an exonic or intronic sequence. To accomplish this, I created synthetic hybrid transcripts that contained the intron of *AMA1* combined with the exons of *MER3* and vice versa. In this manner, I separated the *AMA1* intron from its exons and could test which feature served as a more powerful determinant of RES complex activity. Additionally, I measured the splicing efficiencies of other *AMA1* transcript variants in a further effort to identify a particular pre-mRNA feature that controlled RES complex activity.

Methods

Creation of *AMA1-MER3* Hybrid Transcripts

A PCR sense primer was designed that fused the extreme 3' end of the *MER3* 5' exon sequence to the first nucleotide of the *AMA1* intron sequence. A second primer (antisense) was designed to fuse the 3' end of the *AMA1* intron sequence to the 5' end of the *MER3* second exon. PCR was performed using these primers, Vent DNA polymerase, and the template pRS316AMA1-CUP1 (Spingola *et al*, 2004). The double stranded DNA product contained the full-length *AMA1* intron flanked upstream by *MER3* 5' exon sequence and downstream by *MER3* 3' exon sequence. Overlapping nesting primers of the *MER3* exons were use to extend the flanking regions of the *MER3* exons on both sides of the *AMA1* intron. The final PCR product was 218 nucleotides in length

and contained the 93 nucleotide *AMA1* intron flanked upstream by 64 nucleotides of *MER3* 5' exon and downstream by 61 nucleotides of the *MER3* 3' exon. Plasmid pRS-MER3-CUP1 (previously described in Spingola *et al,* 2004) contains the endogenous *MER3* sequence, but the 3' end of the second exon is truncated and fused to *CUP1*. This plasmid was digested at a unique BlpI site within the *MER3* intron. Both the digested plasmid and 218 bp PCR product were transformed into the yeast strain KH46 (*MATα, ura3-52, leu2-3, 112, trp1-1, lys2, his3-1, ade2-101, cup1Δ::ura-3-52*) where homologous recombination would act to replace the digested *MER3* intron on the plasmid with the *AMA1* intron from the PCR product and create a functional plasmid. The transformed strain was grown on (-) URA plates, which selected for strains with functional plasmids. Plasmids were extracted from surviving yeast colonies and tested for incorporation of the *AMA1* intron by restriction digest analysis and PCR.

A similar strategy was used for creation of A1M3A1, an *AMA1-CUP1* transcript, which contains the *MER3* intron. Here the final nested PCR product of 276 nt contained the 152 nt *MER3* intron flanked by 64 nt of *AMA1* 5' exon and 60 nt of *AMA1* 3' exon. Plasmid pRS316-AMA1–CUP1 was digested at the unique EcoR47 site in the *AMA1* intron. The digested plasmid and extended PCR product were transformed into KH46 and selected on (-) URA plates as described above. Primers required for the construction of these constructs are listed in Table 1. Construction of pRS316-MX-ACT1-CUP1, pRS316-G5A-ACT-CUP1, R1070, and R1130 were described previously (Spingola and Ares, 2000; Spingola *et al.,* 2004). pGB and pGS contain truncated versions of the *AMA1* 5' exon fused to the *AMA1*(intron-exon2)-*CUP1* construct used in pRS316 AMA1-CUP1. These plasmids were derived from pGAC14 (Spingola and Ares, 2000). Plasmid pEN-105 contains a mutated intronic splicing silencer and was previously described (Spingola and Ares, 2000).

RNA and Splicing Assays

RNA isolation and purification, as well as the primer extension assays were conducted as described in Chapter Two.

Table 1. Primers used for construction of *AMA1-MER3* hybrid transcripts

Construct	Primer	Sequence
MER3-AMA1-MER3	M3ex-AMA1int (+)	GGTACAGGAAAAAGAAGTAGACCCTCTCCAAATAGTACGTTATTAAGAGC
	M3ex-AMA1int extd (+)	GTAAGGATGAAAACAAAGTTTGATCGCCTCGGTACAGGAAAAAGAAGTAG
	AMA1int-M3exon (-)	GTAGCAGACTGGTCGTTAAAGTCAATATCTGTAGAAAATATTTG
	AMA1int-M3exon extd (-)	GGTTGGCGGCTATTTTTCTTATTTCTTTTAAATGTAGCAGACTGGTCG
AMA1-MER3-AMA1	AMA1ex-M3int (+)	CACTTATCAAGCTCAGGCACAGCAAGTCTGTGGTAGTAACGAAGCTTAGC
	AMA1ex-M3int extd (+)	GATGAAAATTTAATAGGATTGAAACTTCATTCCACTTATCAAGCTCAGGC
	M3int-AMA1ex (-)	GAATTTCAGAGGACTTATAGGTATTTCTCTGAATGAACATGCAAACCTGC
	M3int-AMA1ex extd (-)	CGCTGAACCCGGTACCGCCGCCGACTGCAAGCAGGTTTGCATGTTC

Creation of *bud13*Δ and *snu17*Δ Diploid Yeast Strains

PCR primers were designed to code for regions of the 5' and 3' UTRs adjacent to the *S. cerevisiae BUD13* ORF fused to the upstream and downstream sequences flanking the

Schizosaccharomyces pombe HIS5+ ORF contained on plasmid pFA6a-HisMX6 (Longtine *et al., 1998*). Using TAQ polymerase, pFA6a-HisMX6, and these primers a PCR product was created that included the full length *HIS5+* ORF surrounded by sequences coding for *BUD13*. Nested PCR primers coding for the 5' and 3' UTR regions of *BUD13* were used to extend the PCR product with additional homology to *BUD13* until nearly 100 nucleotides of the *BUD13* UTRs flanked the *HIS5+* ORF on either end. This 1600 base pair PCR product was transformed into strain KH46 and colonies were selected on media plates lacking histidine. Surviving transformants were confirmed for successful recombination and knockout of the *BUD13* gene by PCR using an additional set of PCR primers that amplified the chromosomal region where *BUD13* was located. A similar strategy was used to create a *snu17*Δ haploid strain in the KH46 background. See Table 2 for a list of primers used to create the *BUD13* and *SNU17* deletion strains. The *NAM8* and *MER1* haploid deletion strains were previously created (Spingola and Ares, 2000; Spingola lab, personal communication).

The haploid deletion strains described above were crossed with KH52 (*MAT*a, *ura3-52, leu2-3, 112, trp1-1, his3-1, ade2-101, cup1*Δ*::ura-3-52*) and selected on (-) HIS (-) LYS plates. The resulting diploids were grown in sporulation media (1% KOAc, pH 7.0) for 3-5 days. A 1.5 ml culture was microfuged for 10 seconds, decanted, washed with H_20, decanted, treated with 0.5 mg/ml lyticase in 1M Soribitol for 5-10 minutes, and then chilled on ice. Tetrads were dissected using a yeast micromanipulator and the spores were germinated on YPD plates. Yeast colonies were selected on (–) HIS plates to isolate strains containing the knockouts (*snu17*Δ, *bud13*Δ, *nam8*Δ, or *mer1*Δ). Survivors were crossed back to KH46 and KH52 strains containing a *URA3* plasmid (pRS316 AMA1-CUP1) and grown on (-) URA (-) HIS selective media to identify the sex phenotypes. Once the knockout *MAT*a haploid strains were identified, they were transformed with a *URA3* plasmid (pRS426). The previously created *MAT*α strains were transformed with a *LEU2* plasmid (R1130) and the strains of opposite sex types were crossed and selected on (-) URA (-) LEU plates to achieve the homozygous diploid deletion strains. Additionally, a KH46 / KH52 diploid and a *bud13*Δ *MAT*a / *snu17*Δ *MAT*α diploid strains were created as control strains.

Tetrad Analysis and Spore Production

Spore production for the deletion and control strains was evaluated by briefly centrifuging 5 ml overnight YPD cultures, washing with H_2O, followed by 3-5 day growth on sporulation media (1% KOAc, pH 7.0). The cultures were then examined at 400x power using a light microscope. The ratio of tetrads to diploid cells was calculated in the various strain backgrounds.

Determination of spore viability was performed in a manner identical to the tetrad dissections performed for the strain construction where diploids were grown in sporulation media (1% KOAc, pH 7.0) for 3-5 days. A 1.5 ml culture was microfuged for 10 seconds, decanted, washed with H_20, decanted, treated with 0.5 mg/ml lyticase in 1M Soribitol for 5-10 minutes, and then chilled on ice. Tetrads were dissected using a yeast micromanipulator and the spores were germinated on YPD plates.

Table 2. Primers used for deletion strain construction

Strain	Primer	Sequence
bud13Δ	Bud 13-1 (+)	GGTGGAAGATAACAACAGGACGTTTATTACCGGATCCCCGGGTTAATTAA
	Bud 13-2 (+)	TGACTTGATTTTGAAAGTTGTTCTCAAGACTCGAATGGTGGAAGATAACA
	Bud 13-3 (+)	GCTTAGAAAATGGCATAAAGAAAATGGCTATTTGACTTGATTTTGAAAG
	Bud 13-4 (+)	GGTATGTGAACGATAACAATGTTTGC
	Bud 13-A (-)	CTTTCCGCATAGTTATATATTATCTCATTTGAATTCGAGCTCGTTTAAAC
	Bud 13-B (-)	AAATGGGGATTGTCAAAGGGTATTTTTTACACAAAGCTTTCCGCATAGTT
	Bud 13-C (-)	AATCGTTGATCTTGTTAAGAAAAAGCTTATAACAAATGGGGATTGTCAA
	Bud 13-D (-)	CTGAGACCTATATAAAGAGGGG
snu17Δ	Budha (+)	GCAGCGTGCAATTCTAGATCAAGAACATAGATAATATAAACAAAATAACACGGATCCCCGGGTTAATTAA
	Shivah 2 (+)	CGAACATTAATTACTCATACAACTCAAAAAGTGCAGCGTGCAATTC
	17KO3 (+)	GTCGAACAAGAAGAGGCACAG
	Vishna (-)	TTTTTTTCTAGGCTATATGAATATAAGATATGCGATGAAAGAAAAAATTATGAATTCGATGCTCGTTTAAAC
	Khrishna (-)	GGATGTAGAATTACAAATATGATATTGATTATTTTTTTCTAGGC
	17KO4 (-)	CCACCTTCTGTTACTCAGG

Meiotic Splicing Time-Course Assay

To promote a synchronized entry into meiosis a sporulation protocol was adapted from Cao *et al.,* (1990). The knockout diploid strains were selected on (-)URA(-)LEU plates and then switched to 3% glycerol plates. From these plates, 5 ml overnight YPD cultures were grown and used to seed 30 ml YPA cultures (containing ampicillin) at a 25-1 or 50-1 dilution. After 24 hours the YPA cultures were briefly centrifuged and washed with 50 ml H_20. After another quick centrifuge, the supernatant was decanted and the yeast pellet was resuspended in 12.5 ml of 1% KOAc @ pH 7.0. These cultures were placed in a 30 degree water bath-shaker. 1.5 ml aliquots were removed from the sporulating cultures at the 0, 3, 6, 9, 12, 24, and 55 hour time points and flash frozen. Also a 3.0 ml aliquot from the remaining YPD culture was frozen for use as a control. Total RNA was extracted from the frozen yeast pellets using the glass bead–hot phenol protocol described in Chapter Two. These RNA samples were treated with RQ1 RNase-free DNase and then phenol-chloroform extracted and ethanol precipitated. The RNA was dried and resuspended in 40 ul H_20. Single strand cDNA was created by performing a 20ul RT reaction at 50 degrees for 60 minutes using 6.0 ug of total RNA, 0.5 ul Super Script (III) RT, 1 ul 10 mM DNTPs, RT buffer, and a primer cocktail (antisense oligos for the 3' exons of *AMA1, MER2,* and *MER3*). A control reaction with no enzyme was performed to confirm the digestion of the genomic DNA by the RQ1 DNase. After the first strand synthesis, the RT was heat inactivated at 95 degrees for 10 minutes. A series of PCR reactions were performed using various quantities of cDNA and extension cycles until it was determined that 2 ul of cDNA in a 50 ul PCR reaction performed for 21 cycles would consistently allow for product accumulation just above the threshold required for visualization on an ethidium bromide stained 2% agarose gel. Digital pictures of the stained gels and ImageQuant 5.0 software allowed for quantification of the DNA bands representing spliced and unspliced mRNA.

Results

Testing RES Complex Activity Using *AMA1-MER3* Hybrid Transcripts

To further explore the relationship between the RES complex and Mer1p-activated splicing, I created two fusion transcripts that contain various features from the *AMA1* and *MER3* transcripts (see Figure 1). While both the *AMA1* and *MER3* transcripts are regulated by Mer1p, they respond differently to loss of RES components (see Chapter Two). In an attempt to identify pre-mRNA sequence requirements for the RES complex, I placed the 93 nucleotide intron from *AMA1* in between the *MER3* 5' exon and the *MER3* 3'exon–*CUP1*fusion contained on pRS316-MER3-CUP1. In a similar manner, I replaced the 93 nucleotide *AMA1* intron normally found between the truncated *AMA1* 5' exon and the *AMA1* 3' exon-*CUP1* fusion contained on pRS316-AMA1-CUP1 construct with the 152 nucleotide *MER3* intron. This was accomplished by use of nested PCR primers, PCR, and homologous recombination in transformed yeast strains. Once completed, the new constructs – pRS316-M3A1M3-CUP1 and pRS316-A1M3A1-CUP1 contained the same general features of the plasmids used for testing the RES complex in Chapter Two, but entire intronic sequences of *AMA1* and *MER3* had been switched with each other.

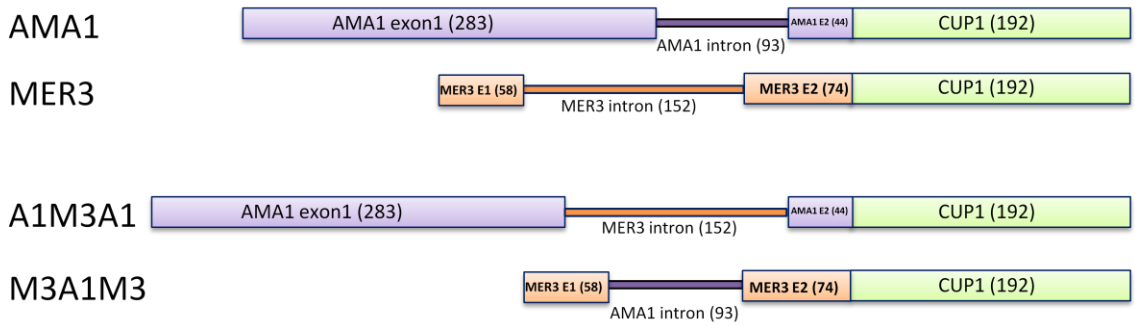

Figure 1. Schematic of *AMA1-MER3* hybrid transcripts. The A1M3A1 construct features the full length *MER3* intron sandwiched in between the *AMA1* exons. The M3A1M3 construct features the full length *AMA1* intron sandwiched in between the *MER3* exons. Length in nucleotides is provided in parenthesis.

The plasmids containing the A1M3A1 and M3A1M3 hybrid transcripts were transformed into wild-type and RES complex deletion strains (*bud13*Δ) along with or without a plasmid coding for Mer1p. Total RNA was harvested from the actively growing strains and primer extension assays were performed to measure splicing efficiencies (see Figure 2). Although not expected, the construct that featured the *MER3* exons and the *AMA1* intron (M3A1M3) spliced very efficiently. A small amount of Mer1p-mediated splicing activation could be observed (Figure 2A compare lanes 3 to 4, 9 to 10, and 11 to 12), but the very high splicing efficiencies observed both with and without the *MER1* plasmid made the contributions of Mer1p to splicing difficult to appreciate.

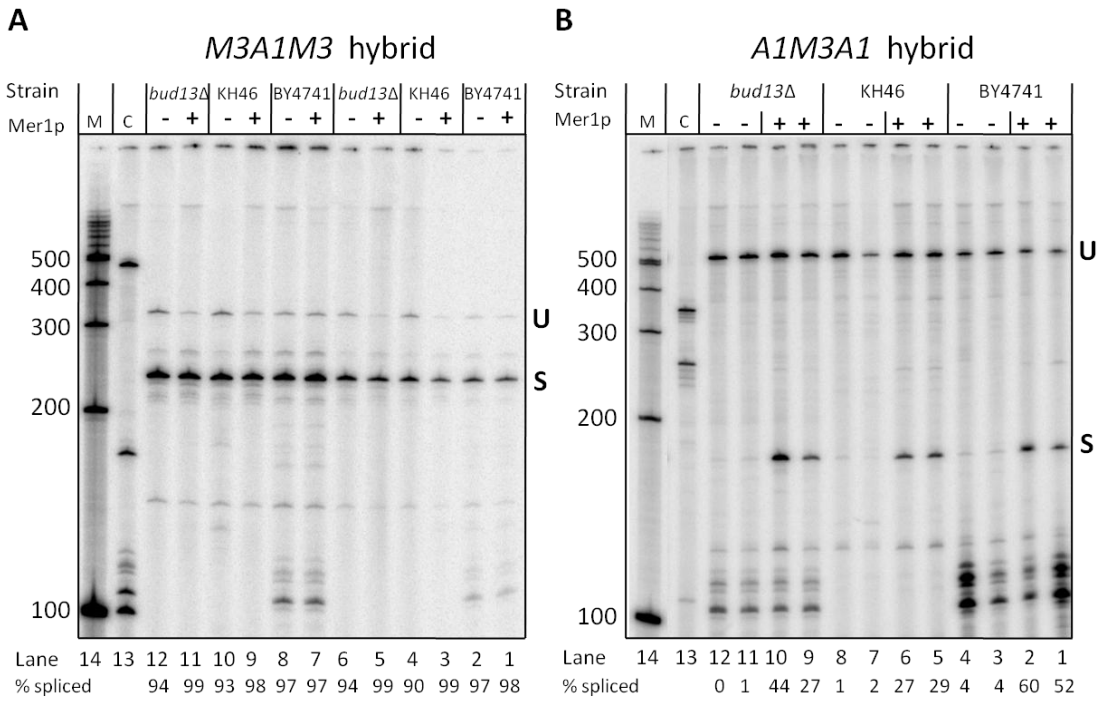

Figure 2. Primer extension analysis of hybrid transcripts *M3A1M3* and *A1M3A1* in wild-type or RES deletion strains with or without constitutive expression of *MER1.* U represents cDNAs from unspliced RNA; S represents cDNA from spliced mRNA. Lane 14 in both panels is 100bp marker. Panel A features the *M3A1M3* construct and the *CUP1* primer (see Figure 1). Lane 13 is a control (C) construct. Panel B features the *A1M3A1* construct and the *CUP1* primer (see Figure 1). Lane 13 is a control (C) construct that is different from Panel A.

Also surprising was the splicing pattern observed with the second hybrid transcript, A1M3A1. This transcript contained the *AMA1* exons and the *MER3* intron; based on this construction the "unspliced" primer extension cDNA was expected to be 559 nucleotides in length and the predicted "spliced" primer extension cDNA was expected to be 152 nucleotides shorter or 407 nucleotides in length. However when tested, this construct created the 559 "unspliced" cDNA and an unexpected "spliced" cDNA that migrated with an apparent length of 180 nucleotides. Thus, an intron approximately 227 nucleotides longer than expected was excised from the construct. This suggests that a cryptic 5' splice was preferred over the non-consensus *MER3* 5' splice site (GUAGUA). Interestingly, this cryptic splice site is Mer1p dependent (compare lanes 2 to 3, 6 to 7, and 10 to 11). Yet a search for an upstream 5'splice site and a nearby Mer1p consensus enhancer sequence did not identify an obvious candidate sequence for splicing.

Because the splicing efficiencies of the hybrid transcripts did not respond to deletions of RES components, a series of additional splicing constructs were employed to further analyze possible pre-mRNA sequence requirements for RES-mediated splicing activation. An additional 5 constructs were transformed into wild-type and RES deletion strains. These included constructs contained on plasmids pG-AMA1-S-CUP1 and pG-AMA1-B-CUP1, which are variants of *AMA1-CUP1* described in Figure 1. Both constructs have shorter 5' exons than *AMA1-CUP1* where *AMA1-B-CUP1*< *AMA1-S-*

CUP1<AMA1-CUP1. Since it was previously demonstrated that a truncated 5'exon of *MER2* enhances splicing efficiencies and alleviates the need for Mer1p, these shorter exons of *AMA1-S-CUP1* and *AMA1-B–CUP1* are expected to enhance splicing efficiencies of the constructs (Nandabalan and Roeder, 1995). Yet the truncations may also eliminate a *cis*-sequence needed for RES activity.

The other three transcripts tested for RES activity were G5A-*ACT1-CUP1*, MX-*ACT1-CUP1*, and EN105-*CUP1*. G5A-*ACT1-CUP1* is an actin transcript fused to *CUP1* (similar in construction to *AMA1-CUP1*). It also contains the G5A 5' splice site mutation, which is known to impair splicing efficiencies (Lesser and Guthrie, 1993; Parker and Guthrie, 1985; Fouser and Friesen, 1986). MX-*ACT1-CUP1* is another actin-*CUP1* transcript variant that contains *MER2's* non-canonical 5' splice site (GUUCGU) and the Mer1p 8 nucleotide enhancer sequence (AUACCCUU) located just downstream of the 5' splice site (Spingola and Ares, 2000). EN105-*CUP1* is the *AMA1-CUP1* construct with a mutated silencer region. Loss of the silencer increases splicing efficiencies and eliminates Mer1p splicing regulation (Spingola and Ares, 2000). Table 3 summarizes the splicing efficiencies for these five constructs described above, as well as, *A1M3A1*, *M3A1M3*, *AMA1-CUP1*, and *MER3-CUP1* constructs.

Table 3. Splicing efficiencies for Mer1p-dependent introns in RES deletion strains

Strain	BY4741		KH46		*bud13*Δ		*snu17*Δ	
Mer1p	-	+	-	+	-	+	-	+
Construct								
AMA1-CUP1	30.5 ± 2.9 *	69.7 ± 3.3 *	NA	NA	14.2 ± 1.8 *	14.9 ± 1.3 *	29.7 ± 4.0 **	21.7 ± 2.7 **
MER3-CUP1	2.9 ± 1.0 *	44.2 ± 1.8 *	NA	NA	1.9 ± 0.4 *	25.4 ± 3.3 *	4.3 ± 1.2 **	27.1 ± 2.8 **
M3A1M3-CUP1	97.6 ± 1.7	97.3 ± 0.7	96.3 ± 1.0	97.35 ± 1.2	92.9 ± 1.2	93.7 ± 5.7	NA	NA
A1M3A1-CUP1	3.8 ± 0.1	55.7 ± 5.9	1.4 ± 0.3	28.1 ±1.1	0.3 ± 0.5	35.8 ± 12.2	NA	NA
AMA1-B-CUP1	NA	NA	72.8 ± 1.6	78.9 ± 3.2	63.4 ± 0.4	62.2 ± 4.2	61.9 ± 1.2	63.6 ± 2.4
AMA1-S-CUP1	NA	NA	53.3 ± 0.5	62.0 ± 1.6	30.8 ± 0.7	32.4 ± 1.3	35.0 ± 0.8	37.0 ± 0.3
MXACT1-CUP1	27.9 ± 0.8	58.1 ± 27.9	50.7 ± 0.4	63.1 ± 1.6	31.1 ± 10.6	47.5 ± 11.9	47.8 ± 4.5	58.8 ± 2.0
G5AACT1CUP1	NA	NA	46.1 ± 0.2	50.9 ± 1.3	23.7 ± 2.2	28.7 ± 0.1	22.4 ± 1.3	22.0 ± 0.8
EN105-CUP1	NA	NA	92.2 ± 3.2	91.4 ± 1.2	65.7 ± 10.5	59.7 ± 7.1	73.7 ± 2.2	69.9 ± 1.4

The in vivo splicing efficiencies (percent spliced) and standard deviations for splicing construct mRNAs with (+Mer1p) and without constitutive expression of *MER1* are averages of 2-6 primer extension reactions. Splicing percentage formula is %=S/(S + U) X 100. * denotes data from Chapter 2. ** denotes data from Spingola *et al.*(2004).

The clearest trend in the splicing data presented in Table 3 is the contribution the RES complex offers to enhance splicing efficiencies of inefficiently spliced transcripts. All constructs tested with the exception of the M3A1M3 transcript demonstrate a splicing enhancement by the RES complex. However, the most remarkable example of the RES contribution to splicing remains *AMA1-CUP1* discussed in Chapter Two. The second most responsive construct to a RES deletion is the G5A-*ACT1-CUP1* transcript. While this construct is not responsive to a loss of Mer1p, the splicing efficiencies are impaired two-fold in both the *bud13*Δ and *snu17*Δ strains compared to wild-type. Both of these

observations are consistent with previous research since this construct does not contain the required Mer1p enhancer sequence, but it does contain a 5' splice site mutation that could be stabilized by the putative RES complex activity (Spingola and Ares, 2000; Dziembowski *et al.*, 2004).

With respect to a *MER1* deletion, *AMA1-CUP1*, *MER3-CUP1*, MX-*ACT1-CUP1* and M3A1M3 were the most responsive. As expected, all four transcripts contain the Mer1p enhancer sequence. Of all the transcripts analyzed it remains the *AMA1-CUP1* transcript that is the most responsive to a loss of either Mer1p or the RES complex components.

Sporulation of RES Deletion Strains

Because the primer extension data presented in Chapter Two demonstrated a requirement for the RES complex for efficient splicing of *AMA1*, it suggests a role for the RES complex during meiosis. Therefore, loss of RES components during meiosis could generate a mutant phenotype featuring decreased spore production or production of inviable spores. To test for such an expected phenotype, I created a both a *snu17*Δ homozygous diploid strain and a *bud13*Δ homozygous diploid strain from a KH46/KH52 background. These strains were monitored for the ability to sporulate efficiently. However, no difference in sporulation or tetrad formation efficiency was observed when these deletion strains were compared to the wild-type KH46/KH52 strain. In contrast, both a *nam8*Δ homozygous diploid strain and a *mer1*Δ homozygous diploid strain created from a KH46/KH52 background both showed diminished spore production.

While the RES deletion strains did form spores at a level comparable to wild-type, it remained possible the spores were defective and inviable. Deletion of *MER1*, for example, causes production of inviable spores (Engebrecht and Roeder, 1990). To test for spore viability in the *bud13*Δ and *snu17*Δ diploid strains, these strains were grown in a liquid sporulation media (1% KoAC, pH 7.0) for 3-5 days and treated with lyticase. Using a micromanipulator, tetrads were dissected and the spores germinated on rich media plates. Table 4 summarizes these results. In both strains tested, many of the tetrad dissections resulted in either 100% or 75% germination rates, that is, 4 or 3 spores survived per tetrad. As such, these strains produce functional spores and no meiotic phenotype is apparent. In summary, the loss of RES components during meiosis does not impair either sporulation efficiency or spore viability.

In a final effort to establish a meiotic function for the RES complex, I questioned whether the splicing efficiencies observed with the plasmid based *AMA1-CUP1* and *MER3-CUP1* constructs during mitosis could be replicated during meiosis with the endogenous *AMA1*. To accomplish this task a meiotic time-course assay was performed to collect yeast samples as the steps of sporulation were taking place. For each meiotic time point collected, total RNA was extracted and the splicing efficiencies of *AMA1*, *MER2*, and *MER3* were measured in wild-type and RES deletion strains.

Table 4. *snu17*Δ and *bud13*Δ spore viability

Strain	Viable Spores per Tetrad	Tetrads
*snu17*Δ	4 (100)%	10
	3 (75%)	8
26 tetrads dissected	2 (50%)	3
	1 (25%)	4
	0 (0%)	1
*bud13*Δ	4 (100)%	6
	3 (75%)	4
14 tetrads dissected	2 (50%)	2
	1 (25%)	2
	0 (0%)	0

To promote synchronization of the yeast cells for a near simultaneous entry into meiosis, the diploid deletion strains (*snu17*Δ*, bud13*Δ*, nam8*Δ, and *mer1*Δ) and control strains (KH46/KH52 and *bud13*Δ*MAT*a/*snu17*Δ*MATα*) were grown on glycerol plates. From these plates, 5 ml YPD cultures were grown overnight and then transferred to YPA media prior to treatment with sporulation media. A control aliquot was collected during the YPD growth and seven more culture aliquots were collect at time points during the sporulation media growth. Purified total RNA was used to create cDNA, which served as a template for semi-quantitative PCR. This low cycle PCR generated products just visible on ethidium bromide stained agarose gels. See Figure 3.

Splicing activation was not observed for the *AMA1*, *MER2*, and *MER3* transcripts contained in the YPD (no sporulation) negative controls. This agrees with the meiotic expression profile reported for Mer1p (Engebrecht and Roeder, 1990). Also, only very faint bands representing unspliced *AMA1* or *MER3* could be seen. In contrast, PCR products representing unspliced *MER2* appeared in much higher quantities. Again this corresponds with previously reported expression profiles for these transcripts; *MER2* is expressed during both mitosis and meiosis, but *AMA1* and *MER3* are only upregulated during meiosis (Engebrecht *et al.*, 1991; Davis *et al.*, 2000; Nakagawa and Ogawa, 1999). Also as expected, in the *mer1*Δ and *nam8*Δ strains, all three transcripts failed to splice (Spingola and Ares, 2000).

The control strains used in this time course assay do demonstrate meiotic regulated splicing; see Figures 3 A, B, and C. Splicing levels peak between 9 and 24 hours after transfer to sporulation media. Similarly, in the RES complex deletion strains, *snu17*Δ and *bud13*Δ, meiotic splicing activation is apparent. In the cases of the *MER2* and *MER3* transcripts (Figures 3 B and C) splicing efficiencies match or exceed the levels observed in the control strains. Yet in the case of the *AMA1* transcript, a reduction in splicing levels could be observed in both the *bud13*Δ and *snu17*Δ strains compared to the wild-type strains.

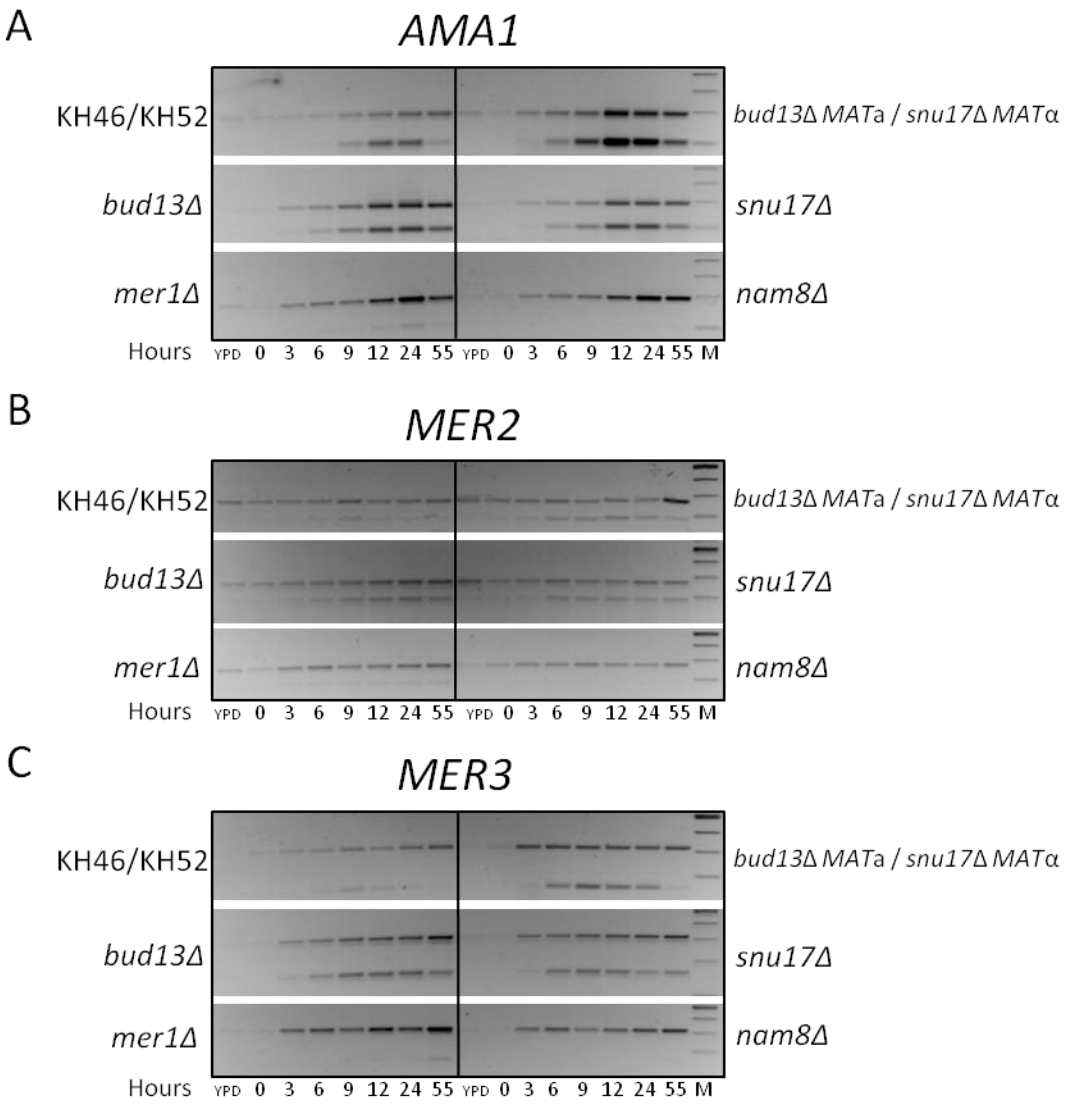

Figure 3. Meiotic time-course assay demonstrating regulated splicing of *AMA1, MER2,* and *MER3* transcripts. Aliquots from yeast cultures in four deletion strains (***bud13***Δ*, **snu17**Δ*, **mer1**Δ*, **nam8**Δ*) and two control stains (KH46/KH52, ***bud13***Δ***MATa***/***snu17***Δ***MATα***) were removed and frozen at hourly time points after transfer to sporulation media. Purified total RNA from aliquots was reversed transcribed into cDNA using primers complmentary to 3' exons. The cDNA served as a template for a semi-quantitative PCR reaction. PCR products were run on 2% agarose gels stained with ethidium bromide. Panels A, B, and C contain PCR products using primers specific for transcripts *AMA1, MER2,* and *MER3* respectively. YPD = a time point taken during growth in rich media. Hours = time after transfer to sporulation media. M = 100 bp marker. In all panels the top band is a PCR product representing unspliced pre-mRNA. A second bottom band (if any) represents spliced pre-mRNA.

Discussion

As yeast enter meiosis many genes are upregulated in order to support the pathways of spore formation and recombination. However, additional fine tuning of meiotic gene expression is achieved by regulated splicing. Indeed, some yeast genes are expressed during mitosis, but it is only during meiosis that their transcripts are properly spliced and permit translation of essential sporulation proteins. For example, *MER2* contains an intron that is only removed during meiosis when Mer1p is expressed. Once spliced, the *MER2* transcript codes for a protein essential for initiation of meiotic recombination. Loss of Mer2p prevents double-strand breaks and the assembly of synaptonemal complexes (Rockmill *et al.,* 1995). Since expression of such a protein during mitosis could be harmful, this meiotic splicing regulation plays a vital role for proper gene expression. During mitosis, when *MER2* transcripts are not spliced, the pre-mRNA is rapidly eliminated by the nonsense-mediated decay process upon its export to the cytoplasm (He *et al*., 1993).

The extent to which splicing regulation controls meiotic gene expression is surprising. Table 5 lists the intron containing genes with a meiosis-specific function. When expressed during mitosis, all of these genes are spliced inefficiently, yet when measured under sporulation conditions, the splicing levels approach 100 percent (Juneau *et al.,* 2007). Perhaps the best example of meiotic regulation imposed by splicing involves the formation of double-stranded breaks (DSBs). While DSBs play a vital role in recombination, they also promote an ordered distribution of homologous chromosomes during Meiosis I. Loss of DSB formation leads to aneuploidy and production of inviable spores. Besides *MER2*, nine other genes are necessary for meiotic DSB formation and three (*REC114, MEI4, REC102*) of these nine genes contain introns (Li *et al.,* 2006). Therefore, 40% of the genes required for meiotic DSB formation contain introns and are spliced inefficiently during mitosis. This low splicing efficiency would effectively prevent DSB formation during mitosis. Furthermore, three of these four intron containing DSB genes code for proteins that isolate in a distinct complex. Mer2p, Mei4p and Rec114p co-localize together, interact via the two-hybrid assay, and co-immunoprecipitate together (Li *et al.,* 2006). In summary, meiotic splicing regulation controls key steps of recombination. Because an entire DSB complex requires meiotic-specific splicing regulation, it minimizes risks of DSB formation during mitosis.

Of the 13 transcripts listed in Table 5 many have sequence variations that justify their low splicing efficiencies observed during mitosis. For example, *MER2, MER3, HOP2,* and *SPO1* possess non-canonical 5' splice sites. Also *MER3, SPO22, MND1, PCH2, SAE3,* and *SPO1* have unusual branch point sequences and *SPO22, REC114, REC102,* and *SAE3* have non-consensus 3' splice sites (Spingola *et al,* 1999). Furthermore, the transcripts *AMA1, REC114,* and *PCH2* contain extended 5' exons which are known to impair splicing efficiencies (Spingola and Ares, 2000). Since the splicing efficiencies of transcripts listed in Table 5 all improve during meiosis, this strongly suggests that meiotically expressed splicing factors serve to improve conditions for spliceosome formation around these poor splicing signals and thereby regulate sporulation.

Table 5. Meiotically Expressed Genes Subject to Splicing Regulation

Official Name	Common Name	Function	ORF Size	Intron Location	Intron Size	Features 5' SS BP 3'SS	% Splicing Efficiency During Mitosis
YGR225w	AMA1	Anaphase Promoting Complex	1875	1184-1276	93	GUACGU AUACUAACAAAU UACAG	4.8
YJR021c	MER2	meiotic recombination	1025	317-396	80	GUUCGU UUACUAACAACU UAUAG	14.9
YGL251C	MER3	meiotic helicase	3716	59-210	152	GUAGUA UGACUAACAUUG UAUAG	0.0
YIL073C	SPO22	meiotic and chromosome synapsis function	3018	56-145	90	GUAUAU CAACUAACAGCU UAAAG	9.8
YGL033W	HOP2	ensures correct synapsis between homologs	727	56-125	70	GUUAAG UUACUAACAAUU AUCAG	22
YGL183C	MND1	meiotic recombination, complexes with Hop2p	743	4-86	83	GUAUGU ACACUAACUUAU AUUAG	40.1
YMR133W	REC114	meiotic recombination	1403	1243-1358	116	GUAUGU AUACUAACUAAC AAAAG	89.0
YLR329W	REC102	meiotic recombination, chromosome synapsis	892	175-271	97	GUAUGU UUACUAACUAUA UGAAG	31.9
YBR186W	PCH2	patchytene checkpoint protein	1808	1552-1664	113	GUAUGU UCACUAACUGUC UAUAG	21.1
YER179W	DMC1	meiotic repair of double-stand breaks	1097	133-224	92	GUAUGU UUACUAACUAAU UAUAG	51.0
YER044C-A	MEI4	meiotic recombination	1315	64-151	88	GUACGU UUACUAACUUUU GACAG	11.3
YHR079C-A	SAE3	meiotic recombinataion	362	114-199	86	GUAUGU UUAUUAACAGAA CAAAG	37.2
YNL012W	SPO1	meiotic spindle pole body duplication	1980	106-189	84	GUAAGU AAACUAACCGAA AUUAG	0.0

Data for table compiled from SGD, Juneau *et al.,* 2007, and personal communication with Kara Juneau.

One such factor, Mer1p, will enhance splicing efficiencies of at least three transcripts (*AMA1*, *MER2*, and *MER3*) listed in Table 5. Another splicing factor, Nam8p is inessential during mitosis, but it is required for efficient splicing of these same three transcripts (Spingola and Ares, 2000). Recently, Nam8p was identified as contributing to the splicing efficiency of another meiotically expressed transcript *SRC1*. The spliced transcript codes for a protein that directs sister chromatid segregation. While *SRC1* plays a role during mitosis and it cannot be considered "meiosis-specific", it is upregulated 5-fold during meiosis. Its dependence on Nam8p for splicing is likely related to its non-canonical 5'splice site (similar to *MER2* or *MER3*) and its lengthy 5' intron (similar to *AMA1* and *REC114*) (Rodrigues-Navarro *et al.*, 2002). However, because the enhanced splicing efficiencies of the majority of transcripts in Table 5 cannot be explained by Mer1p or Nam8p activity, additional meiotic splicing factors are likely undiscovered.

The initial characterizations of RES components Bud13p and Snu17p qualified these proteins as potential meiotic splicing regulators. Not only do they have enhanced expression levels during meiosis, but primer extension data from Chapter Two demonstrated their requirement for the efficient splicing of *AMA1* (Primig *et al.*, 2000). Also the proposed activity for the RES complex predicts it functions to enhance the splicing efficiencies of transcripts with defective splicing signals common to those in Table 5 (Dziembowski *et al*, 2004).

To determine whether the RES complex has a bona fide meiotic splicing regulatory role, I tested whether components of the RES complex are required for meiotic splicing to such a degree that their loss creates a meiotic phenotype. However, tetrad analysis of *BUD13* and *SNU17* deletion strains does not suggest a sporulation defect. In contrast, a mitotic phenotype for the deletions of these genes has been identified. Loss of either gene will cause unipolar and elongated bud formation (Ni and Snyder, 2001).

While the meiotic time-course assay confirmed the requirement for Mer1p and Nam8p during sporulation, the absolute need for Bud13p or Snu17p was not demonstrated. Loss of Bud13p or Snu17p only impaired *AMA1* splicing efficiencies to a limited degree. Poor synchronization of the KH46 strain during sporulation may explain this discrepancy between the meiotic and mitotic experiments, but a more likely explanation centers on sequence differences between the endogenous *AMA1* and the *AMA1-CUP1* construct. It is possible the long 5' exon of *AMA1* diminishes the RES requirement observed previously.

Though an essential meiotic role for the RES complex was not demonstrated, the splicing data presented in Table 3 does support an activity for the RES complex in enhancing the splicing efficiencies of transcripts that contain non-canonical splice site sequences. This minimal contribution by the RES complex during mitosis and meiosis may only generate a noticeable advantage to fitness in a natural setting were intense competition from other yeast and microorganisms makes it more apparent. The budding defect observed in *BUD13* and *SNU17* deletion strains and a slow growth phenotype reported for *snu17*Δ agree with this possibility (Ni and Snyder, 2001; Gottschalk *et al.*, 2001).

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Chapter Five

Pre-mRNA Export and Retention

A long standing question in yeast biology has been: what is the fate of an unspliced pre-mRNA? This was first addressed by the Rosbash Lab in 1989 and a number of times since then (Legrain and Rosbash, 1989; Rutz and Seraphin, 2000; Bousquet-Antonelli *et al.*, 2000; Hilleren and Parker, 2003; Galy *et al.* 2004). It remains a difficult question to answer directly because isolation of mRNA specifically from the nucleus or the cytoplasm is difficult to achieve due to the harsh conditions necessary to disrupt the yeast cell wall. While fluorescent in situ hybridization (FISH) assays have been utilized, the overexpression needed to generate necessary required signal intensities could overwhelm possible nuclear retention mechanisms (Long *et al.* 1995). As a result, some research groups have suggested that a pre-mRNA retention system functions to block pre-mRNA nuclear export (Bousquet-Antonelli *et al.*, 2000; Galy *et al.*, 2004; Palancade *et al.*, 2005), while other groups provide evidence that unspliced mRNAs undergo decay in the cytoplasm (He *et al.*, 1993; Hilleren and Parker, 2003).

Experiments in Chapter Two questioned whether Mer1p functioned to retain pre-mRNAs in the cytoplasm independently of its ability to enhance splicing efficiencies. It was demonstrated that an apparent mRNA retention activity of Mer1p could not be uncoupled from its splicing function. One method of questioning Mer1p's pre-mRNA retention activity relied on creating both splicing and export reporters which allowed for yeast growth on copper containing media plates (see Figure 2, Chapter Two). Of any experiments conducted during this research effort, I believe the implications resulting from these splicing and export reporters are the most significant. For example, Figure 3A in Chapter Two was conducted during mitosis and shows yeast growth (without *MER1* expression) on copper containing plates. Because the yeast strain used in this figure is *cup1*Δ, the growth on the copper plates was supported solely by the plasmid based *AMA1-CUP1* export reporter. This reporter was constructed such that only unspliced transcripts will code for functional Cup1p. This assay, therefore, clearly demonstrates that unspliced mRNAs are exported to the cytoplasm. Furthermore, similar export reporters using *MER2-CUP1* and *MER3-CUP1* fusions also allowed for yeast growth and this served to generalize this observation of unspliced pre-mRNA export (see Figure 3C-Chapter Two).

When the primer extension splicing data for these reporters is compared to the growth on the copper plates, another interesting observation can be made: a large proportion (if not all) of the unspliced reporter transcripts are exported to the cytoplasm. Table 1 below presents the data from Figure 2-Chapter Two supporting this conclusion. The range of copper tolerances that support yeast growth can be inferred from the splicing reporter. When 35% splicing is observed, no growth on copper occurs. Yet when 69% of the transcripts are spliced, robust growth is observed. In the case of the export reporter (which requires unspliced transcript translation for copper resistance), when 37% splicing is observed the remaining 63% of the total transcripts are unspliced and could be

available for translation. Because robust growth is observed, it suggests a large proportion (> 58%) of the unspliced transcripts must export simply to exceed the no growth level defined by the 35% splicing level of the splicing reporter. Since healthy growth was observed, even a higher percentage of unspliced transcripts must have been exported and translated. For the case of the export reporter where Mer1p is present, a 63% splicing efficiency leaves the remaining 37% of transcripts unspliced and available for translation. Here robust growth is not supported, but the threshold of copper resistance is nearly achieved for yeast growth as some growth can be observed; see Chapter Two Figure 3C AMA1-E (Mer1p +). Again because the splicing reporter at a 35% splicing level defines a no growth boundary, a very large percentage, arguably 100% of the unspliced exporter reporter transcripts must be exported to reach the growth threshold.

Table 1. Comparison of Growth and *CUP1* Construct Available for Possible Export

Construct	Mer1p	Splicing %	% *CUP1* Construct Available For Translation	Growth on 150 uM $CuSO_4$ Media Plates
AMA1-CUP1 Splicing Reporter	+	35	35	No
AMA1-CUP1 Splicing Reporter	-	69	69	Yes
AMA1-CUP1 Export Reporter	+	63	37	Threshold
AMA1-CUP1 Export Reporter	-	37	63	Yes

Figure 1 below gives another indication of the growth threshold observed for the *AMA1-CUP1* export reporter with *MER1* on 150 uM $CuSO_4$ media plates. With this titration of copper concentrations it is clear that the exporter reporter with *MER1* will grow on 100 uM $CuSO_4$ and it is nearly growing on 150 uM $CuSO_4$, which suggests a large percentage of the 37 % of the transcripts that are unspliced are being exported to support this growth pattern.

This evidence that a large percentage of the unspliced pre-mRNA population is being exported and translated is significant for several reasons. First it suggests these transcripts are not only evading a nuclear retention system, but it suggests the transcripts are *effectively* evading nuclear retention. Because three transcripts follow this pattern, this evidence casts doubt altogether on a dedicated splicing-independent retention mechanism (Galy *et al,* 2004; Dizembowski *et al.,* 2004*;* Palancade *et al.,* 2005). Furthermore, prior work by the Rosbash lab using a similar export reporter strategy led to the conclusion that unspliced transcripts "leak" to the cytoplasm if they fail to undergo splicing. By their estimates only 5% of an inefficiently spliced mRNA would export to the cytoplasm (Legrain and Rosbash, 1989). In stark contrast, the evidence provided with the *AMA1-CUP1* export reporter suggests an unhindered flood of unspliced transcripts exports to the cytoplasm. These very different results reported with the two reporter systems could mean a pre-mRNA retention mechanism is transcript specific. Yet because the results observed with the *AMA1-CUP1* export reporter are supported by similar results using *MER2-CUP1* and *MER3-CUP1* reporters it suggests that the export reporter utilized in the Rosbash study maybe more of an anomaly rather than a rule. This would be an interesting situation because virtually every subsequent publication that has identified proteins with pre-mRNA retention activities has used the construct originally

created by the Rosbash effort. If this construct behaved abnormally compared to typical yeast constructs, then the results from a number of studies could be called into question. In the pages below I will review these reports that provide evidence both for and against a nuclear pre-mRNA retention mechanism. This topic is of particular interest because a number of new yeast introns have been identified in the last two years and it was recently reported that 45 yeast transcripts either splice inefficiently or not at all during normal growth conditions (Miura *et al.*, 2006; Zhang *et al.*, 2007; Juneau *et al.*, 2007). Therefore, determining the fate of unspliced pre-mRNA remains important for understanding regulation of yeast gene expression.

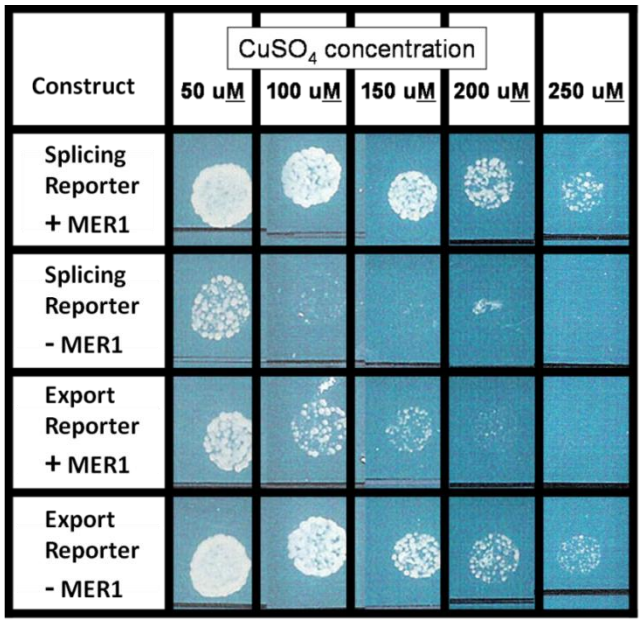

Figure 1. *AMA1-CUP1* splicing and export reporters and growth on a range of copper concentrations. KH46 strains containing the splicing reporter or export reporter along with or without a *MER1* plasmid were grown media plates containing a range of copper concentrations. Growth with strains containing the export reporter require translation of unspliced pre-mRNA. See Chapter Two Materials and Methods for assay details.

Evidence Supporting a Nuclear Retention System

By all accounts the Legrain and Rosbash (1989) report was and remains a landmark study questioning the fate of unspliced mRNA. Interestingly, however, other research groups have cited this study in support of findings both for and against pre-mRNA retention systems (Bousquet-Antonelli *et al.*, 2000; Galy *et al.*, 2004; Dziembowski *et al.*, 2004; Hilleren and Parker, 2003). This is possible because the study generated novel findings, which could be interpreted several ways. Using a series of *LacZ* reporters the authors demonstrated that only a very small percentage of their model pre-mRNA (unspliced) would export to the cytoplasm. This is the normal situation for most yeast transcripts because they splice with very high efficiencies, yet the intron containing reporters in the Legrain and Rosbash study spliced with only 10-20% efficiency. So while it appeared a large pool of unspliced transcripts would be available for export and translation, the β-galactosidase assays suggested that 95% of the available unspliced transcripts were sequestered in the nucleus (Legrain and Rosbash, 1989).

Deletion of the 5' splice site or the branch point sequence of the *LacZ* reporter, however, caused a dramatic increase in the export of the unspliced transcripts with a β-galactosidase activity 55-65% of the wild-type reporter. Smaller increases were noted for deletions to the 3' splice site or for the region between the 5' splice site and the branchpoint. It was further observed that a mutation to the U1 snRNA, *PRP6* or *PRP9* would cause increases to the intron containing reporter's β-gal activity levels. The U1 snRNA mutation involved a nucleotide important for 5' splice site binding and also resulted in loss of splicing efficiency in a *LacZ* splicing reporter. The heat sensitive *PRP6* and *PRP9* mutations caused large increases in unspliced transcript export, a loss of viability and produced a complete block to splicing (Legrain and Rosbash, 1989). Though not known at the time, *PRP6* codes for a tri-snRNP protein, and *PRP9* codes for a U2 snRNP SF3a protein (Abovich *et al.,* 1990).

The results generated from this study suggested the following conclusions: splicing signals and splicing factors work together to retain pre-mRNA in the nucleus, and pre-mRNAs are primarily retained in the nucleus even in the absence of splicing. This led to a model whereby the spliceosome served as the nuclear retention mechanism. It was proposed that early splicing factors such as the U1 snRNP or Prp6p would bind key intronic sequences and sequester the pre-mRNA in the nucleus until the active spliceosome formed and removed the intron. In the case of an inefficiently spliced transcript, these factors would act to prevent the pre-mRNA nuclear exit even though the active spliceosome failed to form (Legrain and Rosbash, 1989).

In the following years, several more studies expanded upon this Spliceosome Retention model. For example, studies exploring nonsense-mediated decay (NMD) demonstrated that pre-mRNA, which "escaped" the nucleus, could be rapidly destroyed if their introns contained a nonsense codon or induced a frameshift resulting in an in-frame nonsense codon (He *et al*., 1993). A variety of mRNA substrates for nonsense-mediated decay were recognized and it was proposed that NMD functioned as a cytoplasmic surveillance mechanism to eliminate aberrant transcripts that came from the nucleus (Gonzalez *et al.*, 2001).

Using the *LacZ* splicing and export reporters created in the Legrain and Rosbash (1989) study, the Mud2p splicing factor was analyzed by Rain and Legrain (1997). They concluded that Mud2p participated to a greater degree in pre-mRNA retention than splicing. It was also demonstrated that many sequences in the branchpoint region were important for pre-mRNA retention. Another group used these *LacZ* reporters while characterizing the Branchpoint Bridging Protein (BBP) and also concluded that BBP played a greater role in pre-mRNA retention than with spliceosome formation. Also a synthetic lethal relationship between *MSL5* (gene coding for BBP) and *UPF1* (a gene required for NMD activity) was reported (Rutz and Seraphin, 2000). Therefore, a picture was emerging where known splicing factors functioned to retain pre-mRNAs in the nucleus. In the event of pre-mRNA leakage to the cytoplasm, a surveillance mechanism, NMD, would act to decay these unspliced transcripts and prevent their translation. The synthetic lethal relationship between components of the nuclear and cytoplasmic quality

control mechanisms suggested they worked in harmony to police the gene expression machinery.

A detailed explanation describing the fate of a nuclear retained pre-mRNA was not proposed in the original study by Legrain and Rosbash (1989) and it was not until a decade later that a nuclear based pre-mRNA decay mechanism was identified (Bousquet-Antonelli *et al.,* 2000). Research on cytoplasmic mRNA decay during the 1990s had uncovered two main pathways of decay. The major pathway required decapping and 5'-3' decay by Xrn1p and the minor mRNA decay pathway occurred in a 3'-5' direction mediated by the exosome (Muhlrad *et al.,* 1994). The exosome is a protein complex containing at least nine exoribonucleases and it is found both in the cytoplasm and the nucleus. By the year 2000, the nuclear exosome had been linked with decay of several types of RNA including: ribosomal, small nucleolar, small nuclear and pre-rRNA spacer fragments, but not pre-mRNA (Bosquet-Antonelli *et al.,* 2000). At that time, however, Bousquet-Antonelli provided evidence that the nuclear exosome played an important role with quality control of nuclear pre-mRNA. In the event of a splicing block or other nuclear retention activity, the nuclear exosome would quickly act to eliminate the pre-mRNA and even mature RNA. It was suggested that the nuclear exosome acted in competition with the spliceosome to process pre-mRNAs. Either the spliceosome would form around a pre-mRNA and the spliced product would be exported or the nuclear exosome would act to degrade the pre-mRNA (Bousquet-Antonelli *et al.,* 2000).

This report acted to further a growing theory that proposed an active nuclear retention and decay mechanism for unspliced pre-mRNAs and other improperly processed mRNAs (Maquat and Carmichael, 2001; Jensen *et al.,* 2003). It also complemented the growing understanding of mRNA export and the need to package a fully processed mRNA into a export competent mRNP (Stutz and Izaurralde, 2003). Other research groups subsequently identified specific pre-mRNA retention factors that presumably worked in conjunction with the exosome activity (Galy *et al.,* 2004; Dziembowski *et al.,* 2004; Palancade *et al.,* 2005). However, this study by Bousquet-Antonelli was refuted by Parker and Hilleren (2003) who made the case that most pre-mRNAs that are not spliced are instead exported to the cytoplasm. Yet, beside the evidence provided by Hilleren and Parker (discussed on page 63), there are several other weaknesses and inconsistencies with this report from Bousquet-Antonelli.

By examining steady state mRNA levels with Northern assays the Bousquet-Antonelli group observed an increase to pre-mRNA levels in an exosome mutant strain (*GAL::rrp41*) and a dramatic decrease in spliced mRNA with little change to pre-mRNA levels in a splicing deficient strain (*prp2-1*). In a combined mutant strain (*GAL::rrp41, prp2-1*) they observed a large decrease in spliced mRNA and a dramatic increase in pre-mRNA. From these observations and others they concluded that the nuclear exosome rapidly decays pre-mRNA. One weakness with this conclusion is that two other studies observe significant increases to pre-mRNA levels in a *prp2-1* mutant strain (Sapra *et al.,* 2004; Pleiss *et al.,* 2007). A second problem involves the reliance on the *GAL*::*rrp41* strain. Since Rrp41p is a component of both the cytoplasmic and nuclear exosomes, the documented increase to unspliced and spliced transcripts could result primarily from a

disruption to the cytoplasmic exosome, which would imply the unspliced transcripts are leaving the nucleus. Recognizing this obvious objection to their work, they support their findings by reporting the expression pattern of the *CYH2* transcript in same mutant backgrounds, as well as, a NMD mutant (*upf1*Δ) (Bousquet-Antonelli *et al.*, 2000). *CYH2* pre-mRNA is subject to cytoplasmic nonsense-mediated decay and therefore leaves the nucleus (He *et al.,* 1993). Because Bosquet-Antonelli demonstrates an increase of *CYH2* pre-mRNA in a NMD mutant, a decrease of *CYH2* spliced mRNA in the splicing mutant and loss of both spliced and unspliced product in the double mutant (*prp2-1, upf1*Δ), the research group concludes the nuclear exosome must be responsible for the decay. However, they fail to justify or explain any mechanism for the retention of the *CYH2* pre-mRNA in the nucleus other than the splicing block. Unfortunately, it is not obvious that a splicing block would sequester this pre-mRNA species which is believed to avoid commitment complex formation and efficiently export to the cytoplasm (He *et al.,* 1993; Swida *et al.* 1988). Therefore, some of the primary conclusions of this study concerning the existence of a regulated and robust nuclear pre-mRNA turnover mechanism are left in question.

Nonetheless, some other research groups did accept these findings and continued to provide support for a dedicated nuclear pre-mRNA retention and decay mechanism that functions in addition to the spliceosome (Galy *et al*, 2004; Casolari and Silver, 2004; Saguez *et al.,* 2005; Akhtar and Gasser, 2007). Specifically, an additional three pre-mRNA nuclear retention factors have been identified in yeast since the Bosquet-Antonelli paper proposed a specific function for the nuclear exosome with pre-mRNA decay. These nuclear proteins (Mlp1p, Pml1p and Pml39p) are unlike the previously identified splicing factors that have retention-like qualities because these recently characterized proteins have no reported splicing roles. Pml1p is part of the RES complex along with the splicing factors Bud13p and Snu17p. Mlp1p and Pml39p are proteins associated with the periphery of the nuclear pore complex (Galy *et al.,* 2004; Dziembowski *et al.*, 2004; Palancade *et al.,* 2005). Interestingly, the primary assay used to justify each of these protein's pre-mRNA retention activities is the β-gal export and splicing reporter system developed by the Rosbash lab in 1989. As mentioned above, these reporters contain a synthetic intron that has a very low splicing efficiency (10-20%) and results in a large accumulation of unspliced transcript.

I suggest that the repeated use of this export assay with its peculiar inefficiently spliced transcript may lead to excessive or false claims of pre-mRNA retention activities. For example, it is possible this *LacZ* reporter contains specific *cis* sequences besides the known splicing signals that control its splicing patterns or specific nuclear sequestration. Other intron bearing transcripts such as *RPL30* or *YRA1* are known to undergo autoregulation that controls their splicing efficiencies (Warner *et al.,* 1985; Vilardell and Warner, 1997; Preker *et al.,* 2002). Yet beyond the assay used to determine a pre-mRNA retention factor, there are several other inconsistencies with the notion of a dedicated pre-mRNA nuclear retention and decay system. First, if the exosome rapidly decays pre-mRNAs that fail to undergo splicing, then large accumulations of unspliced pre-mRNAs should not appear on Northern blots. Yet the Rosbash *LacZ* reporter and other transcripts do accumulate as the unspliced version. Second, Mlp1p, the first splicing independent

pre-mRNA retention factor identified, has numerous other reported activities including: sumoylation, telomere silencing, transcription regulation, Nab2p docking, and Npl3p docking (Zhao *et al.,* 2004; Galy *et al.,* 2000; Green *et al.,*2003; Casolari *et al.,* 2005). Furthermore, Mlp1p also interacts with a very similar filamentous protein, Mlp2p, that also has been identified with many other diverse activities (Niepel *et al.,* 2005). While this non-essential protein appears to play a role contributing to gene expression, these additional reported transcriptional activities diminish the probability that the primary role of the Mlp1p involves pre-mRNA retention as reported by Galy *et al.*(2004). Any retention activity by these nuclear periphery proteins is likely a secondary or tertiary effect. Supporting this notion, a recent report suggested the docking function of Mlp1p for Nab2p and Nlp3p was related more generally to mRNP quality control including transcripts without introns. It was demonstrated that Mlp1p could retain mRNPs in an export mutant background (*yra1-8*). These data support a more general quality control function over mRNP export by functioning as a transient docking platform for mRNPs ready for export rather than the specific retention of intron containing transcripts (Vinciguerra *et al.,* 2005).

Still other published data conflicts with a nuclear pre-mRNA retention model that is separate from the established splicing mechanisms. For example, while yeast and metazoans share a surprisingly similar export system, there is no evidence for a pre-mRNA retention model in metazoans (Reed and Hurt, 2002). Also, though much more common in humans, alternative splicing does exist in yeast. The Saccharomyces Genome Database recognizes 10 transcripts with two introns and at least three more are indentified in other studies (Hong *et al.,* 2008; Davis *et al.,* 2000; Miura *et al.,* 2006). An active retention and decay system in yeast should allow only transcripts with all introns removed to exit the nucleus, but splicing data suggests that alternative splicing patterns do exist (Miura *et al.,* 2006). Finally, there are a number of examples that are presented below that suggest in the absence of splicing, intron containing transcripts exit the nucleus.

Evidence for Pre-mRNA Export

The data generated from the *Ama1-Cup1* export reporter assay in Chapter Two demonstrate that pre-mRNAs which fail to splice will exit the nucleus. This finding is inconsistent with the recently proposed pre-mRNA retention and decay mechanism consisting of the nuclear exosome, Mpl1p, Pml1p, Pml39p, and other proteins (Sommer and Nehrbass, 2005). Interestingly, a search of the literature provides data that suggests other unspliced yeast transcripts readily avoid proposed nuclear retention mechanisms. For example, a research effort that questioned whether the nonsense-mediated decay system recognizes nonsense codons in yeast introns revealed that several yeast pre-mRNAs are indeed substrates for nonsense-mediated decay. This demonstration consisted of Northern blot shut-off assays conducted in a wild-type and a nonsense-mediated decay deficient (*upf1*Δ) yeast strain. A significant pre-mRNA accumulation for the transcripts *CYH2, MER2,* and *RP51B* occurred when NMD was inactivated and resulted in dramatic increases to their half-lives. Also, while the *CYH2* and *RP51B* transcripts appear to splice efficiently in the wild-type strains, the large pre-mRNA accumulation in the *upf1*Δ strain suggests instead that the splicing is actually inefficient

(He *et al.*, 1993). Because NMD is a cytoplasmic event, the large pre-mRNA accumulations in the *upf1*Δ strain demonstrate an unfettered pre-mRNA nuclear export (Atkin *et al.* 1997; Maderazo *et al., 2003*; He *et al.*, 1993). Proponents of the pre-mRNA Retention and Decay model suggest that pre-mRNA retention will require intact 5' splice site and branch point sequences; while *MER2* has a non-consensus 5' splice site, *CHY2* and *RP51B* (and also *AMA1*) have consensus splicing sequences (Bousquet-Antonelli *et al.*, 2000; Galy *et al.*, 2004; Hong *et al.*, 2008).

Nonsense-mediated decay was originally recognized in a yeast strain harboring *URA3* mutant alleles that contained nonsense mutations within the ORF (Losson and Lacroute, 1979). Since that time, the NMD mechanism was observed to act upon nonsense codons within pre-mRNA introns and transcripts undergoing leaky scanning or that contain extended 3' UTRs or upstream ORFs (Gonzalez *et al.*, 2001). An effort to identify the extent of NMD regulation in yeast was made by Levivelt and Culbertson (1999). They tested 6218 yeast transcripts and identified 529 mRNAs that are significantly upregulated in NMD deficient strains. On average the mRNA abundance increased 2.4 fold when NMD was disabled. 27 of these 529 transcripts contain introns and are listed in Table 2. Since significant export of an unspliced transcript containing a nonsense codon in the intron could explain the NMD regulation, I examined these 27 transcripts and found in-frame intronic nonsense codons in every case but one. The single exception, *MTR2*, is an unusual example because the intron is upstream of the ORF. Table 2, therefore, provides 26 candidate transcripts from the Levivelt and Culbertson study whose expression patterns in nonsense-mediated decay mutant strains suggest these transcripts in the unspliced form are being exported to the cytoplasm in large numbers. Also provided in Table 2 are six additional intron-containing transcripts identified in a more recent large scale study documenting NMD regulation (He *et al.*, 2003).

To be clear, the data in Table 2 is not direct proof that unspliced transcripts are leaving the nucleus in large numbers; instead it is merely suggestive that this is occurring. To confirm that this nonsense-mediated decay microarray data reflects significant pre-mRNA nuclear export, Northern blots using intronic probes should be performed in wild-type and NMD mutant strains. A large signal increase for the NMD deficient strains compared to wild-type would indicate unrestricted pre-mRNA nuclear export. Interestingly, however, there already exists data that further suggest these NMD-regulated intron-containing transcripts of Table 2 are exported unspliced to the cytoplasm. Juneau and colleagues recently performed an extensive yeast-tiling microarray in an effort to indentify novel introns. In the course of validating their assays, they used RT-PCR to measure splicing efficiencies of both known and suspected intron-containing transcripts. Their data identify 45 yeast transcripts that are inefficiently spliced during mitosis (Juneau *et al.*, 2007). Table 3 lists these yeast transcripts and their splicing efficiencies (K. Juneau, personal communication). Because 14 of the 33 NMD regulated transcripts of Table 2 were identified by Juneau *et al.* (2007) as inefficiently spliced, this combined data does suggest many of transcripts are effectively evading a pre-mRNA nuclear retention and decay mechanism. Table 3 shows that nine of these transcripts do not splice during mitosis and yet they are substrates for the cytoplasmic NMD regulation. It is also worth noting that NMD regulation can be so effective against

unspliced transcripts that Northern blots, primer extension, or RT-PCR splicing data may report higher than actual splicing efficiencies. The *CYH2* transcript is known example; in wild-type strains the splicing efficiency approaches 100%, yet in a *upf1*Δ strain, this efficiency is closer to 50%. It remains possible then that transcripts listed in Table 2, but not listed in Table 3 could still have high percentages of unspliced transcripts being exported to the cytoplasm and subjected to rapid decay by NMD in a manner similar to *CYH2* (He *et al.*, 1993).

Table 2. Intron Containing Transcripts Subject to Nonsense-Mediated Decay

Official Name	Common Name	Function	ORF size	Intron Location	Intron Size	Features	First Nonsense Codon Location	Reference
YML133c	unchar	mitocondrial location	4224	795-893	99	GCAUGU UACUAAC CAG	814-816	Lelivelt and Culbertson, 1999; SGD
YNL162w	RPL42A	protein in 60s rib subunit	833	5-516	512	GUAUGU UACUAAC UAG	13-15	Lelivelt and Culbertson, 1999; SGD
YLL067c	unchar	possible helicase	3717	288-386	99	GCAUGU UACUAAC CAG	307-309	Lelivelt and Culbertson, 1999; SGD
YFL034c-A	RPL22B	protein in 60s rib subunit	690	13-333	321	GUACGU UACUAAC CAG	19-21	Lelivelt and Culbertson, 1999; SGD
YHL050c	unchar	possible helicase	2866	642-1413	772	GCAUGU UACUAAC CAG	1066-1068	Lelivelt and Culbertson, 1999; SGD
YLL066c	unchar	possible helicase	3717	288-386	99	GCAUGU UACUAAC CAG	307-309	Lelivelt and Culbertson, 1999; SGD
YPL283c	YRF1-7	helicase	5728	20-167	148	GUACGU UACUAAC UAG	64-66	Lelivelt and Culbertson, 1999; SGD
YGR296w	YFR1-3	helicase	5728	20-167	148	GUACGU UACUAAC UAG	64-66	Lelivelt and Culbertson, 1999; SGD
YNL339c	YFR1-6	helicase	5728	20-167	148	GUACGU UACUAAC UAG	64-66	Lelivelt and Culbertson, 1999; SGD
YJL225c	unchar	possible helicase	5665	1162-1549	388	GUAUGU UACUAAC CAG	1180-1182	Lelivelt and Culbertson, 1999; SGD
YOR318c	Dubious ORF	unknown	653	5-351	347	GCAUGU UACUAAC CAG	61-63	Lelivelt and Culbertson, 1999; SGD
YDL125c	HNT1	interacts with Kin28p	588	98-208	111	GUAUGU UACUAAC CAG	112-114	Lelivelt and Culbertson, 1999; SGD
YDL115C	IWR1	meiotic unknown function	1132	83-152	70	GUAUGU GACUAAC CAG	130-132	Lelivelt and Culbertson, 1999; SGD
YIL177c	unchar	possible helicase	5665	1162-1549	388	GUAUGU UACUAAC CAG	1180-1182	Lelivelt and Culbertson, 1999; SGD
YHR218w	unchar	possible helicase	1911	603-701	99	GCAUGU UACUAAC CAG	622-624	Lelivelt and Culbertson, 1999; SGD
YNL246w	VPs75	vacuolor protein sorting	890	33-127	95	GUAUGU UACUAAC CAG	73-75	Lelivelt and Culbertson, 1999; SGD
YGR183c	QCR9	subunit cytochrome-C reductase	414	4-216	213	GUAUGU UACUAAC UAG	43-45	Lelivelt and Culbertson, 1999; SGD
YPL175w	SPT14	glycosyl transferase	1459	18-117	100	GUAUGU UACUAAC UAG	25-27	Lelivelt and Culbertson, 1999; SGD
YDL012c	unchar	Plasma membrane protein	410	46-131	86	GUACGU UACUAAC CAG	52-54	Lelivelt and Culbertson, 1999; SGD
YNL004W	HRB1	mRNA export factor	1707	31-372	342	GUAUGU UACUAAU UAG	73-75	Lelivelt and Culbertson, 1999; SGD
YJR079w	unchar	mitocondrial function	1035	144-848	705	GCAUGU UACUAAC UAG	250-252	Lelivelt and Culbertson, 1999; SGD
YJR021c	MER2	meiotic recombination	1025	317-396	80	GUUCGU UACUAAC UAG	394-396	Lelivelt and Culbertson, 1999; SGD
YML056c	IMD4	dehydrogenase	1983	461-868	408	GUAUGU UACUAAC CAG	469-471	Lelivelt and Culbertson, 1999; SGD
YDR005c	MAF1	negative regulator of Pol III	1268	007-86	80	GUAUGU UACUAAC UAG	52-54	Lelivelt and Culbertson, 1999; SGD
YLR306w	UBC12	ubiquitin enzyme	701	4-137	134	GUACGU UACUAAC UAG	49-51	Lelivelt and Culbertson, 1999; SGD
YBL111c	unchar	possible helicase	2103	795-893	99	GCAUGU UACUAAC CAG	814-816	Lelivelt and Culbertson, 1999; SGD
YKL186C	MTR2	mRNA export	555	5' INTRON	154	GUACGU AACUAAC CAG	5' INTRON	Davis et al., 2000;Juneau et al., 2007; SGD
YGL251C	MER3	meiotic helicase	3716	59-210	152	GUAGUA GACUAAC UAG	73-75	He et al., 2003;SGD
YJL024C	APS3	subunit of AP-3 clathrin complex	662	23-99	77	GUAUGU UACUAAC UAG	28-30	He et al., 2003;SGD
YKR004C	ECM9	unknown	1238	227-330	104	GUAUGU UACUAAC UAG	241-243	He et al., 2003;SGD
YLLO57C	JLP1	sulfonate catabolism	1239	???	???	?????	????	He et al., 2003;SGD
YPR153W	YPR153W	Unknown	557	6-139	134	GUAUGU AACUAAC CAG	70-72	He et al., 2003;SGD
YLR173W	YLR173W	Unknown	1827	353-1315	963	GUAAGU Not Clear	1313-1315	He et al., 2003;SGD

Table 3. Inefficiently Spliced Yeast Transcripts

% Spliced	Official Name	Common Name	Regulated by NMD
89.4	YMR133W	REC114	
84.8	YBR215W	HPC2	
75.2	YMR201C	RAD14	
74.6	YBR119W	MUD1	
72.2	YEL003W	GIM4	
69.2	YDL115C	IWR1	YES
68.7	YPL175W	SPT14	
68.2	YDL012C		YES
66.1	YPL031C	PHO85	
65.0	YNL038W	GPI15	
61.1	YKL002W	DID4	
60.8	YDL108W	KIN28	
55.3	YHR076W	PTC7	
53.0	YPR153W	YPR153W	YES
51.0	YER179W	DMC1	
50.1	YOL047C		
40.1	YGL183C	MND1	
38.8	YBL091C-A	SCS22	
38.6	YBL059W		
37.2	YHR079C-A	SAE3	
31.9	YLR329W	REC102	
22.2	YGL033W	HOP2	
21.1	YBR186W	PCH2	
14.9	YJR021C	MER2	YES
11.3	YER044C-A	MEI4	
9.0	YIL073C	SPO22	
4.8	YGR225W	AMA1	
0.0	YNL012W	SPO1	
0.0	YGL251C	MER3	YES
0.0	YFL031W	HAC1	
0.0	YHL050C		YES
0.0	YIL177C		YES
0.0	YJL225C		YES
0.0	YLR464W		
0.0	YEL076C-A		
0.0	YLL066C		YES
0.0	YLL067C		YES
0.0	YML133C		YES
0.0	YHR218W		YES
0.0	YBL111C		YES
0.0	YJR112W-A		
0.0	YBR219C		
0.0	YJR079W		YES
0.0	YLR054C	OSW2	
0.0	YLR445W		

Splicing Percentages are from personal communication with Kara Juneau.

Beyond data from NMD studies, there exists additional examples in the literature of unspliced transcripts that may export freely to the cytoplasm and avoid nuclear retention. For example, regulated expression of the export factor Yra1p has been linked to inefficient splicing caused by autoregulation. Because the unspliced form of *YRA1* dramatically accumulates in a *xrn1*Δ strain (Xrn1p is a cytoplasmic 5'-3' exoriboncuclease) it suggests a significant amount of *YRA1* pre-mRNA exports to the

cytoplasm (Preker and Guthrie, 2006). In support of this evidence, it was recently observed that *Yra1* pre-mRNA levels are controlled by the cytoplasmic decapping activator Edc3p. In *edc3*Δ strains the half-live of *YRA1* pre-mRNA is at least four-fold greater than in wild-type strains (Dong *et al.,* 2007). Another example of a pre-mRNA that avoids nuclear retention and decays mechanisms is *RPL30.* Like *YRA1,* the splicing of *RPL30* is controlled by autoregulation and overexpression of *RPL30* causes an accumulation of pre-mRNA. Fluorescent in situ hybridization demonstrates this pre-mRNA accumulation occurs in the cytoplasm. Surprisingly, sucrose gradient analysis demonstrates that only a small percentage of this pre-mRNA associates with ribosomes suggesting very little is translated. In addition to this cytoplasmic sequestration, the pre-mRNA is also regulated by NMD (Vilardell *et al.,* 2000). Finally, there exists a recently identified intron containing transcript *PTC7* that not only splices inefficiently (55.3 %), but also lacks a nonsense codon within its intron or second exon. It is therefore possible this transcript codes for two isoforms and is not subject to NMD regulation (Kara Juneau, personal communication; Zhang *et al*, 2007).

Perhaps the most direct rebuke or counter claim to the pre-mRNA nuclear retention and decay model was presented by Hilleren and Parker (2003). In this study they provide evidence that most mRNA decay (whether a pre-mRNA, lariat 2nd exon intermediate or spliced mRNA) occurs in the cytoplasm. While their model concedes that the nuclear exosome does contribute to the decay process, it generally assumes rapid processing and export of these mRNA species to the cytoplasm. The model supports many of the previous observations about pre-mRNAs that avoid spliceosome assembly and are subject to NMD, but questions previous work concerning stalled splicing intermediates. In particular, Hilleren and Parker are critical of evidence provided by Bousquet-Antonelli *et al.* (2000) and their conclusion that a decrease in mRNA or pre-mRNA steady state levels is a direct result of enhanced nuclear exosome decay. They contend that such conclusions must be substantiated with decay rates measurements (which were not completed by Bosuquet-Antonelli). To correctly access the contribution of the nuclear exosome to pre-mRNA decay Hilleren and Parker created a pulse-chase reporter system that could measure decay rates of splicing mutants in a variety of RNA decay mutant backgrounds. The splicing mutants analyzed included constructs that fail to assemble spliceosomes and constructs that prevent the second catalytic step of splicing and result in lariat intermediates (Hilleren and Parker, 2003).

For the 5’ splice site mutant and branch point sequence mutant reporters tested by this system, splicing was not observed. The decay profile for these pre-mRNAs featured a steady deadenylation followed by 5’-3’ decay. This pattern was similar to the wild-type reporter and was previously demonstrated as the primary mRNA cytoplasmic decay pattern (Muhlrad *et al.,* 1994). On the other hand, the nuclear exosome decay pathway described by Bosuquet-Antonelli primarily proceeds in a 3’-5’ direction (Bosuquet-Antonelli *et al.* (2000). These pulse-chase transcription reporters therefore indicate that pre-mRNAs that fail to assemble in active spliceosomes are exported to the cytoplasm where they undergo decay. Additional conformation for the cytoplasmic location of decay was generated in numerous mutant strains defective for nuclear or cytoplasmic mRNA decay factors (Hilleren and Parker, 2003).

To block splicing at the second transesterification reaction a 3' splice site mutant construct was used. For this reporter a 5'-3' decay pattern occurred concurrent with deadenylation. This is the expected pattern for a cap-less transcript undergoing cytoplasmic decay. Subsequent experiments demonstrated this decay pattern was not dependent of the nuclear exosome component Rrp6p, but the decay was dependent on the debranching enzyme and the cytoplasmic 5'-3' exoribonuclease, Xrn1p. Collectively, the experiments suggest a pattern where most mRNA transcripts whether they are spliced, unspliced or an arrested intermediate, efficiently export to the cytoplasm and are primarily subjected to a 5'-3' decay mechanism. These results largely disagree with the results presented by Bosquet-Antonelli, which suggested the nuclear exosome specifically and rapidly targets unspliced transcripts and splicing intermediates for decay. However, they do agree with the *AMA1-CUP1* export reporter results discussed above and in Chapter Two.

Despite this research by Hilleren and Parker, support for a nuclear pre-mRNA retention model continued as several putative pre-mRNA nuclear retention factors were identified in 2004 and later (Galy *et al.* 2004, Dizembowski, *et al.,* 2004; Palancade, *et al.,* 2005). While the role and importance of pre-mRNA nuclear retention factors continues to be discussed in the most recent reviews describing yeast gene expression (Akhtar and Gasser, 2007; Sommer and Nehrbass, 2005), I believe the data from the *AMA1-CUP1* export reporter assay and other examples discussed above discounts the likelihood a retention system, independent of the spliceosome, functions in yeast to retain pre-mRNA in the nucleus. Instead, an efficient splicing machinery acting both co-transcriptionally and post-transcriptionally splices most transcripts efficiently. For those transcripts with poor splicing signals or interfering secondary structures or inhibitory trans factors, splicing efficiencies will be lower. These transcripts will likely be treated as "intronless" and become coated with numerous export factors, which will serve as escorts through the nuclear pore to the cytoplasm. Here the NMD system will destroy many of the aberrant transcripts as the ribosome pauses at an internal stop codon during the first round of translation. Some pre-mRNAs will avoid both the spliceosome and the NMD machinery and will be translated into truncated isoforms.

Rather than controlling a dedicated pre-mRNA retention system that blocks export of intron-bearing transcripts that have failed to splice, the exosome and nuclear pore proteins such as Mlp1p, Mlp2p, and Pm139p could be participating in a less discriminating quality control mechanism. Perhaps all mRNAs are subject to exosome decay. The susceptibility of particular mRNAs to exosome attack could then be more a function of time in the nucleus and protection afforded by proper mRNP packaging (Fasken and Corbett, 2005). Evidence from several studies support this model. For example, mutations to any of a number of export factors (*MEX67, RAT7, GLE1, RAT8,* or *RIP1*) will cause hyperadenylation of a transcript and accumulation at transcription foci (Hilleren and Parker, 2001; Jensen *et al.,* 2001). Yet hypoadenylation will also result in transcription foci accumulation. In both cases, this sequestration at the point of transcription is caused by the exosome. Mutations to key nuclear exosome components such as *RRP6, RRP4* or *MTR4* will disrupt the foci accumulation and presumably allow

these 3' end mutant transcripts to export (Hilleren *et al.*, 2001). However, this exosome activity is not limited to only aberrant transcripts. Using a *nup116*Δ strain that blocks mRNA export, but does not cause hyperadenylation, Das and colleagues demonstrated that the exosome will attack normal mRNAs and decrease their half-lives (Das *et al., 2002*). Ironically, there are recent reports that a nuclear exosome mutant (*rrp6*Δ) will cause mRNA nuclear accumulation (Galy *et al.*, 2004; Hieronymus *et al.*, 2004). Previous reports had assumed loss of exosome activity resulted in increased nuclear export. These new data imply the nuclear pore will retain mRNA (or become overwhelmed) when the exosome is disabled (Hilleren *et al.*, 2001; Hieronymus *et al.*, 2004).

One problem about making general statements concerning mRNA decay, nuclear retention and export is that specific transcript sequences do play very important roles in determining the fate of individual transcripts. Several simple examples emphasize this point. A single nucleotide change to a *CHY2* transcript (G1A in the 5' splice site of the intron) can block splicing almost completely (Newman *et al.*, 1985). A single nucleotide change in an *LYS2* mRNA can lead to exosome targeting and decay (Das *et al.*, 2006). A stem loop structure in the intron of the *RPS22B* transcript is targeted by Rnt1p, an endonuclease that specifically targets a sequence specific hairpin cap (AGNN) (Danin-Kreiselman *et al.*, 2003). Furthermore, a global analysis of yeast export factors paints a similar picture. Microarray co-immunoprecipitation studies suggest the putative export factors Mex67p and Yra1p bind to only 1150 and 1000 mRNAs respectively. Because this represents only 16% of the yeast genome, it suggests multiple export factors are utilized for mRNA export in a sequence-specific manner (Hieronymus and Silver, 2003). Without a general export receptor or adapter protein in control, nuclear export may have to be considered on a case-by-case basis where each transcript accumulates a unique mix of bound export factors sufficient to negotiate the hydrophobic regions of the nuclear pore interior (Tran and Wente, 2006).

The conflicting data concerning a dedicated pre-mRNA retention and decay system suggests it is not a general mechanism. It is more likely that certain transcripts have sequences that create stronger affinities with nuclear-based proteins. These transcripts will export at a slower rate and be subject to prolonged attack by the exosome. The alternative is a pre-mRNA retention system that acts when splicing fails. This is unlikely because this system would need to recognize intronic sequences that the spliceosome could not. The retention would further require binding to these sequences (or to proteins already bound to the intron). This binding would result in a competition between splicing and retention and would likely be reflected in an accumulation of pre-mRNA as the spliceosome or exosome processes the retained transcripts. This accumulation of pre-mRNA caused by a robust retention mechanism conflicts with current splicing data that indicates the large majority of yeast transcripts splice very efficiently meaning that very little pre-mRNA accumulation occurs in a native *in vivo* context (Juneau *et al., 2007*). Resolving the questions of pre-mRNA nuclear retention may require a much greater understanding of the nuclear pores and their relationship to quality control and even the newly reported links to transcriptional regulation (Ishii *et al.*, 2002; Brown and Silver, 2007).

One way to resolve questions about the existence, effectiveness, or specificity of a pre-mRNA nuclear retention mechanism would be to use Northern blots (or a similar analysis) with anti-sense primers specific to introns and compare wild-type pre-mRNA levels to those in a conditionally depleted *XRN1* yeast strain. A detailed study of the approximate 300 intron-containing transcripts could successfully identify the nuclear or cytoplasmic fate of unspliced pre-mRNAs and bring some closure to this long standing question in yeast biology.

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