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# MECHANISM OF SPLICING REGULATION BY THE MEIOSIS ENHANCER FACTOR Mer1p IN YEAST *Saccharomyces cerevisiae*

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MECHANISM OF SPLICING REGULATION BY THE MEIOSIS ENHANCER

FACTOR Mer1p IN YEAST *Saccharomyces cerevisiae*

by

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A DISSERTATION

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in

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St. Louis, May 2007

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## ABSTRACT

In eukaryotes, genes are presented in a series of coding and non-coding DNA regions (exons/introns) that are transcribed into a premature RNA (pre-mRNA). Introns can be removed from the mature pre-mRNA, before its translation into proteins, in a process called splicing. The splicing reaction occurs in two highly regulated transesterification reactions inside of the cell nucleus, and it is catalyzed by the Spliceosome, involving the binding and release of five small nuclear ribonucleoprotein particles (snRNPs). While some introns are constitutively spliced, others can be alternatively spliced, giving different exon combinations and therefore different proteins, increasing the protein diversity of the species. In humans, misregulation of alternative splicing can result in the production of aberrant proteins, some of which may produce cancer or other severe diseases.

In yeast, alternative splicing is regulated by different splicing factors, such as Mer1p. Mer1p is expressed during meiosis in the yeast *Saccharomyces cerevisiae* and activates the splicing in at least three different genes (*AMA1*, *MER2*, and *MER3*), which contain a conserved intronic splicing enhancer sequence. Previous results have shown that Mer1p is able to interact with the pre-mRNA and with specific proteins of the U1 and U2 snRNPs. However, the specific molecular mechanisms by which Mer1p activates splicing remained unknown. The objective of this work is to determine how Mer1p regulates the splicing of its targets, and how different splicing factors modulate Mer1p activity.

Using biochemistry and genetics, the data presented in this work indicate that Mer1p recruits the snRNPs U1, U2 and U6, to pre-mRNA. This recruitment of the snRNPs is dependent of the U1 snRNP protein Nam8p and the U2 snRNP protein Snu17p, but independent on the branchpoint region or ATP. Furthermore, Mer1p accelerates and stabilizes the formation of the early complexes of the spliceosome.

Finally, U1 and U2 are recruited to the pre-mRNA at the same time, emerging a new alternative hypothesis of splicing regulation that can be applied to other enhancer regulators and that differs from the classical model of stepwise assembly of the snRNP.

Dedicado con mucho cariño a mis padres Enrique y Cristina y a mis hermanos, Enrique, Carolina, Cristina, Guillermo, David, Juan Jose, Rocio y Virginia, por estar siempre conmigo y apoyarme en todo momento. También se lo dedico a Marie-Chistine, por su cariño y dedicación

A ellos GRACIAS.

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*CHAPTER I*

**INTRODUCTION**

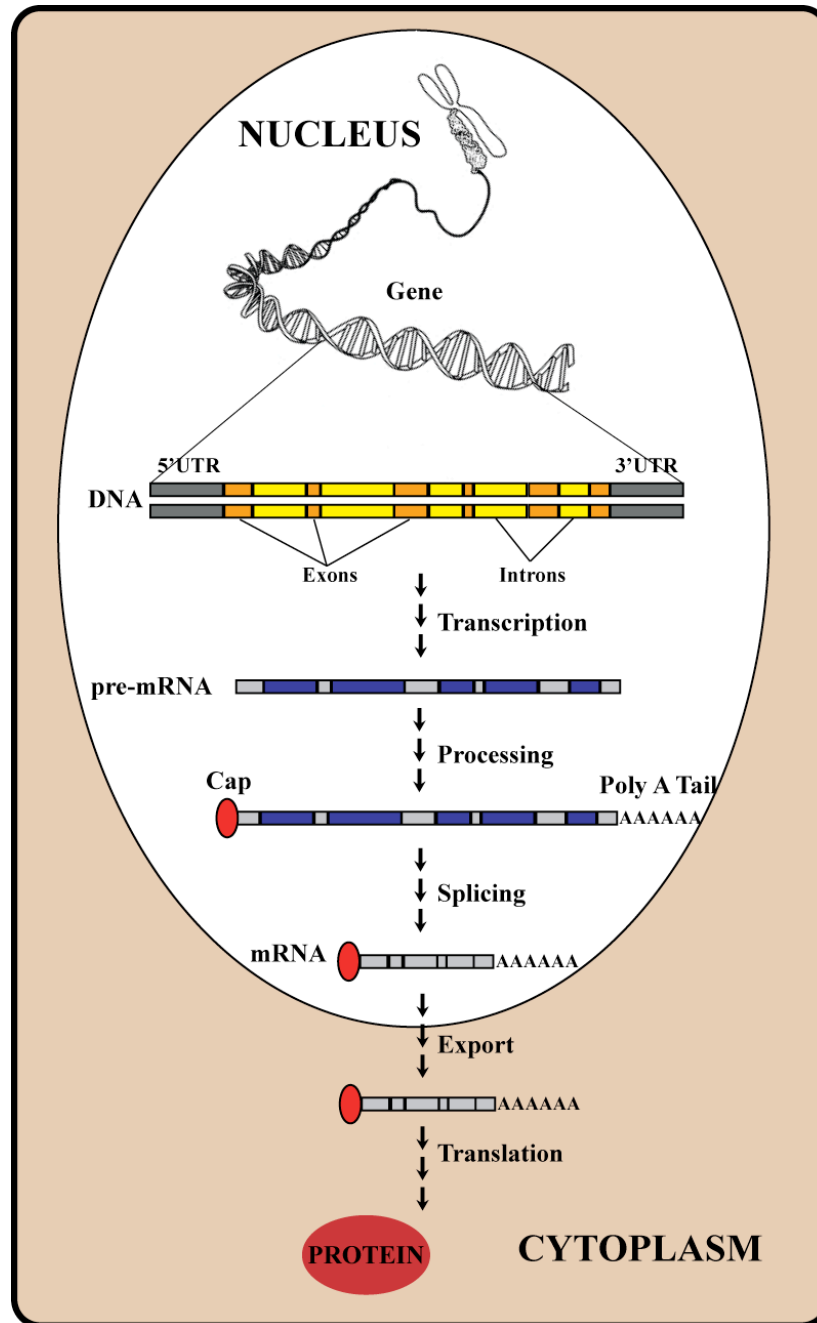
## 1.1 Pre-mRNA Splicing

The genomic information encoded in the genes is transcribed into an intermediary RNA before it can be translated into proteins. The transcription reaction occurs inside of the cell nucleus and is carried out by the RNA polymerase II (only for mRNAs), which creates a primary transcript or precursor mRNA (pre-mRNA). The pre-mRNA undergoes a series of modifications before it can be exported to the cytoplasm as a mature RNA (mRNA), where it can be translated into protein (*Figure 1.1*).

Approximately 90% of human genes and 4% of yeast genes contain non-coding sequence or introns that interrupt the coding sequence or exons (Spingola, Grate et al. 1999), and which need to be removed from the primary transcript. The process of intron removal and proper exon joining is called splicing, and it occurs inside of the cell nucleus. The splicing process is a highly conserved mechanism among species, with some particularity in each case. For example, all yeast introns contain highly conserved sequence elements that provide the proper signals for the splicing reaction. These sequences are the 5' splice site (5' ss), the branchpoint sequence (bps), and the 3' splice site (3' ss). Interestingly, these sequences are more conserved in lower eukaryotes (the yeast *Saccharomyces cerevisiae*) than in higher eukaryotes. In yeast, the 5' splice site is almost always GUAYGU while in humans, only the first GU sequence is highly conserved. The branchpoint sequence is usually UACUAAC in yeast and CAAUCAU in metazoan (although it can be more degenerate), where the last adenosine is highly conserved in both situations. Finally, the 3' splice site is a short sequence YAG, which in some organisms is preceded by a conserved pyrimidine track located between the

branchpoint sequence and the 3' splice site. This pyrimidine track is not that apparent in the yeast *Saccharomyces cerevisiae*.

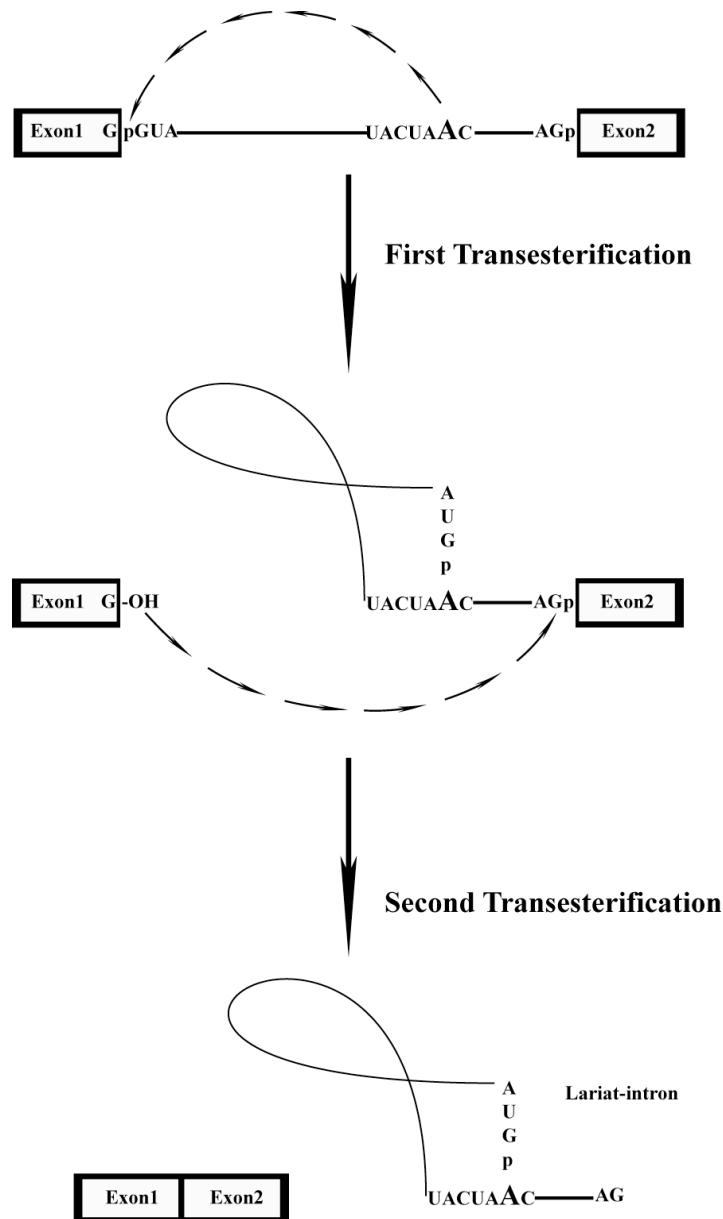
In addition to these well characterized signals, introns and exons may contain additional sequences that are also involved in the splicing process and can act as positive or negative regulators. These sequences are named enhancer or silencer elements.



**Figure 1.1** Processing of the genomic information. After the transcription reaction, the pre-mRNA needs to remove the intronic sequences to form the mature RNA (mRNA) in a process called splicing. The splicing reaction occurs inside of the cell nucleus before the mRNA can be exported to the cytoplasm and translated into proteins.

## 1.2 Splicing Reaction

The splicing reaction occurs in two highly regulated ATP-independent transesterifications reactions (Reed and Hurt 2002) (*Figure 1.2*). During the first transesterification a 2' hydroxyl group from the conserved adenosine residue located within the branchpoint sequence (UACUAAC) carries out a nucleophilic attack over the 5' splice site (GUAYGU). Consequently a free 3' hydroxyl group on the 5' exon and a lariat-intron-3' exon splicing intermediate are formed. In the second transesterification reaction, the 3' hydroxyl on the 5' exon attacks the 3' splice site (YAG), leading to the ligation of the two exons and the release of the lariat-intron product (Staley and Guthrie 1998; Reed and Hurt 2002). In yeast, the two transesterifications reactions are catalyzed by a 40S ribonucleoprotein complex described for the first time in 1985 by Brody and Abelson as the spliceosome (Brody and Abelson 1985). In humans, purification and electron microscopic visualization of the functional spliceosome identified a larger particle (60S). The difference in size between the yeast and the human spliceosome are due to a more complex spliceosome organization in humans, and the presence of additional factors such as the Serine/Arginine-residue proteins (SR family of proteins, Fabrizio, Esser et al. 1994; Zhou, Licklider et al. 2002). Since the splicing signals are less conserved in higher eukaryotes, SR proteins are required to assist in the identification of the proper splicing signals of a given transcript. Indeed, SR proteins can interact with the U1 particle during the early steps of spliceosome assembly, putting in close proximity essential sequences of the pre-mRNA required for the proper splicing efficiency of some human genes (Kent and MacMillan 2002).



**Figure 1.2** Overview of the splicing reaction. The splicing reaction takes place in two ATP-independent transesterifications. At the end of the second transesterification, exons are joined leaving a lariat-intron product that will be degraded.



## 1.3 Splicing Machinery

### 1.3.1 Spliceosome

Other than the difference in size between the human and yeast spliceosome, the basic structure and organization is conserved. In both cases, the spliceosome is formed of five small nuclear ribonucleoprotein particles or snRNPs (U1, U2, U4, U5 and U6), and each snRNPs is composed of a small nuclear RNA (U1, U2, U4, U5 and U6) and a number of proteins, some of which are shared among the snRNPs. For example, while U1, U2, U4 and U5 snRNAs bind to a common core of Sm proteins (B, D1, D2, D3, F and G) (Branlant, Krol et al. 1982; Zhang, Abovich et al. 2001), U6 snRNA binds to like-Sm (Lsm) protein core, composed of seven proteins (Lsm2 to Lsm8) (Beggs 2005; Mayes, Verdone et al. 1999). In addition, each snRNA binds to a specific group of proteins, many of them named Prp (Pre-mRNA Processing proteins). Moreover, there are over 75 non-snRNP proteins associated with the spliceosome that are also required for the formation of the spliceosome. Some of these proteins are members of DExD/H-box RNA helicases, which are required for the formation of the active spliceosome, and might interact directly with the splicing machinery (Tanner and Linder 2001; Cordin, Banroques et al. 2006). Finally, there is a group of non-snRNP factors that bind directly to the pre-mRNA and influence the activity of the spliceosome. Among these factors are the yeast proteins Mud2p and Bbp1p, which recognize and bind to the branchpoint region, or the cap binding proteins Cbp20p and Cbp80p, which bind to the 5' exon and modulate the binding of U1 snRNP. (Spliceosomal proteins are reviewed in Jurica and Moore 2003)

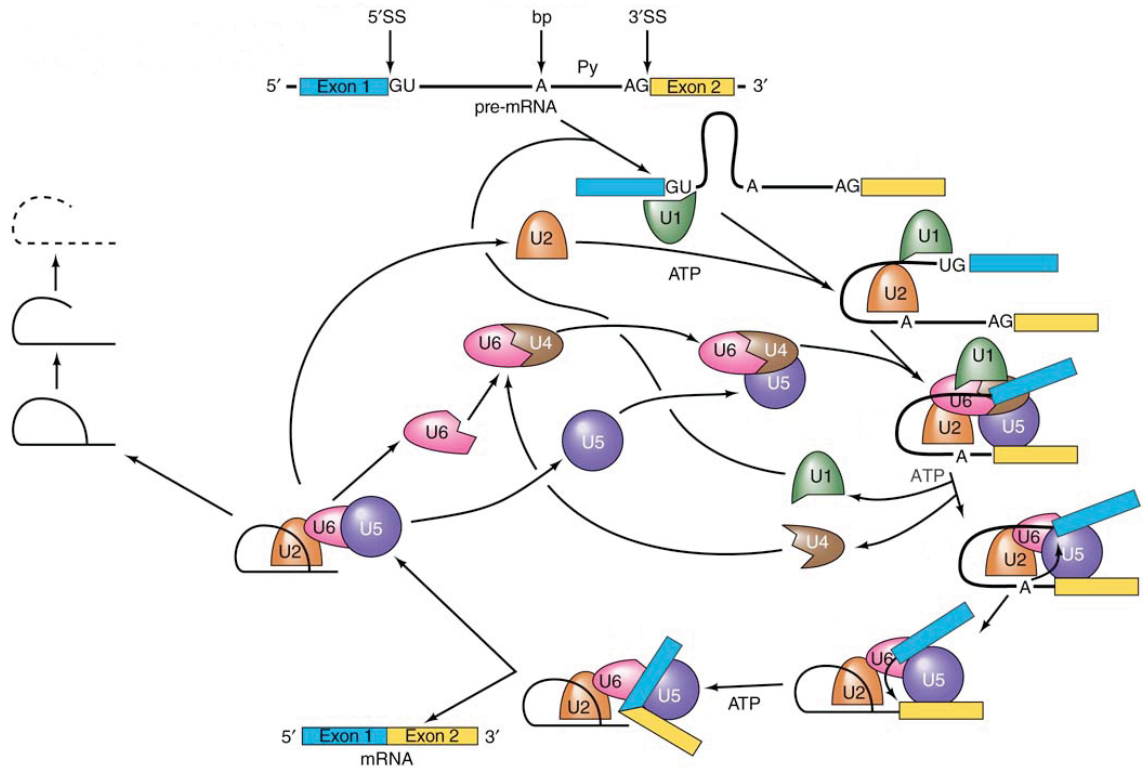
The splicing reaction involves the binding and release of the five snRNPs and

protein factors (*Figure 1.3*). It also involves the formation and disruption of RNA helices, RNA-RNA, RNA-protein and protein-protein interactions, between snRNPs and between snRNPs and the pre-mRNA. Most of these rearrangements are ATP-dependent, but in some cases the reaction requires the hydrolysis of GTP (Nilsen 1994; Nilsen 2003).

The current model for spliceosome formation *in vivo* includes an ordered stepwise assembly of the snRNPs that occurs co-transcriptionally (*Figure 1.3*) (Lacadie and Rosbash 2005; Lacadie, Tardiff et al. 2006; Tardiff and Rosbash 2006) that is initiated by the binding of U1 snRNP to the pre-mRNA shortly after the RNA polymerase transcribes the 5' splice site. This binding is mediated by base pairing interactions between the 5' end of U1 snRNA with the conserved sequences at the 5' splice site. This association forms the commitment complex I in yeast, or early (E) complex in mammals. Subsequently, the branchpoint sequence is recognized by the branchpoint binding protein Bbp1p/SF1 in a sequence-specific reaction, forming commitment complex II or A complex in metazoans. Then the U2 snRNP binds to the complex to form the yeast pre-spliceosome or B complex in mammals. The binding of U2 snRNP to the pre-mRNA involves the recognition of the branchpoint sequence by base pairing with U2 snRNA (Seraphin and Rosbash 1991). Finally, the tri-snRNP complex of U5 and the base paired U4-U6 snRNPs stably joins the pre-spliceosome.

After the binding of the tri-snRNP, the U4-U6 snRNA duplexes unwind, and the U4 and U1 snRNPs are released from the spliceosome complex (*Figure 1.3*). Now U6 snRNA can form base pair interactions with the 5' splice site and with a region of U2 snRNA located close to the binding between U2 and the branchpoint sequence (Patel and Steitz 2003). Once all these RNA rearrangements occur, the spliceosome is completely

active to execute the first transesterification reaction that leads to the cleavage of the 5' exon from the intron and produces the lariat-intron-3' exon intermediate. After the first transesterification reaction, a new rearrangement of the catalytically active spliceosome must follow in order to allow the second transesterification reaction, which leads to splicing of the exons and excision of the intron. (Staley and Guthrie 1998; Hastings and Krainer 2001). In *S. cerevisiae*, after the exon junction, the lariat intron is debranched by Dbr1p protein and is finally degraded (Chapman and Boeke 1991).



**Figure 1.3** Spliceosome assembly and recycling pathway. Evidence from *in vitro* and *in vivo* studies supports a stepwise model where all the spliceosome elements are assembled to the pre-mRNA sequentially manner (Weaver 2002).

### 1.3.2 Spliceosome Assembly

Even though recent evidence supports the stepwise assembly model *in vivo* (Tardiff and Rosbash 2006), results from experiments from *in vitro* studies have suggested that the spliceosome could be working as a holoenzyme, a pre-assembled complex. The “Holospliceosome” or Penta-snRNP model was first described by the Abelson’s group in 2002 when they were able to isolate, under low salt conditions, a functional complex containing all the snRNPs (Stevens, Ryan et al. 2002) and additional factors associated specifically with the penta-snRNP, including eight proteins of the Prp19-complex (which are specific components of the active spliceosome) (Chen, Yu et al. 2002; Chan, Kao et al. 2003; Chen, Kao et al. 2006). Additional support for this model came from the finding that U2 snRNP was able to associate with the tri-snRNP without a pre-mRNA (Konarska and Sharp 1988), and that the U1 snRNA was able to form base pairs with the 5’ splice sites in a penta-snRNP context (Malca, Shomron et al. 2003). Evidence against the penta-snRNP formation comes from the finding that *in vitro*, the formation of the functional spliceosomal A complex is possible, even in the absence of the tri-snRNP (blocking the possibility of a penta-snRNP formation) (Behzadnia, Hartmuth et al. 2006).

In both models, there is a consensus that during the spliceosome assembly RNA-RNA, RNA-protein and protein-protein interactions play a key role in the dynamic progression of the reaction, and that during the splicing reaction one interaction is required to be disrupted in order to progress to form another.

### 1.3.2.1 Commitment Complex Formation

Spliceosome formation is initiated with the 5' splice site recognition by U1 snRNP, forming the commitment complex I (CCI). It is designated commitment complex because once the U1 snRNP is bound to the pre-mRNA, it commits the pre-mRNA to the splicing pathway. CCI is stabilized by the base pair interaction between the 5' end of the U1 snRNA and the conserved 5' splice site sequence (Rosbash and Seraphin 1991). In yeast, U1-C protein interacts with the 5' splice site before its base pairing with the U1 (Du and Rosbash 2002). Additionally, crosslinking experiments have shown direct interactions between several U1 snRNP proteins (U1-70K, Snu56p, Prp40, Nam8p, Prp39p, U1-C, and Sm core proteins) and the pre-mRNA at the 5' splice site region during the formation of CCI, and therefore the stabilization of the U1 snRNP with the pre-mRNA requires proteins-RNA interactions and RNA-RNA interactions (Gottschalk, Tang et al. 1998; Zhang and Rosbash 1999; Jurica and Moore 2003).

Interestingly, some components of U1 snRNPs have additional roles besides their splicing regulation. For example, the U1 snRNP protein Prp40p has been associated with the nuclear export machinery, indicating that spliceosome components can act at different stages during the cell cycle and in different cellular events (Murphy, Olson et al. 2004). Additional factors involved during the formation of the CCI include the yeast Cap Binding Complex (yCBC), which increases the efficiency and stability of the U1 binding to the pre-mRNA (Lewis, Gorlich et al. 1996; Lewis, Izaurralde et al. 1996). Its function was determined by a genetic screen to find components that showed synthetic lethality with the yCBC *cbp20-Δ* and *cbp80-Δ* double mutations. During the screening, a group of U1 snRNPs components was found to be synthetic lethal with the yCBP deletions.

Finally, this result, together with the characterization of physical interactions between the yCBC components of the commitment complex (Snu56p as part of U1 snRNP) and other splicing factors (Mud2p), suggested that yCBC could be not only directly regulating the spliceosome formation, but could also be regulating the later steps of the spliceosome assembly (Fortes, Kufel et al. 1999).

After the formation of CCI, Bbp1p recognizes and binds to the highly conserved branchpoint sequence. This interaction is mediated through the KH-domain at the N-terminal region of Bbp1p, and together with Mud2p assists in the recruitment of U2 snRNP to the branchpoint sequence (*Figure 1.4*) (Berglund, Fleming et al. 1998; Peled-Zehavi, Berglund et al. 2001). In addition, Mud2p interacts directly and simultaneously with the pre-mRNA and the U2 snRNP protein Prp11p. Surprisingly, even though Mud2p does not bind directly to the bps (binds to the 3' region of the bps) it requires the branchpoint sequence and Bbp1p in order to bind to the pre-mRNA (Rutz and Seraphin 1999). Finally, Mud2p also increases the binding affinity of Bbp1p to branchpoint sequence, suggesting cooperative binding among the different elements of the splicing machinery (Abovich, Liao et al. 1994; Berglund, Abovich et al. 1998). The association of Bbp1p and Mud2p to the pre-mRNA forms commitment complex II (CCII) or A complex in metazoans (metazoans require additional factors, such as U2AF<sub>35</sub>, to form the A complex) (Abovich and Rosbash 1997; Fortes, Kufel et al. 1999).

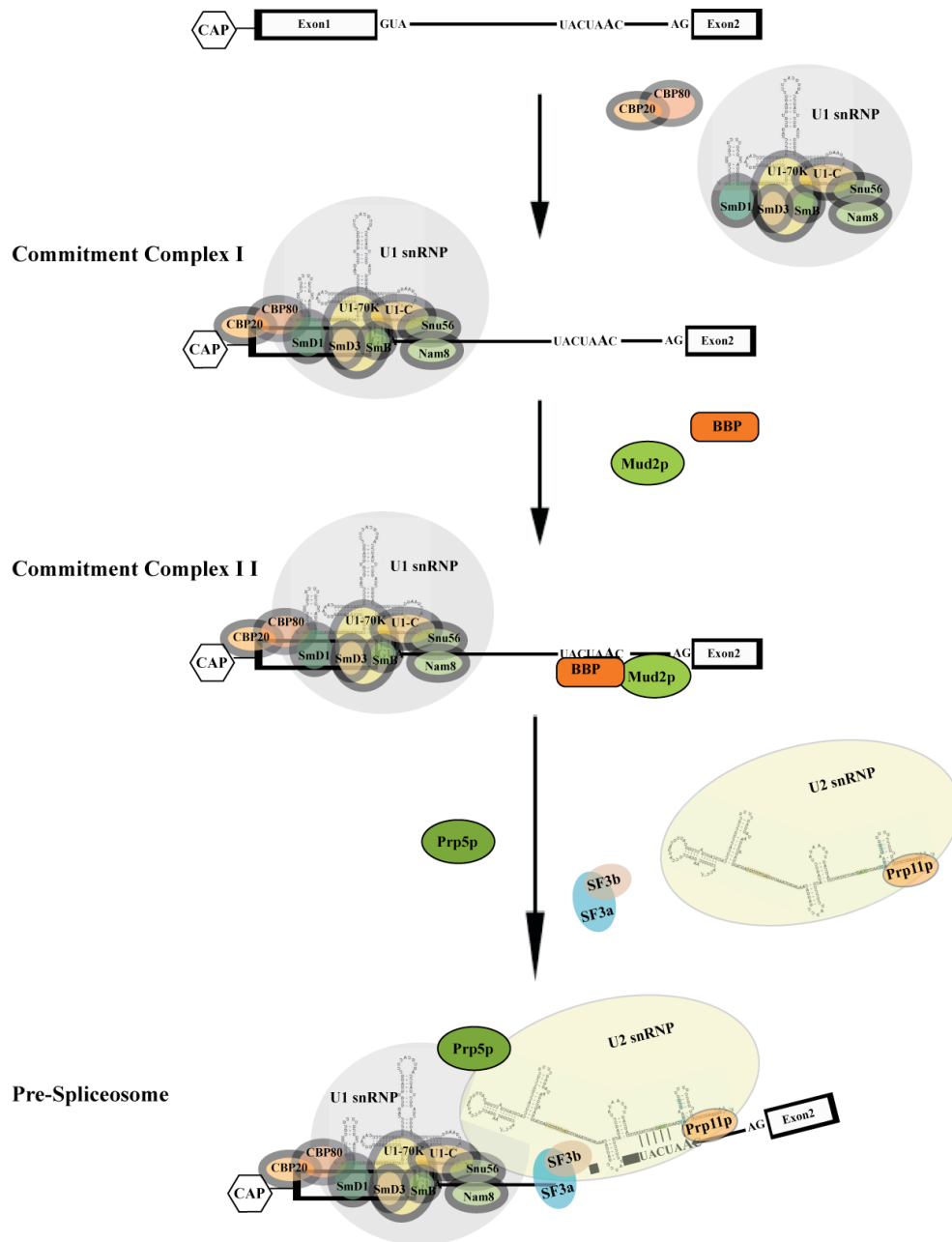
### **1.3.2.2 The Pre-Spliceosome**

After the binding of Bbp1p and Mud2p, the U2 snRNP is recruited to the branchpoint region forming the pre-spliceosome complex. Interestingly, *in vivo* the

recognition of the branch site region by the U2 snRNP does not involve the base pairing of U2 snRNA and the pre-mRNA (Rain and Legrain 1997). Indeed, U2 snRNP is first associated with the branch site region in an ATP-dependent manner, and then U2 snRNA base pairs with the branchpoint sequence, displacing Bbp1p from the pre-mRNA. The process of disruption and formation of new base pairs during the pre-spliceosome formation is regulated by the ATPases, Sub2p and Prp5p. Sub2p is an essential DECD box putative ATPase involved in the transition from CCII to pre-spliceosome and requires Mud2p for its function (Kistler and Guthrie 2001; Libri, Graziani et al. 2001). Sub2p is also involved in mRNA export, constituting another example in which the splicing reaction works as a part of complex nuclear network of events and not as an isolated event (Jensen, Boulay et al. 2001; Zhang and Green 2001).

Before the splicing reaction can progress to the formation of the pre-spliceosome, the U2 snRNP particles SF3a and SF3b need to bind to the upstream region of the branchpoint sequence. Moreover, both factors are required for the proper conformation of the U2 snRNA binding to the branchpoint sequence, and the stable formation of the pre-spliceosome complex (Wiest, O'Day et al. 1996). In addition, the stable addition of U2 snRNP to the pre-mRNA is ATP-dependent and requires the DEXD/H box ATPase Prp5p (Xu, Newnham et al. 2004), although the pre-spliceosome formation can also occur in the absence of ATP, but only when the U2-associated splicing factor Cus2p protein is missing or when the downstream conserved region of U2 snRNA has been removed (Perriman and Ares 2000; Perriman, Barta et al. 2003).





**Figure 1.4** Assembly of the commitment complex and pre-spliceosome. Once U1 snRNP binds to the pre-mRNA, the pre-mRNA is committed to splice. snRNP proteins as well as snRNAs and non-snRNP proteins are required for the proper recognition, binding and stabilization of the different complexes during the spliceosome assembly.

### 1.3.2.3 Tri-snRNP Addition and RNA Rearrangements

Following the U2 snRNP binding, U4/U6-U5 tri-snRNP binds to the pre-spliceosome, forming new interactions, while rearranging others (*Figure 1.4*). For example, the U5 snRNA interacts in an ATP-dependent reaction with the 5' splice site region while U1 is still bound there. These new base pairs are located in a different 5' splice site region than the base pairs between the U1 snRNA and the pre-mRNA, and they allow interactions between U1 and U5 snRNPs (Newman, Teigelkamp et al. 1995; Fromont-Racine, Rain et al. 1997). Also new base pairs between the 5' end of U2 snRNA and the 3' end of U6 snRNA are formed. At the same time, the 5' splice site region of U6 RNA, which formed base pairs with the U4 snRNA, then forms new base pairs with the U2 snRNA. The U6 snRNA also forms RNA-RNA interactions with itself. The unwinding process of U4 from U6 is mediated by the DExD/H-box ATPase RNA-helicase Brr2p (Kim and Rossi 1999; Raghunathan and Guthrie 1998), and it is an important requirement for the progression of the spliceosome.

After the addition of the tri-snRNP and the cross-intron interactions have occurred, U1 and U4 snRNPs are displaced, allowing the binding of U6 snRNA with the 5' splice site. This process of separating the base pairs between U1 snRNA and the 5' splice site requires the activity of the ATPase helicase Prp28p (Staley and Guthrie 1999). In the active spliceosome, U6 snRNA base pairs with U2 snRNA (bound at the same time to the bps) at the same time that it base pairs with the 5' splice site, (Kim and Abelson 1996).

In addition to the helicases, there are other splicing factors involved in the rearrangement of the active spliceosome, such as the Prp19-associated complex and the DExD/H-box protein Prp2p, which function in the unwinding of U4 and U6.

Finally, during the last step of the splicing reaction, the release and recycling of the snRNPs require more RNA-RNA rearrangements. U6 needs to leave the 5' splice site, U2, the branchpoint sequence, and U5, the exons (Staley and Guthrie 1998).

All of these rearrangements that occur along the splicing reaction are driven by several splicing factors that have been classified into 3 different groups. Group I contains factors with a RNA recognition motif; Group II factors are the ATPases DEx/H box proteins; and Group III factors are the GTPases (Staley and Guthrie 1998). Group I includes Mud2p/U2AF<sub>65</sub> because of their ability to assist in the base pairing of U2 with the branchpoint sequence through its RRM motifs (Abovich, Liao et al. 1994; Valcarcel, Gaur et al. 1996). The Group II factors are composed of ATPases DEx/H box proteins (Brr2p), which are believed to unwind the RNA helix of snRNAs and RNA-RNA interactions between snRNAs (reviewed in Tanner and Linder ; Tanner and Linder 2001).

### **1.3.3 The Catalytic Core**

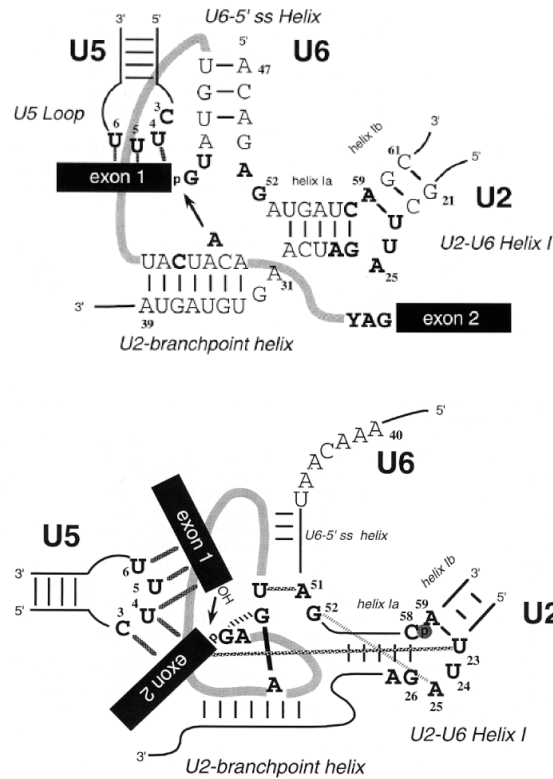
Once all the rearrangements are completed, the active spliceosome catalyzes the first transesterification reaction, which is believed to be catalyzed by the snRNAs and not the proteins of the snRNPs (Nilsen 2000; Valadkhan and Manley 2001). After the first transesterification reaction, significant conformational changes must occur before the second catalytic reaction takes place, including the displacement of the branch lariat and the alignment of both exons (*Figure 1.5*). The second catalytic step requires the three

consensus intron sequences (the 3' splice site (YAG) being the most critical region). The splicing signals interact among themselves to facilitate the 3' splice site identification, and in this way they enhance the fidelity of splicing and ensure the coupling between the first and second transesterification (Umen and Guthrie 1995). Interestingly, mutation in the first G of the 5' splice site affects the efficiency of the second step. The explanation for this effect is the consequence of disruption in the interaction between the first G of the 5' splice site and the last G of the 3' splice site (Parker and Siliciano 1993; Chanfreau, Legrain et al. 1994). Additionally, the U6 snRNA, and to a lesser extent the U2 snRNA, are required for the second transesterification reaction. Moreover, the U5 snRNA plays an important role in this second step because it is responsible for the proper alignment between exons (Newman 1997). After the first catalytic reaction, the conformation of U5 snRNA changes in such a manner that its conserved U rich loop (located at the end of a stem structure) is in close proximity to the reactive groups of the 5' exon and 3' exon (*Figure 1.5*). This U5 loop has the ability to interact with the reactive groups in a non-specific sequential manner, and therefore it brings together both exons.

Additionally, there is a set of specific proteins required for the second catalytic step. Some of them belong to the superfamily of DEXH ATPases (such as Prp16p), while others are ATP-independent proteins required for the proper 3' splice site selection (Slu7p), or snRNP stability (Prp8p) during the second transesterification (reviewed in Umen and Guthrie 1995).

At the end of the second transesterification, the mature mRNA is released, leaving the lariat intron and a complex that contains the U2, U5 and U6 snRNPs. Then the U2, U5 and U6 snRNPs complex dissociates. The snRNPs are recycled for future splicing

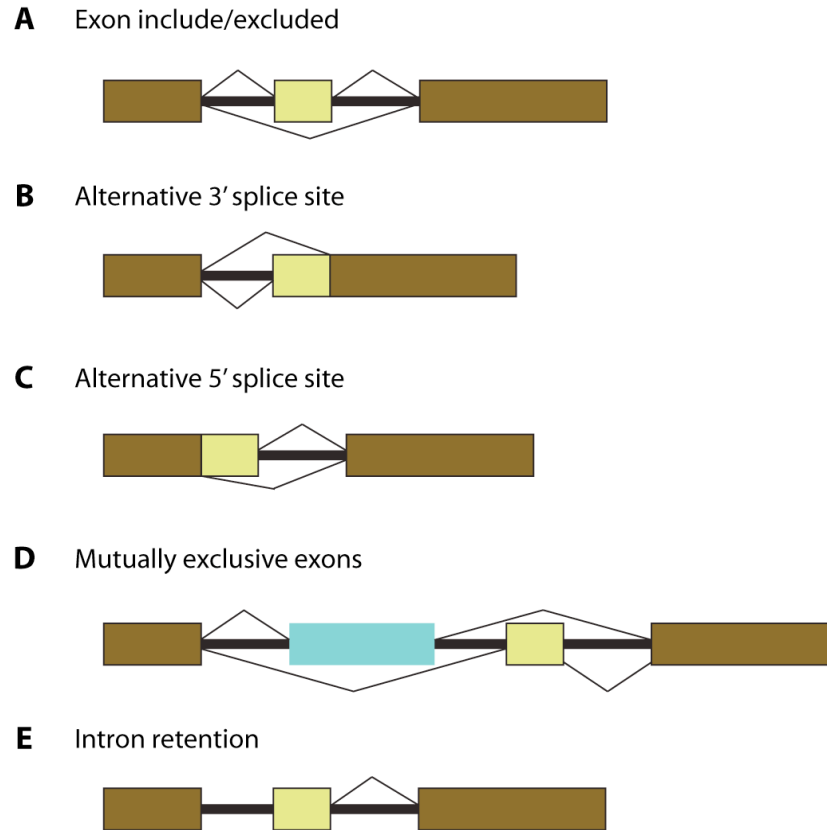
reactions and the lariat intron is degraded by exonucleases with the help of the debranching enzyme Dbr1p (Chapman and Boeke 1991; Khalid, Damha et al. 2005).



**Figure 1.5** RNA rearrangement during the splicing reaction. Representation of RNA-RNA interactions prior to the first transesterification reaction (top), and the second transesterification reaction (bottom). The transition from the first transesterification reaction to the second requires several RNA-RNA rearrangements including the formation of new base pairs between the U5 snRNA and exon 2 (required for the proper alignment between exons) (Umen and Guthrie 1995).

#### **1.4 Alternative Splicing**

The process by which some exons/introns of some pre-mRNAs are included while others are excluded from the mRNA is denoted alternative splicing (*Figure 1.6*). In humans, between 40-74% of the genes undergo an alternative splicing process and up to 80% of alternative spliced genes experience changes in the final protein (Modrek and Lee 2002). This number increases if additional results from different studies of alternative splicing, such as microarrays, are taken into account. The importance of alternative splicing is that up to 50% of human genetic diseases are related to mutations in elements that regulate accurate splicing (Faustino and Cooper 2003). For example, cystic fibrosis has been linked with mutations in cis-acting elements and trans-acting factors that lead to aberrant splicing of pre-mRNA and, therefore, an abnormal protein production. As occurs in metazoan, some yeast exons are constitutively spliced while others can be alternatively spliced, producing different exon/intron combinations and, therefore, different proteins form. In all the species, the result of alternative splicing is an increase in the proteomic diversity, which is considered one of the major contributors to protein diversity in metazoan organisms. In some, alternative splicing working as an evolutionary tool can provide the organisms with new advantages (Graveley 2001).



**Figure 1.6** Variants of alternative splicing. (A) An exon can be included or excluded from the final mRNA. (B, C) The alternative use of 3' or 5' splice site can affect the length of the final mRNA. (D) In some cases, the inclusion of a cassette exon can cause the exclusion of the next one. (E) If an intron is retained, the mature transcript can be exported and translated, giving a new protein isoform. The combination of some or all of these effects can take place in the same pre-mRNA during the splicing process (Black 2003).



Alternative splicing includes conserved and species-specific mechanisms of action, and its regulation involves the participation of positive (enhancer) and negative (repressor) cis and trans-acting elements (Thanaraj, Clark et al. 2003). These enhancer or repressor elements can assist in the selection of the 5' splice site, as well as the 3' splice site (Black D., 2003), therefore selecting for the inclusion or exclusion of a given intron/exon.

Interestingly, although the spliceosome machinery is well studied, the molecular details of how regulators alter splice site recognition remains largely unknown. The effects of alternative splicing are important in many cellular and developmental processes, including sex determination, apoptosis, axon guidance, cell excitation and contraction, and many others (Faustino and Cooper 2003). Changes in splice site choice result in changes in the assembly of the spliceosome. As occurs in metazoans, the selection of specific splice sites in yeast can be regulated by altering the binding site of the initial factors to the pre-mRNA and the formation of early spliceosome complexes. Once the CCI is formed, the bound intron or exon is committed to be spliced (Black 2003; Graveley 2001).

During the process of alternative splicing, the 5' and 3' consensus sequences are generally not sufficient for the assembling of the spliceosome and additional information and interactions are required to activate the use of a specific site. In this sense many non-splice site regulatory sequences can modulate the spliceosome assembly. These auxiliary sequences are highly variable and can act positively to stimulate spliceosome assembly (splicing enhancer sequences), or negatively, acting as splicing silencers or repressors that block the spliceosome assembly. Moreover, these cis-elements can be located in the

exon (exonic splicing elements) or in the intron (intronic splicing elements). The intronic splicing elements are often found within the polypyrimidine tract or immediately adjacent to the branchpoint or 5' splice site, and in some cases they can act from a distance.

Finally, there are several different splicing factors that can work in trans to regulate the splice site selection (Black 2003). As an example of trans-acting factors, there are the exonic splicing regulatory proteins of the SR-family (Blencowe 2000). SR proteins are essential regulatory factors, with a common C-terminal domain (structure of one or more arginine(R)/serine(S) dipeptides), and a N-terminal RNA recognition motif that binds to RNA. Other splicing factors involved in the splicing reaction also contain RS domains but serve in different roles than the SR proteins. Such factors are the mammalian U2 auxiliary factor U2AF, which recognizes the branchpoint sequence and the U1 snRNP protein U1-70K (Graveley 2000; Zhang, Abovich et al. 2001).

#### **1.4.1 Mechanism of Alternative Splicing Regulation**

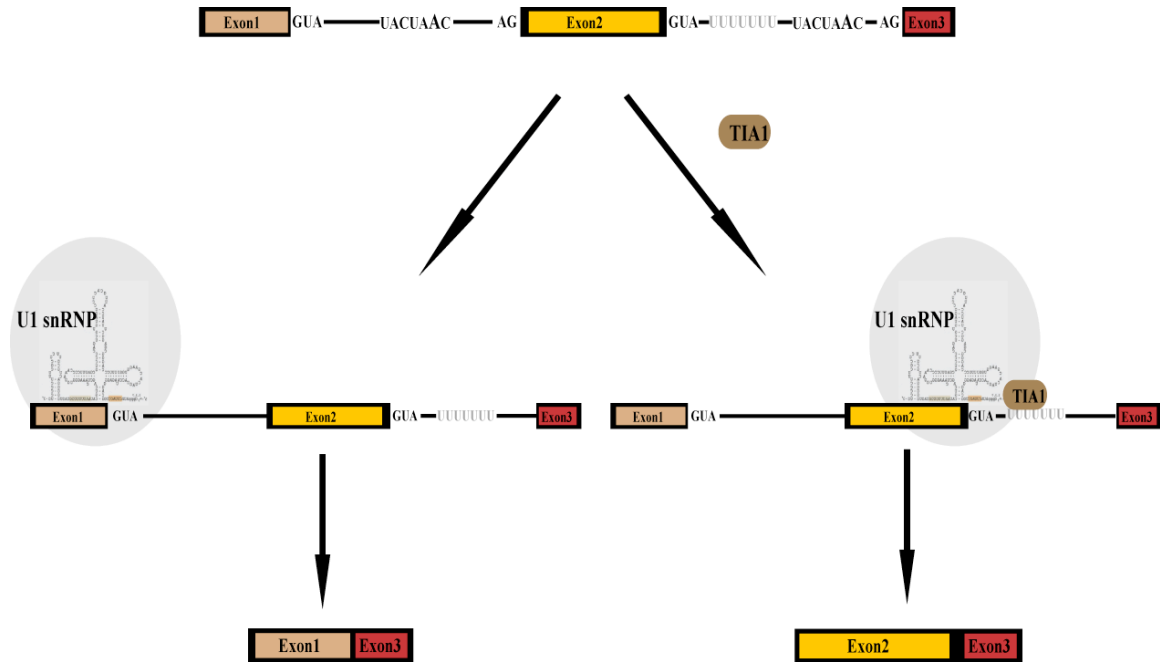
The majority of the information obtained regarding splicing activators comes from studies in SR proteins. SR proteins can bind to exons containing enhancer sequence elements (ESE), and activate specific 5' or 3' splice sites (Tacke and Manley 1999), such in the case of the *Drosophila* sex-specific gene *DSX*, where the ESE element is formed by a purine-rich sequence. In this case, the SR proteins in combination with additional female-specific regulators (*TRA* and *TRA2*) assist in the recruitment of the splicing factor U2AF<sub>65</sub> to a weak polypyrimidine track located near the 3' splice site. In addition, biochemical experiments have shown interactions between the RS domains of the SR proteins with the U1-70K protein, suggesting a way to activate a specific 5' splice site

(Du and Rosbash 2002). This result also indicates that splicing regulatory elements could modulate the spliceosome assembly by direct interactions with their components.

Additional ways to stimulate the use of alternative splice sites can come through intronic splicing enhancers (ISEs). This is the case of *TIA1*, an apoptosis-promoting factor and homologue of the yeast U1 protein Nam8p, TIA1p binds to a uracil-rich ISE and promotes the recruitment of U1 snRNP to weak 5' splice sites by interacting directly with the specific U1-C protein (*Figure 1.7*) (Forch, Puig et al. 2000; Forch, Puig et al. 2002).

In some cases, alternative splicing regulation involves silencer elements. For example, SR proteins have the ability to recognize and bind to an intronic sequence near the 3' splice site of the L1 pre-mRNA. Once the SR proteins bind to this region, the 3' splice site is no longer available for the splicing machinery and a new 3' splice site must be selected.

Both negative and positive regulation can act at the same time in the same pre-mRNA and it is the balance between enhancers/activators and silencers/repressors that determines many of the alternative splicing events.



**Figure 1.7** TIA1p splicing regulation. TIA1p recognizes and binds to U-rich sequences located in the intron near the 5' splice site. Once TIA1p is bound, it promotes the assembly of the spliceosome machinery to its proximal 5' splice site by interacting directly with the U1 snRNP.

### 1.4.2 Misregulation of Alternative Splicing in Human Diseases

Current studies using global approaches, such as *in vitro* binding and *in vitro* functional experiments or *in vivo* functional selection, have been used to identify general regulatory sequences of alternative splicing. These techniques, in combination with computational approaches, have been used to search for more specific motifs involved in the regulation of alternative splicing. One successful approach that has provided valuable information about alternative splicing regulation started with a particular splicing regulator and defined which pre-mRNAs were regulated under its expression, as well as at which step of the splicing reaction it was involved. The information collected from the different approaches has linked misregulation of the alternative splicing reaction with several human genetic diseases such as cystic fibrosis or myotonic dystrophy (see *Table 1.1* for details, Matlin, Clark et al. 2005).

Interestingly, about 10% of the mutations that occur in the human genome affect the splicing sites, leading to abnormal exon/intron definition and resulting in an inappropriate inclusion or exclusion of a given exon/intron. In some cases, mutations can cause a gain or loss of splicing function depending on where and when during the cell cycle they occur. Mutations could create cryptic splicing sites, ESEs, ESSs, ISEs, ISSs and lead to the activation of specific splice sites, therefore, contributing to the splicing of the wrong exon/intron producing the erroneous mRNA. Even single-nucleotide mutations can activate or weaken splice sites. Mutations that inactivate cis-acting splicing elements, such as the canonical splice sites, can lead to a loss of splicing function (such in the splicing for  $\beta$ -globin). Mutations can also affect trans-acting splicing regulators involved

in the regulation of splicing, such in the case of myotonic dystrophy (Faustino and Cooper 2003).

<b>Disease</b>	<b>Alteration</b>	<b>Cause</b>	<b>Symptoms</b>
Familial isolated growth hormone deficiency type II	Disruption in alternative splice site	Mutation in the GH gene producing miss balance in the isoform ratio	Short stature
Frasier syndrome	Disruption in alternative splice site	Change in the +KTS/-KTS ratio	Urogenital disorders involving kidney and gonad developmental defects
Frontotemporal dementia and Parkinsonism linked to Chromosome 17	Disruption in alternative splice site	Mutations in the MAPT gene; alteration in the ratio of tau isoforms	Aggregation of the microtubule-associated protein tau in neurons; Alzheimer
Atypical cystic fibrosis	Disruption in alternative splice site	Loss of function of the cystic fibrosis transmembrane regulator (CFTR) gene caused by exon 9 skipping	Severe pulmonary and pancreatic disease; Male infertility
Retinitis pigmentosa	Basal splicing machinery	Alteration in genes involved in the function of the U4/U6·U5 tri-snRNP	Progressive retinal degeneration.
Spinal muscular atrophy	Basal splicing machinery	Disruption of an ESE in exon 7 causing the exon to be skipped in SMN2 mRNAs	Progressive loss of spinal cord motor neurons; paralysis of muscles.
Myotonic dystrophy	Regulator of alternative splicing	Increase number CTG triplet repeat. Overexpression of splicing regulator, resulting in misregulated splicing of its target pre-mRNAs	Skeletal muscle hyperexcitability; cardiac conduction defects; cataracts; muscle dysfunction; testicular atrophy; neuropsychiatric and cognitive disturbances
Neoplasia and Malignancy	Regulator of alternative splicing	Trans-acting regulatory factors	Anaplasia, invasion, and metastasis
$\beta^+$ -thalassemia	Activation alternative splice site	Activation of a cryptic 3' ss in the $\beta$ -globin gene	Severe severe anemia and sometimes death

**Table 1.1** Misregulation of splicing in human diseases (Cooper and Mattox 1997; Faustino and Cooper 2003).

Furthermore, some mutations can exhibit high phenotypic variability depending on cell type and individuals, and in some situations (Cystic Fibrosis) the phenotypic diversity is a combined effect of cis-acting splicing elements and trans-acting factors (Nissim-Rafinia and Kerem 2002). Because alternative splicing can produce several protein isoforms at the same time, it is the unbalanced ratio of these different isoforms that can cause improper cellular function. For example, the human disease Alzheimer's is caused, in part, by a variation in the ratio of the different *tau* proteins isoforms, caused by a mutation in a cis-acting splicing element (Hong, Zhukareva et al. 1998).

*Can gene therapy prevent genetic diseases associated with alternative splicing?*

There are several treatments focusing on blocking the effects of misregulation in alternative splicing, including drugs designed against specific isoforms of the protein based on their unique sequence, or alterations in the gene expression of specific mRNAs, so that the correct isoform of the protein is predominant over a pool of isoforms. In cases where the gene is regulated by a specific splicing factor, it is possible to affect the function of such a factor and in this way to block its effects. Finally, the use of oligonucleotide-based compounds as an alternative treatment has been tested. Oligonucleotide-based compounds have the ability to bind specifically to cis-acting regions in the pre-mRNA and inhibit or activate specific splicing sites by blocking or recruiting splicing elements. In all these cases, the creation of a specific treatment against a splicing defect requires first a deep understanding of how the splicing reaction works and how it is regulated at different stages in different cells.



### 1.5 Splicing during Meiosis

During starvation periods the budding yeast *Saccharomyces cerevisiae* goes through the meiosis process, inducing the expression of over 100 genes (Burns, Grimwade et al. 1994). These genes, which can be expressed at very low levels during vegetative growth, have been identified based on their ability to produce specific meiosis defects when they are mutated, or increase their expression when the cell enters into meiosis. The activation of sporulation genes occurs at distinct times during the developmental process. The timing of their expression has been categorized as early, middle, and late genes, depending on when their transcription take place during sporulation (*Table 1.2*). Briefly, early genes are expressed at the initial stage of meiosis (beginning of Prophase I) and are usually involved in DNA synthesis and DNA recombination. This group of genes is the best studied. Middle and late genes are more difficult to classify due to their unique time of expression and strain-specificity. Middle genes would be those ones expressed during later prophase I, while late genes would be expressed at the end of cell division and sporulation. Most of the sporulation genes are regulated at the transcription level during the early process of meiosis, and the products of *IME1* and *IME2* are believed to be responsible for the induction of the transcription of these genes (reviewed in Mitchell 1994). In some cases, some meiotic genes are modulated post-transcriptionally, like *MER2*, which is regulated by the enhancer regulator Mer1p. Mer1p acts at the posttranscriptional level, and its gene product is required for efficient splicing of *AMA1*, *MER2* and *MER3* transcript.

<b>Time of Expression</b>	<b>Gene</b>	<b>Function</b>
<b>Early</b>	DMC1	Recombination
	HOP1	Recombination
	IME1	Transcription
	IME2, SME1	Transcription
	IME4	Transcription
	MEI4	Recombination
	MEK1, MRE4	Recombination
	MER1	Splicing
	REC102	Recombination
	REC104	Recombination
	REC114	Recombination
	RED1	Recombination
	RIM4	IME2 expression
	SPO11	Recombination
	SPO13	Meiosis I division
	SPO16	Sporulation efficiency
	ZIP1	Synaptonemal complex formation
<b>Middle</b>	SIT2	Central core RNA polymerase II
	SIT3	Unknown
	SIT4	G1/S transition
	SPO12	Meiosis I division
	SPS1	Post-meiotic events
	SPS2	Spore wall formation
	SPS3	Unknown
	SPS4	Unknown
<b>Mid-late</b>	DIT1	Spore wall formation
	DIT2	Spore wall formation
<b>Late</b>	SGA1	Glucoamylase; starvation
	SPR1	B-Glucanase
	SPR2	Unknown
	SPR3	Bud neck microfilament
	SPS100	Spore wall maturation
	SPS101	Unknown

**Table 1.2** Sporulation gene expression (Mitchell 1994).

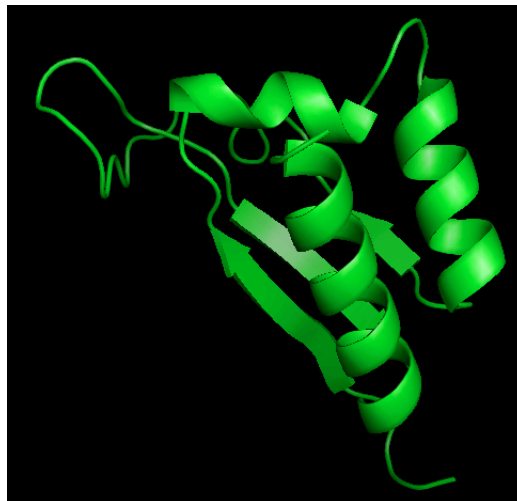
### 1.5.1 Mer1p

Mer1p is a 31 kDa splicing factor expressed during prophase I of meiosis, and it is not required for cell viability (Engbrecht, Voelkel-Meiman et al. 1991). *MER1* mutants are defective in meiosis recombination, producing inviable spores. Mer1p activates splicing of introns containing a conserved intronic splicing enhancer sequence located between the 5' splice site and the branchpoint sequence (approximately 25 nt from the 5' splice site). This enhancer element (AYACCCUY) has been found in at least three different genes: *AMA1*, *MER2* and *MER3* (Davis, Grate et al. 2000; Engbrecht, Voelkel-Meiman et al. 1991; Nakagawa and Ogawa 1999), some of which contain a weak 5' splice site (*MER3*), a large 5' exon (*MER2*) or a silencer sequence (*AMA1*). Mutations in the enhancer element block the ability of Mer1p to activate splicing of *MER3*, *MER2* and *AMA1* indicating that the enhancer is necessary for Mer1p-regulated splicing (Spingola and Ares 2000).

It has been observed that at least one of the U1 snRNP proteins (Nam8p) is required for Mer1p to activate splicing, and that Mer1p co-immunoprecipitates with U1 snRNA, indicating a physical interaction between Mer1p and the spliceosome machinery (Spingola and Ares 2000). Although Nam8p is a U1 snRNP protein, its deletion does not block the binding of Mer1p with the U1 snRNA, suggesting that the requirement of Nam8p for Mer1p function could be independent of Mer1p-U1 snRNP interaction (Spingola and Ares 2000).

The U1 snRNP protein Nam8p is an RNA binding protein essential for meiosis but not for vegetative growth, and it is required for proper splicing efficiency of *MER2*, *MER3* and *AMA1* pre-mRNAs (Nakagawa and Ogawa 1997; Nakagawa and Ogawa

1999). Nam8p crosslinks near the 5' splice site (Zhang and Rosbash 1999), and helps to stabilize CCI on uncapped pre-mRNA or pre-mRNA with a weak 5' splice site (Puig, Gottschalk et al. 1999). Nam8p deletion also affects the structure of U1 snRNP, but does not affect the formation of CCI. When Nam8p is deleted, the stability of two essential U1 snRNP proteins, Snu71p and Snu56p, are affected within the U1 snRNP complex (Gottschalk, Tang et al. 1998). Therefore, Nam8p stabilizes the association of Snu71p and Snu56p with the U1 snRNP. Moreover, Mer1p has an evolutionary conserved KH-domain (homology to hnRNP-K protein) that is a RNA-binding motif located at the C-terminal region, and it is involved in the recognition of the enhancer sequence (*Figure 1.8*).



**Figure 1.8** Predicted Mer1p KH-domain by Swiss-model (Schwede, Kopp et al. 2003). The left external loop followed by the small  $\alpha$ -helix is predicted to be the RNA-binding regions.

The KH-domain can be replaced by an alternative RNA-binding motif, and still the N-terminal of Mer1p is able to activate splicing (Spingola, Armisen et al. 2004). This result, together with the fact that only the N-terminal region of Mer1p interacts with U1 snRNA, suggest that while the N-terminal domain is required for binding with the U1 snRNP, the C-terminal of Mer1p is required for the binding with the enhancer element. Therefore, both domains contain independent functions.

In addition to activate the splicing of pre-mRNA with a long 5' exon or a weak 5' splicing site, Mer1p can also activate splicing of introns with mutations in the branchpoint sequence (Spingola, Armisen et al. 2004). This suggests that Mer1p could be acting at several steps during the spliceosome assembly, not only helping in the formation/stabilization of CCI. Supporting the hypothesis that Mer1p acts at several steps during the splicing pathway, two-hybrid interactions between Mer1p and different proteins of U1 snRNP, U2 snRNP, and non-snRNP proteins were performed (*Table 1.3*). Surprisingly, while Mer1p was not able to interact with Nam8p (despite its requirement for Mer1p activity), Mer1p was able to interact with two Nam8p-associated proteins of the U1 snRNP, Snu56p and Snu71p. Also, the two-hybrid experiment indicated a weaker interaction between Mer1p and Bbp1p or Mud2p, two accessory splicing factors involved in the recognition of the branchpoint sequence before the formation of CCII, and between Mer1p and proteins of the U2 snRNP such Prp9p. These results support a model where Mer1p accelerates or stabilizes the formation of commitment complexes and pre-spliceosome.

### Mer1p interactions

Protein Name	Function
Rho2p	Non-essential small GTPase. Establishment of cell polarity and in microtubule assembly
Uhs1p	Cytoplasmic serine/threonine protein kinase; Role in G1/S phase progression
Rec107p	Early stages of meiotic recombination; Alteration chromatin structure at DNA double-stranded break sites and in coordination between the initiation of recombination and the first division of meiosis
Srb4p	Subunit of the RNA polymerase II mediator complex; Essential for transcriptional regulation
Mrp8p	Mitochondrial ribosomal protein
Snu56p/Mud10p	Component of U1 snRNP; interacts with mRNA in commitment complex
Snu71p	Component of U1 snRNP
Prp11p	Subunit of the SF3a splicing factor complex, required for spliceosome assembly
Luc7p	Essential protein associated with the U1 snRNP complex; splicing factor involved in recognition of 5' splice site
Mud2p	Protein involved in early pre-mRNA splicing; component of the commitment complex; interacts with BBP splicing factor and Sub2p
Msl5p	Component of the commitment complex; essential protein that interacts with Mud2p and Prp40p, forming a bridge between the intron ends; also involved in nuclear retention of pre-mRNA
Prp9p	Subunit of the SF3a splicing factor complex, required for spliceosome assembly; acts after the formation of the U1 snRNP-pre-mRNA complex
Rad52p	Protein that stimulates strand exchange; involved in the repair of double-strand breaks in DNA during vegetative growth and meiosis
Spo13p	Meiosis-specific protein, involved in maintaining sister chromatid cohesion during meiosis I as well as promoting proper attachment of kinetochores to the spindle during meiosis I and meiosis II

**Table 1.3** Mer1p interactions. Mer1p interacts with many proteins involved in several cellular processes (SGD)

## 1.6 Dissertation Overview

While the role of the splicing regulator Mer1p during meiosis has been previously determined, its molecular mechanisms of splicing activation remain largely unknown. Genetic interactions as well as biochemical experiments have shown that Mer1p interacts with the snRNPs and that the non-essential U1 snRNP protein Nam8p is required for Mer1p function. Although these results support a model where Mer1p could stabilize the formation of commitment complex by interacting with the U1 snRNPs, they do not demonstrate that Mer1p facilitates the binding of U1 snRNP or other snRNPs to pre-mRNA to form commitment complexes or any other complex. Moreover, they might be that more splicing factors are involved in Mer1p activity that still remain unknown, as do the steps by which Mer1p is regulating splicing.

The objective of my dissertation is to determine how Mer1p regulates the splicing of pre-mRNA, containing the Mer1p-enhancer element. The goal of this work is to elucidate the molecular mechanism of Mer1p activation and to characterize the interaction between Mer1p and the spliceosome machinery. The results from this work will help to understand better the role of splicing enhancer elements during the splicing reaction. The data presented in this work demonstrate that Mer1p recruits U1 snRNP and U2 snRNP to pre-mRNAs containing the Mer1p-enhancer element and that this process requires the U1 snRNP protein, Nam8p, and the U2 snRNP protein, Snu17p. These findings suggest a model of Mer1p-splicing activation by direct interaction and recruitment of the snRNPs to the pre-mRNA.

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*CHAPTER II*

**EXPERIMENTAL PROCEDURES**

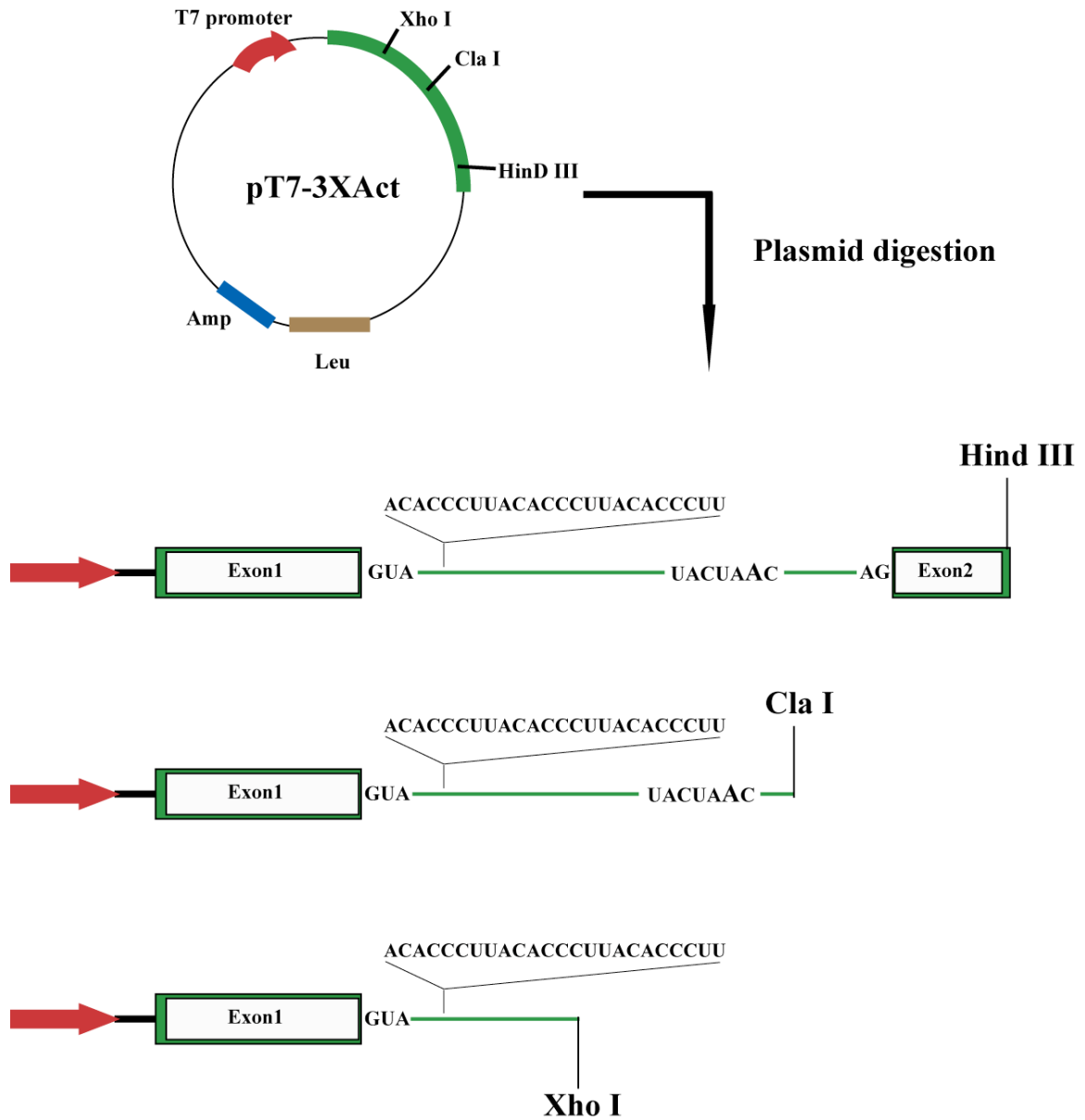
## 2.1 Splicing Extracts

Splicing extracts were prepared by the liquid nitrogen grinding procedure as described (Umen and Guthrie 1995) using the wild type yeast strains (*KH46* and *KH52*), Nam8 deletion (*nam8Δ*) strain, Snu17 deletion (*snu17Δ*) strain, and Bud13 deletion (*bud13Δ*) strain. These strains carried either a constitutive *MER1* expression plasmid, pRS426FLAGMER1, or empty pRS426 vector. One liter cultures were grown in YEPD to an O.D. 600 of 2.5-3.5. Cells were harvested, washed and resuspended in cold grinding buffer before freezing them in liquid nitrogen. Frozen pellets were grounded to a fine powder, quick thawed and centrifuged at different speeds to remove ribosome and other cellular components such as cell wall. Extracts were dialyzed against cold buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 M EDTA, 20% glycerol) for a total of 3 hours and centrifuged one more time to remove any precipitate. Flash freeze aliquots were made for future use.

Splicing extracts were analyzed for total protein content by the Bradford protein assay procedure, measuring the absorbance at 595 nm and then determining the amount of protein using a protein standard curve. Splicing activity of the extracts was evaluated by *in vitro* splicing reaction.

## **2.2 *In vitro* Transcription Reaction**

For *in vitro* transcription reactions pT7 plasmids were linearized by digestion with Hind III, Cla I, or Xho I (*Figure 2.1*). Run off transcripts from Hind III digested plasmids included the 5' exon, intron, and 3' exon; transcripts from Xho I digested plasmids included the 5' exon and approximately the first 70 nt of the intron and lacked a branchpoint sequence; transcripts from Cla I digested plasmids lacked the 3' splice site, but contained the branchpoint sequence. Radiolabeled RNAs for *in vitro* splicing reactions and commitment assays were transcribed with T7 RNA polymerase to a specific activity of approximately 6000 cpm/fmol using pT7 digested with Hind III as a template. Transcription of biotinylated RNA was performed using a mix of rNTPs with a ratio of 1:6 biotinylated UTP (biotin-16-UTP) versus rUTP. Transcripts were PAGE purified.



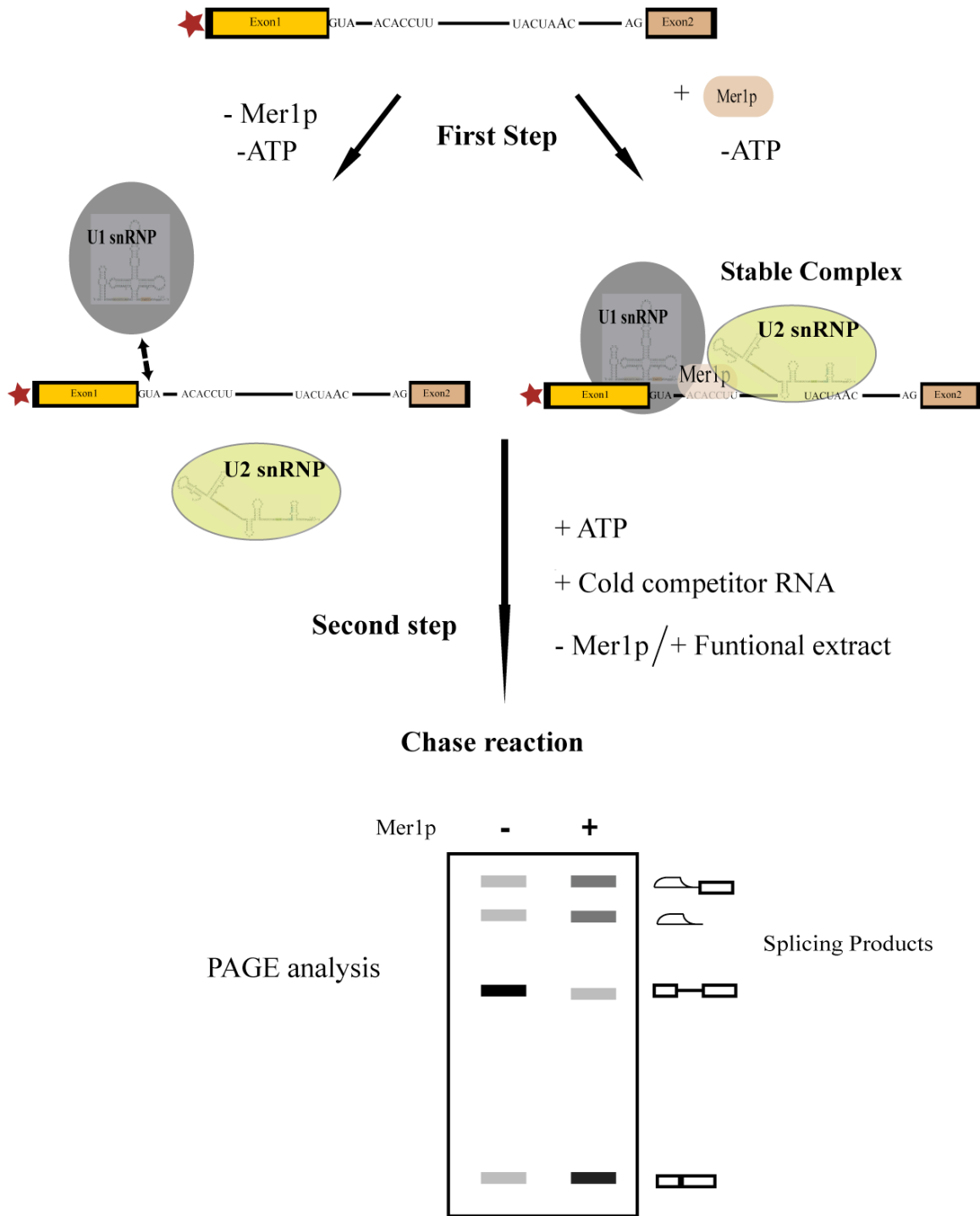
**Figure 2.1** Templates used during *in vitro* transcription reactions. Plasmid pT73XAct has three copies of *MER1* enhancer element (ACACCCTT). While digesting the vector with XhoI removes the branch point region and the 3' splice site, digestion with ClaI removes only the 3' splice site from the template.



### 2.3 Commitment Assay

*In vitro* splicing commitment assays were performed in two steps or reactions (Figure 2.2). During the first reaction, radiolabeled pre-mRNA (from run-off transcription of Hind III digested plasmid) was incubated with ATP-depleted splicing extracts containing or lacking Mer1p. ATP was depleted from the extracts by pre-incubating them with dextrose prior to addition of pre-mRNA. Dextrose is used as a substrate for hexokinase in the extracts, converting ATP into ADP. After incubation of the first extract at 23 °C, a second functional extract was added with ATP and a 400-fold molar excess of unlabeled (cold) competitor pre-mRNA and incubated at 16 °C. Reactions were then incubated for additional time and terminated by addition of stop buffer. Radiolabeled RNA was recovered, PAGE analysis and phosphorimaging were conducted to determine the extent of splicing.

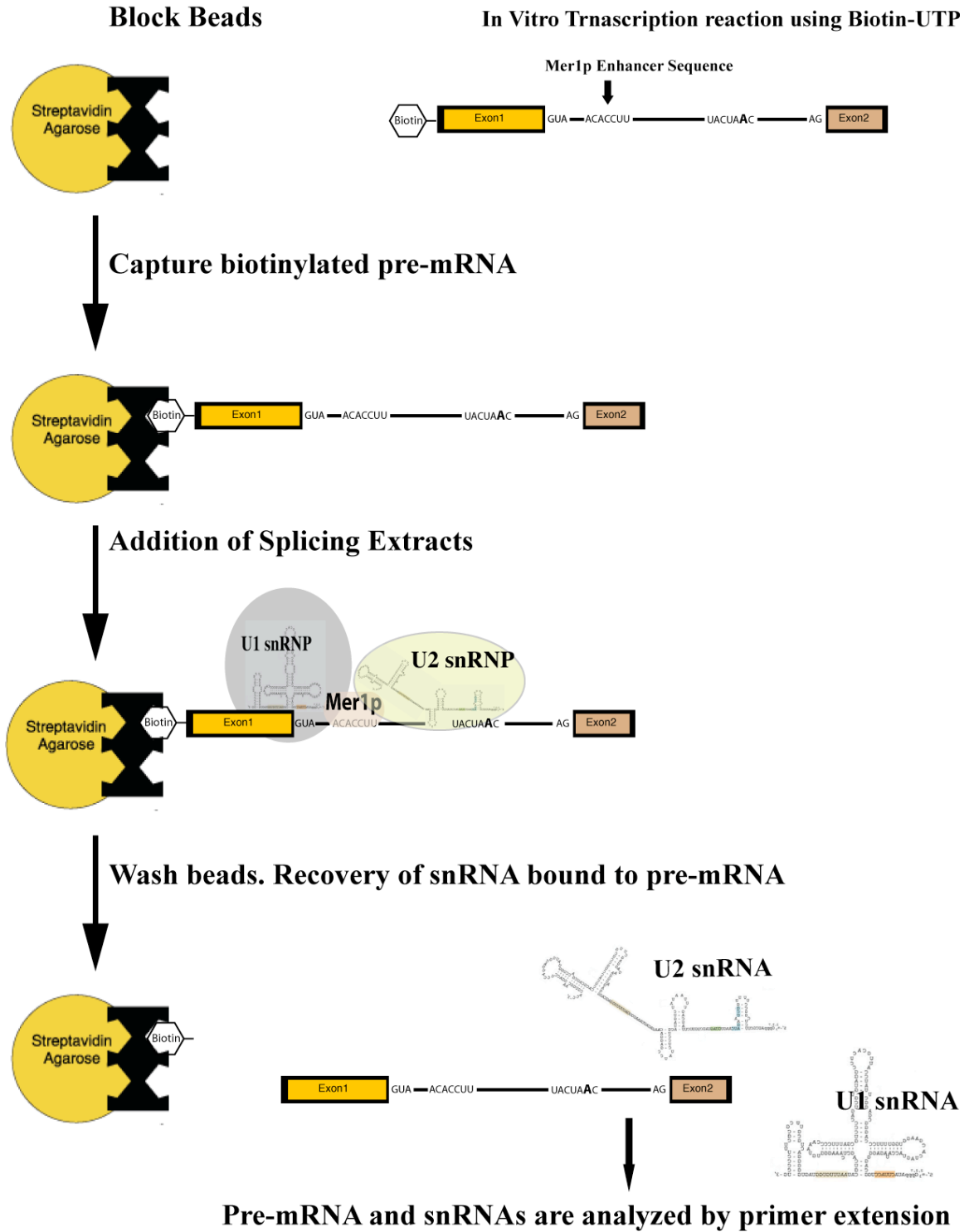
*In vitro* splicing commitment assays were also performed by inactivating the U2 snRNP instead of depleting ATP. The U2 snRNP was inactivated with an antisense oligonucleotide specific against the U2 snRNA. The hybrid formed between U2 snRNA and the oligonucleotide was recognized by the RNase H and therefore degraded. Oligo was added only before the first step and in quantities that would affect only the first reaction but not the second.



**Figure 2.2** Commitment assay.

## 2.4 RNA Affinity Chromatography Assay

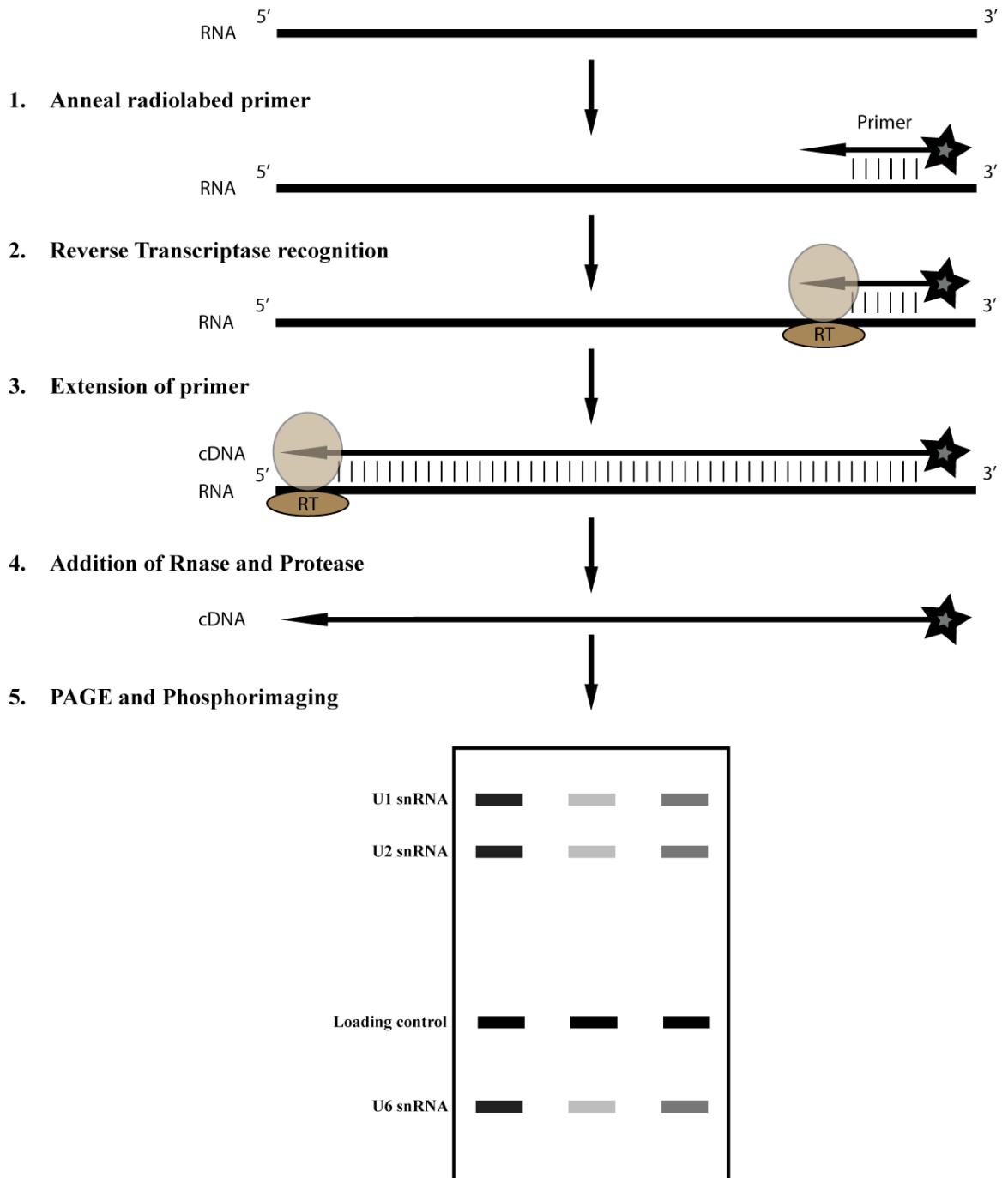
The high affinity and specificity of streptavidin–biotin interactions can be used to immobilize molecules to a solid support to capture complexes. Using this principle, RNA affinity chromatography was performed, where the biotin group was incorporated into the actin pre-mRNA, while the streptavidin group was attached to a solid agarose bead. *In vitro* transcribed biotinylated pre-mRNA was incubated with pre-blocked streptavidin agarose beads. After incubation, beads were washed to remove any unbound biotinylated pre-mRNA, and splicing extracts, with or without Mer1p, were added to the beads. The splicing reaction, containing the pre-mRNA attached to the beads and the extracts, was incubated for 20 minutes before the beads were washed to remove unbound cellular components. RNA was recovered, followed by EtOH precipitation, and analyzed by primer extension using specific oligos against U1 snRNA, U2 snRNA, U6 snRNA and actin pre-mRNA. For ATP depletion, dextrose was added to the reaction prior to addition to the beads. For snRNA depletion experiments, extracts were pre-incubated with U1 or U2 antisense oligos and then added to the reaction (*Figure 2.3*).



*Figure 2.3* RNA affinity chromatography assay.

## **2.5 Primer Extension Analysis**

Primer extension analyses were used to quantify RNA levels (*Figure 2.4*) (Spingola, Armisen et al. 2004). In an initial step, a radiolabeled primer is annealed to the RNA template in a heat and flash cold process. Then, during the extension step, the reverse transcriptase recognizes the primer and starting from the 3' end of the primer incorporates dNTPs, creating a radiolabeled cDNA copy of the RNA template that ends at the 5' end of the template molecule. Once the reaction is completed, products are treated with proteinases and RNases, and the cDNAs are EtOH precipitated and analyzed by PAGE. Finally, phosphorimaging visualizes the different product sizes, the intensity of the band is proportional to the amount of initial template in the reaction.



**Figure 2.4** Primer extension analysis.

## 2.6 References

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*CHAPTER III*

**Mer1p FUNCTION DEPENDS ON  
THE CONSERVED U2 snRNP  
PROTEIN Snu17p**



### 3.1 Introduction

There are several types of experimental evidence that support a mechanism of splicing activation by Mer1p through its interactions with the splicing machinery. For example, Mer1p co-immunoprecipitates with U1 snRNA, and it forms two-hybrid interactions with the essential U1 snRNP proteins Snu56p and Snu71p (Spingola, Armisen et al. 2004). In addition, Mer1p requires the non-essential U1 snRNP protein Nam8p. Interestingly, Nam8p does not interact directly with Mer1p and its deletion blocks the ability of Mer1p to activate splicing of introns containing Mer1p-enhancer element (Spingola and Ares 2000).

In addition to Nam8p, other non-essential splicing factors have been tested for their ability to regulate Mer1p activity. Several yeast deletion strains for non-essential snRNP proteins and non-snRNP proteins were tested including the following yeast strains: *snu66Δ*, *ntc20Δ* and *cdc40Δ* as a part of the U4/U5-U6 tri-snRNP complex, *lea1Δ* and *cus2Δ* as part of the U2 snRNP; *cbp20Δ* as a component of the nuclear cap binding complex; and *mud2Δ* as part of the CCII. None of these factors showed complete disruption of Mer1p activity (Spingola, Armisen et al. 2004), and therefore it was hypothesized that the previous result with *nam8Δ* was due to a specific effect of the missing Nam8p, and was not due to a general defect in the stability of the commitment complex or on spliceosome formation.

During the search for new splicing factors required for Mer1p activity, two new snRNP proteins were found. Both of them belong to the U2 snRNP particle. The first one, Prp11p, is an essential splicing factor, that interacts with two additional U2 snRNP proteins (Prp9p and Prp21p) to form the SF3a splicing factor complex, which is

necessary for the addition of the U2 snRNP to the pre-mRNA (Ruby, Chang et al. 1993; Wiest, O'Day et al. 1996). The second factor, Snu17p, is a non-essential U2 snRNP protein associated with the SF3b complex (Gottschalk, Bartels et al. 2001; Wang, He et al. 2005), which has also been associated with the RES complex (Dziembowski, Ventura et al. 2004). Snu17p is required for the first catalytic step of splicing reaction and for the branch site recognition region during the spliceosome formation.

The results show that while Prp11p presents a strong two-hybrid interaction with Mer1p, it fails to show any interaction in co-immunoprecipitation experiments, suggesting the possibility that additional factors could be involved in the Mer1p-splicing regulation. Moreover, when Prp11p is not a component of the SF3a subunit, it fails to interact with Mer1p, indicating that the interaction between Prp11p and Mer1p only occurs in a very specific context. Interestingly, and as occurred with Nam8p, Snu17p does not make a two-hybrid interaction with Mer1p, but its deletion results in a loss of splicing activation by Mer1p. Finally, *in vivo* experiments show that *SNU17* deletion does not have the same effect in all the Mer1p-dependent introns. Indeed, a more severe effect was observed in the Mer1p activity when the splicing efficiency of *AMA1* or *MER2* pre-mRNAs was analyzed, compared to the splicing efficiency of *MER3*, in a *snu17Δ* background.

### 3.2 Materials and Methods

**Yeast strains and plasmids.** The yeast strains used for these studies and for isolating total RNA for primer extension analysis were *KH46* (MAT $\alpha$  ura3-52 leu2-3 112 trp1-1 lys2 his3-1 ade2-101 cup1D::ura-3 $\pm$ 52), gene deletion in *BY4741* (MAT  $\alpha$  his3- $\Delta$ 1 leu2- $\Delta$ 0 met15- $\Delta$ 0 ura3- $\Delta$ 0) (Invitrogen) and the temperature sensitive *prp11-1 yeast strain*. For the two-hybrid assay, yeast strain *L40* (MAT  $\alpha$  his3 $\Delta$ 200 trp1-901 leu2-3112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lexAop-lacZ) GAL4) was used. Yeast strains *KH46* and *snu17 $\Delta$*  were co-transformed by the LiOAc method (Hill, Donald et al. 1991) with the following plasmids: R1070 (constitutively expresses Mer1p) or its parental empty vector R1130 (gifts from G.S. Roeder described in Engebercht et al., 1991) and pRS316/cupAMA1-E or pRS316/cupMER2 or pRS316/cupMER3. Yeast strain *prp11-1* was co-transformed with pRS426FlagMER1 or its parental empty vector pRS426 and pRS316/cupAMA1-E.

**RNA and Splicing Assays.** RNA was isolated from yeast, containing plasmids, by a glass beads method (Derker and Parker 1993) from 5ml cultures of synthetic complete dextrose (SCD) medium lacking the appropriate amino acids and/or base. Splicing of each of the cloned introns *in vivo* was analyzed by primer extension of total RNA using a 5' radiolabeled CUP primer, which is complementary to the second exon, followed by PAGE. Typically, 5–10  $\mu$ g of total RNA were annealed to 0.2 ng of 5' <sup>32</sup>P-labeled primer in a 10  $\mu$ L reaction by first heating the sample to 90 °C for 3 min and then slowly cooling from 65 °C to 42 °C. To the annealed mix were added 10  $\mu$ L of 50 mM Tris (pH 8.0), 80 mM KCl, 20 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 0.2 mM dNTP, 100  $\mu$ g

actinomycin D, and 20 U of SuperScriptII Reverse Transcriptase (Invitogene). Reactions were incubated at 42 °C for 60 min and terminated by adding 10 µL of 0.6 M NaOAc (pH 5.0), 60 mM EDTA, 1 mg/mL RNaseA with further incubation for 10 min at 42 °C. To this were added 10 µL of 0.6 M NaOAc (pH 5.0), 0.2% SDS, 20 mg proteinase K, and they were incubated for another 10 min at 42 °C. Samples were then precipitated on dry ice after the addition of 3 volumes of ethanol. Precipitates were centrifuged 5 min at 14,000 rpm, the supernatants were removed, and the pellets were dried. Pellets were redissolved in 3 µL of water and 3 µL of formamide/20 mM EDTA/0.25% bromophenol blue and xylene cyanol and then denatured for 5 min at 90 °C. The reverse transcription products were electrophoresed through 6% polyacrylamide, 7.5 M urea gels. Gels were dried and splicing efficiency was quantitated with a Molecular Dynamics Phosphorimager. Primer extension analysis was performed on duplicate or triplicate samples. The formula  $S/(S + U)$ , where S is spliced product and U is unspliced pre-mRNA, was used to calculate splicing efficiency. Primer sequences were specific for plasmid-encoded mRNA and bind to the *CUP1* sequences in the second exon. The *CUP1* primer sequence is 5'-GGCACTCATGACCTTCATTTTGG.

RT-PCR was performed in two steps. RNA isolated was first reverse transcribed into cDNA in a 20 µL reaction by primer extension using CUP1 primer. The reverse transcription products were amplified by PCR in a 50 µL reaction using the following cycle: initial step of 5 min at 95 °C, and then twenty-four times 1 min at 95 °C, 1 min at 50 °C, 1 min at 72 °C, with a final step of 7 min at 72 °C and using B<sup>+</sup> (5'-GGAAGAGCTCATGTCCACTTATCAAGCTCAGGC) and CUP1 primers. PCR products were analyzed in a 2% agarose gel.

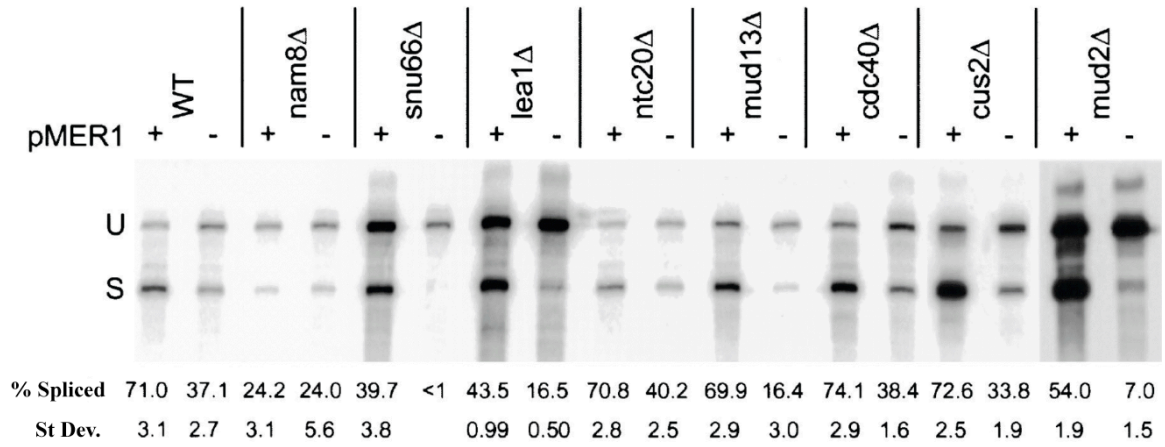
***Two-hybrid plasmids and co-immunoprecipitation assays.*** The construction of the U2 snRNP genes in pACT2 plasmids has been described (Pauling, McPheeters et al. 2000; Igel, Wells et al. 1998). The *MER1* ORF or activation domain fragment of *MER1* was amplified by PCR and ligated in pBTM116 to form pBTM-MER1 or pBTM-AD, which fuses the *LexA* DNA binding domain to Mer1p. Colorimetric assays were performed (Igel, Wells et al. 1998; Pauling, McPheeters et al. 2000) with strain *L40* carrying pBTM and pACT2 derivatives after 3-4 days of growth on selective media. Co-immunoprecipitation experiments were performed as described before (Spingola and Ares 2000) using HA-tagged Prp11p expression plasmid, as well as Flag tagged Mer1p.

### 3.3 Results

***Snu17p is required for efficient Mer1p activity.*** Previous work identified the U1 snRNP protein Nam8p as an essential requirement for Mer1p activity. In addition, Mer1p co-immunoprecipitated with the U1 snRNA (Spingola and Ares 2000) suggesting that Mer1p could be activating splicing by interacting with several spliceosome components. In order to identify new splicing factors involved in the regulation Mer1p-activity, several deletions of non-essential splicing factors were tested for their ability to block Mer1p activation of splicing (including components of the tri-snRNP and commitment complex) (Spingola and Ares 2000). Yeast deletion strains were transformed with pMer1 or control vector and the pRS316CUP-AMA1 vector, which had Mer1p-regulated yeast gene *AMA1*. RNA was extracted and the splicing efficiencies for *AMA1* pre-mRNA were analyzed by primer extension (*Figure 3.1*). None of these gene deletions showed significant effects on Mer1p-activation splicing (Spingola, Armisen et al. 2004). Searching for non-essential spliceosome components involved in Mer1p regulation, additional non-essential U2 snRNP proteins were tested, including Snu17p (*Figure 3.2*). Yeast strain *snu17Δ* was co-transformed with Mer1p expressing vector (R1070) or empty vector (R1130) and *AMA1* expressing plasmid. RNA was isolated and primer extension was performed using a specific primer against the 3' region of the transcript. Primer extension products were separated by PAGE and splicing efficiency was calculated by the formula  $S/(S + U)$ . In the absence of Snu17p, Mer1p could not activate the splicing of *AMA1* pre-mRNA. Similar result was obtained when *MER2* reporter pre-mRNA was used instead of *AMA1* (*Figure 3.2*).

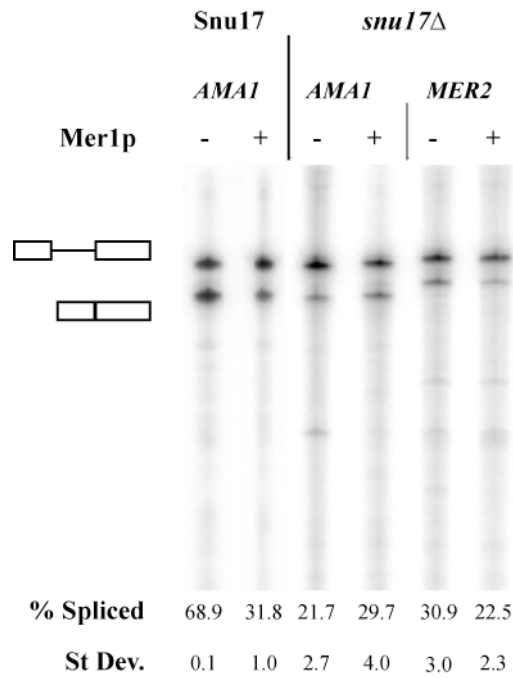
Since the loss of Mer1p activity did not occur with the deletion of the other non-essential U2 snRNP proteins, Cus2p and Lea1p (*Figure 3.1*) (Spingola and Ares 2000), this result suggested that, as occurred with Nam8p, the splicing defect was due to a specific effect of the protein loss and not due to a general defect in spliceosome formation or stabilization. Surprisingly, *snu17Δ* partially disrupts Mer1p activity on *MER3* (*Figure 3.3*). While the *AMA1* and *MER2* pre-mRNAs splicing activity by Mer1p was fully dependent on the presence of Snu17p, in *MER3* pre-mRNA splicing activity was not totally defective in a *snu17Δ* yeast deletion strain (*Figure 3.3* and *Table 3.1*). In the presence of Snu17p, Mer1p was able to activate splicing up to 15-fold for *MER3* pre-mRNA, but in the absence of Snu17p, the splicing activity went down less than half (*Table 3.1*). Interestingly, this effect did not take place when, instead of deleting Snu17p, other splicing factors and non-splicing factors were tested (Bud13p and Pml1p) (Scherrer and Spingola 2006). In both cases, Mer1p was able to increase the splicing efficiency of *MER3* pre-mRNA similar to the wildtype levels.

This result suggests that Snu17p regulates Mer1p activity and its effect on Mer1p splicing regulation differ from pre-mRNA to pre-mRNA. Therefore, the regulation of Mer1p activity could be a combination of effects from the splicing factors and transcript characteristics.

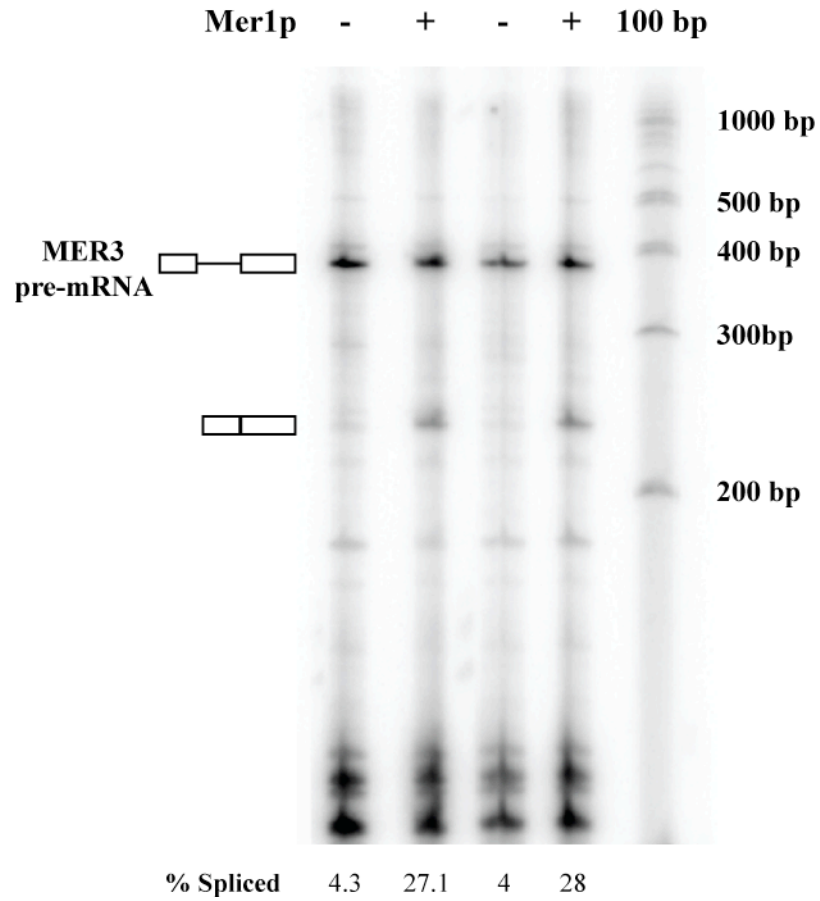


**Figure 3.1** Splicing efficiencies of the *AMAI* reporter pre-mRNA in different gene deleted yeast strains. The deleted genes were non-essential splicing factors (Spingola and Ares 2000). Splicing percentage was calculated by the formula:  $[S/(S + U)] * 100$





**Figure 3.2** Snu17p is essential for Mer1p activity in *AMA1* and *MER2* pre-mRNAs. Primer extension analysis of *AMA1*, *MER2* reporter mRNAs from *SNU17* and *snu17*Δ yeast strains containing a *MER1* expression plasmid (+ pMER1) or a control plasmid (-). Percent spliced is reported as the average of several replicate samples (Spingola, Armisen et al. 2004).



**Figure 3.3** Snu17p loss partially modulates Mer1p activity in *MER3* pre-mRNA. Primer extension analyses of *MER3* reporter mRNAs from *snu17Δ* yeast containing a *MER1* expression plasmid (+ pMER1) or a control plasmid (-). Percent spliced is calculated by the formula  $[S/(S + U)] \times 100$ .

Yeast Strain	% RNA Spliced		Splicing Activation
	MER3	MER3 + Mer1p	
BY4741	2.9 +/- 1	44.2 +/- 1.8	15.2 fold
<i>bud13Δ</i>	1.9 +/- 0.4	25.4 +/- 3.3	13.3
<i>pml1Δ</i>	1.9 +/- 0.6	25 +/- 3	13.2
<i>snu17Δ</i>	4.3 +/- 1.2	27.1 +/- 2.8	6.3

**Table 3.1** *In vivo* splicing activation of *MER3* reporter by Mer1p. Fold increase levels were determined by dividing the % RNA spliced + Mer1p by % RNA spliced - Mer1p (from Scherrer and Spingola 2006).

***Mer1p must interact with Prp11p within the SF3a subunit to activate splicing.***

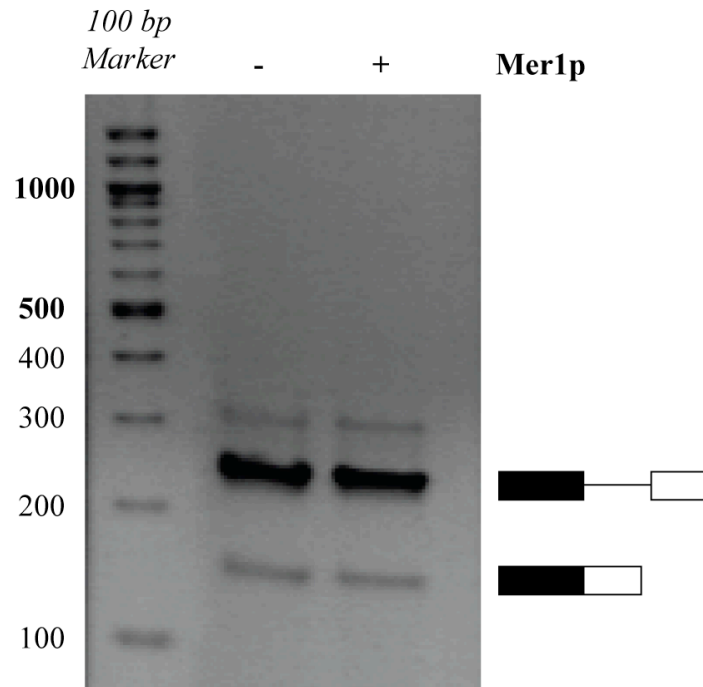
The study of functional requirements for Mer1p splicing activation identified two snRNP proteins, the U1 snRNP protein Nam8p (Spingola and Ares 2000) and the U2 snRNP protein Snu17p. To test for possible protein-protein interactions between Mer1p and U1 and U2 snRNP proteins, two-hybrid assays were performed (*Table 3.2*). Although Nam8p and Snu17p were required for Mer1p to activate splicing, there was no detectable two-hybrid interaction between Nam8p or Snu17p and Mer1p. Remarkably, two essential U1 snRNP proteins, Snu71p and Snu56p, that were destabilized from the U1 snRNP when Nam8p was absent (Gottschalk, Bartels et al. 2001), gave a positive signal in the two-hybrid assay with Mer1p (Spingola, Armisen et al. 2004). Since Snu71p and Snu56p are essential splicing factors it is not possible to uncouple their interaction from their function, and therefore any effect that they could have over Mer1p activity remains unknown.

Mer1p also interacted weakly with the U1 snRNP protein Luc7p and two accessory splicing factors Mud2p and Bbp1p (implicated in recognizing the branchpoint region prior to the U2 snRNP interaction). Continuing the screening for spliceosome proteins that interact with Mer1p, several U2 snRNP proteins were tested and, surprisingly, only intact Prp11p interacted strongly with Mer1p (*Table 3.2*). A second U2 snRNP protein (Prp9p) that interacts with Prp11p (Legrain and Chapon 1993), also weakly interacted with Mer1p. Since Prp9p interacts with Prp11p in the SF3a subunit, the weak two-hybrid signal between Mer1p and Prp9p could be the result of an interaction that was bridged by Prp11p, as opposed to a direct interaction between Mer1p and Prp9p. Moreover, since Mud2p and Bbp1p interact with Prp11p, the weak interactions seen between Mer1p and Bbp1p or Mud2p might also be indirectly mediated by Prp11p (Legrain and Chapon 1993; Abovich and Rosbash 1997). This hypothesis seems unlikely since Snu17p is also part of the SF3a complex and did not present any two-hybrid interaction with Mer1p. Additionally, strong positives from the two-hybrid analysis were repeated using a bait construct containing the activation domain fragment of Mer1p. The results mirrored those obtained with the full-length Mer1p (*Table 3.2*) indicating that the Mer1p activation domain was the primary mediator of the interactions observed with these splicing factors. The strong interactions noted by the two-hybrid assays did not distinguish whether the interactions were direct or mediated by other proteins. To analyze whether the strong interaction between Prp11p and Mer1p was the result of direct interaction between the two proteins, co-immunoprecipitation experiments were performed using HA-tagged Prp11p and Flag-tagged Mer1p to capture any Mer1p-Prp11p complex, and anti-flag antibody or polyclonal antibody against Prp11p to detect

any interaction between Mer1p and Prp11p. Mer1p did not co-immunoprecipitate with Prp11p. Thus, although two-hybrid interactions do not always reliably predict direct protein-protein interactions, the two-hybrid results support the hypothesis that Mer1p interacts with the U1 snRNP proteins, as well as with other components of the commitment complexes and the pre-spliceosome. In addition, and to further characterize the interaction between Prp11p and Mer1p, a genetic study was performed using the temperature sensitive mutant *prp11-1*. *Prp11-1* mutation prevents the association of Prp11p with the spliceosome (Schappert and Friesen 1991), so it is no longer part of the U2 snRNP. Moreover, *prp11-1* fails to interact with Mer1p during the two-hybrid assay (Table 3.2). *Prp11-1* strain was transformed with the Mer1p expression vector or the empty vector and with *AMAI* expression vector PGE. RNA was isolated at the permissive temperature and the splicing efficiency of *AMAI* was measured by reverse transcriptase PCR. Even in the presence of Prp11-1p, Mer1p could not activate the splicing of *AMAI* (Figure 3.4), suggesting that Mer1p-splicing activation could occur through its interaction with the splicing machinery and that Prp11p properly associated with the U2 particle is required for Mer1p activity.

<b>pACT+</b>	<b>Blue intensity with pBTM-MER1</b>
Vector	-
NAM8	-
SNU56	++
SNU71	+++
MER1	-
U1C	-
LUC7	+
MUD2	+
BBP	++
CUS1	-
CUS2	-
HSH49	-
HSH155	-
SUB2	-
PRP9	+
PRP11	++++
prp11-1	-
PRP5	-
PRP21	-
SNU17	-
	<b>Blue intensity with pBTM-AD</b>
SNU56	++
SNU71	+++
PRP11	++++

**Table 3.2** Two-hybrid interactions between Mer1p and different splicing factors. Minus signs indicate no accumulation of blue color. Plus signs indicate development of blue color with more plus signs corresponding to darker blue color development (Spingola, Armisen et al. 2004).



**Figure 3.4** RT-PCR of *AMAI* splicing products from a *prp11-1* yeast strain. The unspliced *AMAI* gene gives a product band of 222nt while the spliced *AMAI* gives a product of 129nt.

### 3.4 Discussion

Previous works identified interactions between proteins of the U1 snRNP and Mer1p (Spingola and Ares 2000; Spingola, Armisen et al. 2004). In fact, two essential U1 snRNP proteins, Snu71p and Snu56p, were identified as strong candidates that interacted with Mer1p in the two-hybrid assay. Additionally, Mer1p also interacted weakly with the U1 snRNP protein Luc7p. Surprisingly, the non-essential U1 snRNP protein Nam8p that was required for Mer1p activity did not present any detectable two-hybrid interaction with Mer1p. Moreover, several accessory splicing factors (Mud2p and Bbp1p) and U2 snRNP proteins, Prp9p and Prp11p, presented interactions with Mer1p. In this chapter, we report that only the wild type Prp11p is able to interact with Mer1p, while the temperature sensitive mutant *prp11-1* abolishes such interaction (*Table 3.2*). Attempts to confirm direct interaction between Mer1p and Prp11p by co-immunoprecipitation failed, suggesting that a transient association between both proteins might occur, or that an additional splicing factor is required for their interaction. Independent of the nature of association between Mer1p and Prp11p, the two-hybrid interactions support the hypothesis that Mer1p regulates splicing by interacting with components of the spliceosome and, therefore, Mer1p could regulate the splicing of Mer1p-dependent introns at different stages during the spliceosome formation. Moreover, Mer1p cannot activate splicing without the non-essential U2 snRNP protein Snu17p and, as occurred with Nam8p, Mer1p does not make two-hybrid interaction with Snu17p. Snu17p enters splicing complexes as part of the U2 snRNP and is thought to function in spliceosome assembly after addition of the tri-snRNP but before U1 snRNP release (Gottschalk, Bartels et al. 2001). A possible metazoan homolog of Snu17p has been identified as p14,

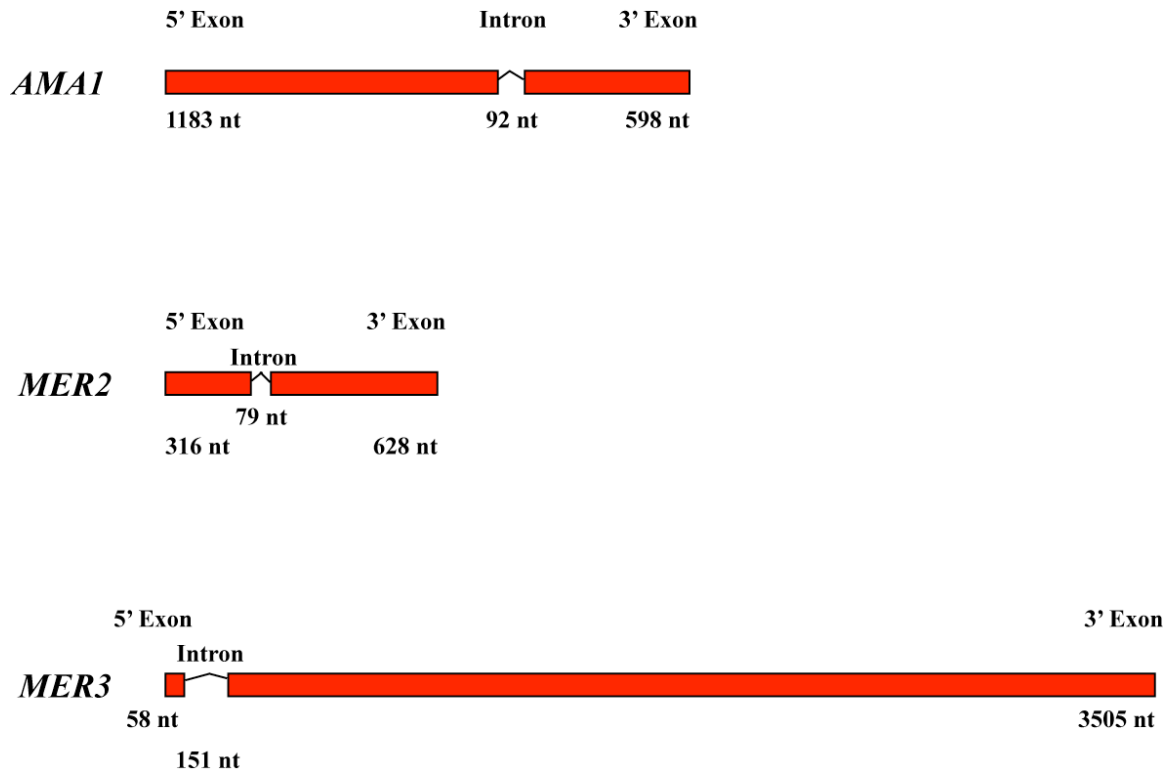


which can be cross-linked to the pre-mRNA branchpoint nucleotide (Will, Schneider et al. 2001). When Snu17p is present, U1 and U4 are rapidly displaced from the assembling spliceosome after the binding of the tri-snRNP. While non-essential, the loss of Snu17p retards progression of spliceosome assembly and leads to the formation of a complex that contains all five snRNPs (Gottschalk, Bartels et al. 2001). Hence, Snu17p may be involved in displacing both the U1 and U4 snRNPs from assembling spliceosomes.

*Why does *snu17Δ* have different effects on specific Mer1p dependent introns?*

The splicing efficiency of *AMAI* reporter pre-mRNA is greatly affected in the *snu17Δ* yeast deletion strain, even in the presence of Mer1p, indicating that in the absence of Snu17p, Mer1p cannot activate the splicing of *AMAI*. The same result is obtained with the *MER2* pre-mRNA reporter. Surprisingly, this is not the situation for the *MER3* pre-mRNA reporter, where the deletion of *SNU17* only partially prevents Mer1p-activation. Since previous results support the hypothesis of Mer1p interacting at different stages during spliceosome formation, the difference in Mer1p regulation between *MER3* and *AMAI* could reflect the different stages at which Mer1p regulates splicing in different pre-mRNAs. In addition, these results suggest that the splicing regulation of Mer1p is not entirely dependent on its interaction with the splicing machinery, and that additional gene-specific elements play an important role in Mer1p function. The study of *MER3* and *AMAI* pre-mRNAs reveals two main features that clearly differentiate both pre-mRNAs (Figure 3.5). While *MER3* contains a small 5' exon, the 5' exon of *AMAI* is very large. Additionally, *MER3* possesses an extremely large 3' exon compared to the *AMAI* 3' exon. These differences in exon size could affect the splicing efficiencies of *AMAI* and

*MER3* in a *snu17Δ* yeast strain when Mer1p is present. It been reported that splicing efficiency is affected by the extension of the 3' exon (Turnbull-Ross, Else et al. 1988). The shorter 3' exon seems to affect negatively the splicing efficiency, presumably because of its influence over the second step of the splicing reaction. In this sense, any effect that Snu17p might have over the early spliceosome complex stability on *MER3* transcript could be compensated by a more stable second step due to a larger 3' exon. Moreover, the difference in exon size could modulate the timing at which Mer1p is working and/or affecting specific splicing factors involved in Mer1p-regulation. Different exon sizes can adopt different spatial conformations and structures, which can result in differences in spliceosome assembly and therefore different interactions with Mer1p. Finally, it has been reported that reduction in the large 5' exon of *AMA1* increases its splicing efficiency in a wild type strain and *nam8Δ* strain, and that *MER2* becomes Mer1p-independent when its 5' exon is shortened (Nandabalan and Roeder 1995; Spingola and Ares 2000). Since the cap-binding complex (CBC) increases the efficiency and stability of the U1 binding to the pre-mRNA, and it is able to interact directly with Mud2p (Lewis, Izaurralde et al. 1996), maybe a shorter 5' exon would increase the interaction between CBC and the different splicing complexes making them more stable and able to overcome any destabilizations caused by the disruption of *SNUI7* gene.



**Figure 3.5** Schematic representation of *AMAI*, *MER2*, and *MER3* genes.

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*CHAPTER IV*

**MECHANISMS OF PRE-MRNA  
SPLICING REGULATION BY  
THE MEIOSIS-SPECIFIC  
FACTOR Mer1p**

Mechanisms of pre-mRNA splicing regulation by the meiosis-specific factor Mer1p

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Running title: Mechanisms of Mer1p-regulated splicing

#### **4.1 Abstract**

In the yeast *Saccharomyces cerevisiae* Mer1p is expressed only during meiosis and regulates the splicing of at least three mRNAs: *AMA1*, *MER2*, and *MER3*. *In vitro*, Mer1p increases the levels of snRNAs bound to pre-mRNA. This increase is dependent on the nonessential U1 snRNP protein Nam8p and the Mer1p intronic enhancer, but not on a 5' splice site-U1 snRNA interaction. Mer1p also recruits the U2 snRNP to commitment complexes without a requirement for the branchpoint sequence or ATP hydrolysis. Surprisingly, the inactivation of U2 snRNP reduced the ability of Mer1p to enhance the binding of U1. Time course experiments indicate that Mer1p increases the rate of U1 snRNA binding to pre-mRNA, and commitment assays show that Mer1p regulates splicing prior to hydrolysis of ATP. These data indicate that the Mer1p splicing regulator functions early in the spliceosome assembly pathway by recruiting snRNPs to pre-mRNA and stabilizing early splicing complexes involving U1 and U2.

#### **4.2 Introduction**

In the yeast *Saccharomyces cerevisiae*, the vast majority of mRNA splicing occurs constitutively and is not regulated. Only a handful of genes contain multiple introns or are alternatively spliced (Spingola et al., 1999; Davis et al., 2000). This is in contrast to human primary transcripts, which almost exclusively contain multiple introns whose alternative splicing must be tightly controlled (Croft et al., 2000). Nonetheless, splicing of some introns in yeast is regulated, and these introns provide an ideal system to study the mechanism of splicing regulation in a highly tractable system. For example, during prophase I of meiosis the splicing regulator Mer1p is produced in the yeast



*Saccharomyces cerevisiae* (Engebrecht et al., 1991). This protein contains an evolutionarily conserved KH domain RNA binding motif (Siomi et al., 1993) and activates the splicing of at least three genes: *AMA1*, *MER2*, and *MER3* (Engebrecht et al., 1991; Nakagawa & Ogawa, 1999; Davis et al., 2000). Proteins produced from the spliced mRNAs of each of these three genes are essential for the completion of meiosis and the development of viable spores. Without Mer1p, these pre-mRNAs are poorly spliced and spores are inviable.

Splicing occurs in the nucleus and involves over one hundred proteins and five small nuclear RNAs (snRNAs) (reviewed in Staley & Guthrie, 1998; Brow, 2002; Jurica & Moore, 2003; Butcher & Brow, 2005). These snRNAs bind several proteins to form five small nuclear ribonuclear protein particles (snRNPs) called U1, U2, U4, U5, and U6. *In vitro*, these snRNPs bind to pre-mRNA in a specific order to form a mature spliceosome (Pikielny et al., 1986; Cheng & Abelson, 1987). However, a penta-snRNP has also been isolated from yeast and shown to be active *in vitro* as well, suggesting that *in vivo* a holoenzyme might be recruited to introns (Stevens et al., 2002). Recent chromatin immunoprecipitation studies support the sequential accretion model for spliceosome assembly *in vivo* (Gornemann et al., 2005; Lacadie & Rosbash, 2005; Tardiff & Rosbash, 2006). In this model, first U1 snRNP binds to pre-mRNA to form the commitment complex (CC) (Seraphin & Rosbash, 1989). The formation of this complex involves base pairing between the 5' splice site sequence of the pre-mRNA and the 5' end of U1 snRNA (Seraphin et al., 1988) and involves the interactions of several snRNP proteins with the intron (Puig et al., 1999; Zhang & Rosbash, 1999). The U2 snRNP binds to the CC to form the pre-spliceosome (PS) (Perriman & Ares, 2000).

Formation of the PS relies on the base pairing of the intronic branchpoint sequence (bps) with the bps interacting sequence (bpis) on U2 snRNA as well as snRNP protein-intron interactions (Parker et al., 1987). Several negative splicing regulators, most notably the *Drosophila* PSI protein and yeast RPL30 protein, function at these early stages (Siebel et al., 1992; Siebel et al., 1994; Vilardell & Warner, 1994). After formation of the PS, the remaining snRNPs bind as a tri-snRNP. Structural rearrangements ensue U1 to be released from the assembly, U4 to unwind from U6 snRNA, and U2 to base pair with U6 snRNA. At this stage, U6 base pairs to the 5' splice site. The transesterification reactions that are catalyzed by the spliceosome now occur: first the 2' hydroxyl of the branchpoint nucleotide reacts with the 5' splice site to generate a free 5' exon and lariat branched intron that is still connected to the 3' exon. Then the 3' hydroxyl group of the 5' exon reacts with the 3' splice site to connect the two exons and to excise the intron in a lariat form.

The molecular mechanisms underlying positive splicing regulation remain largely unknown. Generally, positive regulation, or activation of splice sites, is thought to involve the recruitment of snRNPs or accessory splicing factors to splice sites by regulators that are bound to the pre-mRNA (reviewed in Black, 2003). This facilitates the formation of active spliceosomes that select splice sites that would otherwise not be selected. It remains unknown when during spliceosome assembly or during the splicing reactions that regulation can or cannot occur and what spliceosome assembly intermediates can or cannot be targets for regulating splicing. In these studies we use RNA affinity chromatography assays and commitment assays to analyze the function of Mer1p *in vitro*. We show that Mer1p accelerates the formation of commitment

complexes and increases their stability. Mer1p can only recruit and stabilize the binding of snRNAs to pre-mRNA if the nonessential U1 snRNP protein, Nam8p, and the Mer1p intronic splicing enhancer element are present. Moreover, we show that Mer1p can also recruit the U2 snRNP to pre-mRNA lacking a branchpoint sequence and without ATP hydrolysis. Surprisingly, the loss of functional U2 reduced the ability of Mer1p to recruit U1, suggesting that U1 and U2 may be simultaneously recruited to pre-mRNA by Mer1p or that the binding of U2 stabilizes the commitment complex. These data indicate that a cell-cycle specific regulator controls splicing by promoting the formation of and increasing the stability of early splicing complexes that involve U1 and U2 snRNPs. Moreover, Mer1p must be able to interact with U1, U2, and the intronic enhancer element to activate the formation of spliceosomes.

#### 4.3 Results

*Mer1p increases the amount of snRNAs bound to enhancer-containing pre-mRNA.* Previously we had demonstrated that Mer1p can co-immunoprecipitate U1 snRNA (Spingola & Ares, 2000). While this implies that Mer1p activates splicing by interacting with the U1 snRNP, it does not demonstrate that Mer1p facilitates the binding of U1 to pre-mRNA to form commitment complexes. Here we have utilized RNA affinity chromatography, to demonstrate that Mer1p increases the amount of snRNAs that bind to pre-mRNA. In previous studies we have shown that Mer1p can activate the splicing of an actin pre-mRNA that has been altered to contain a Mer1p enhancer element (ACACCCUU) and a weakened 5' splice site (GTTCGT or GTATAT, underlined nts deviate from wild type sequence GTATGT) *in vivo* (Spingola & Ares, 2000; Spingola et

al., 2004). A biotinylated derivative of these RNAs that contains three repeats of the enhancer element with a wild type 5' splice site sequence and branchpoint sequence, but lacking a 3' splice site and second exon, was transcribed *in vitro* (bt-3X-Act pre-mRNA). Previous work has shown that Mer1p rescues a variety of splicing defects: weak 5' splice sites, large 5' exons, weak branchpoint sequences, and even an intronic splicing silencer (Nandabalan et al., 1993; Nandabalan & Roeder, 1995; Spingola & Ares, 2000; Spingola et al., 2004). We chose the 3X-Act construct with a wild-type 5' splice site for affinity assays because it splices *in vitro* and binds snRNAs, whereas 3X-Act with a 5' splice site mutation does neither (not shown).

Bt-3X-Act pre-mRNA was immobilized on streptavidin-agarose beads and then incubated with splicing extracts that were prepared from haploid cells that either contained or lacked a plasmid for the constitutive expression of *MER1*. After washing unbound extract off the beads, bound snRNAs were eluted and analyzed by primer extension and PAGE. To compare the amounts of bound snRNAs in each reaction, any variability in agarose beads and RNA recovery during the wash and elution steps must first be considered. Hence, the bands in each lane of a gel were normalized to the actin recovery band in the same lane by taking the ratio of the snRNA band to the actin band. After this correction to account for minor differences in RNA recovery from the beads, the fold-increase of snRNA binding by Mer1p can be made by taking the ratio of snRNA/actin with Mer1p to snRNA/actin without Mer1p. The results in *Figure 4.1A* indicate that extracts that contained Mer1p led to more U1, U2 and U6 snRNAs binding to the immobilized pre-mRNA. Mer1p reproducibly caused a 2-3-fold increase in the levels of snRNAs bound to the pre-mRNA. Similar results were also obtained with a

biotinylated *AMAI* pre-mRNA (data not shown). Background binding of snRNAs to beads is common in RNA affinity experiments with yeast pre-mRNAs (Ruby et al., 1990), even after stringent washing. Although the levels of snRNAs bound to pre-mRNA-coated beads are not dramatically higher than background, the signals are consistently above background and the increase in snRNAs bound to Mer1p is very reproducible.

The results shown in *Figure 4.1A* do not distinguish whether Mer1p recruits all of the snRNAs or just U1 snRNA to the pre-mRNA, which is required for subsequent binding of U2 and U6. Furthermore, these results do not distinguish whether Mer1p increases the rate of binding of these snRNAs or if it stabilizes the binding of these snRNPs, which in turn allows higher order splicing complexes to form. These possibilities are addressed later.

To reduce any variability between splicing extracts, isogenic strains were used that differed only by the constitutive expression of *MER1*. Cultures of the two strains were grown simultaneously to identical cell densities, and extracts were prepared side-by-side. Then they were tested to show that (1) they contain the same amounts of snRNAs by primer extension (for example see lanes 1-2 of *Figure 4.1A*), (2) they have the same amount of total protein by a Bradford protein assay (data not shown), and (3) they have equivalent splicing activity on Actin pre-mRNA by a standard *in vitro* splicing assay (Lin et al., 1985) (data not shown).

As an important control to verify that the increased binding of snRNAs to pre-mRNA was due to Mer1p in the extracts, we tested whether extracts containing Mer1p would increase the binding of snRNAs to a similar pre-mRNA that lacked a Mer1p

enhancer element. To this end, bt-Act transcripts, which are identical to bt-3X-Act transcripts except that they lack the triple repeat of the enhancer element, were employed in the RNA affinity assay. *Figure 4.1B* shows that the ability of extracts with Mer1p to increase binding of snRNAs to pre-mRNA was abolished if the pre-mRNA lacked the enhancer element. *In vitro*, the Mer1p-dependent increase in snRNAs bound to immobilized bt-3X-Act pre-mRNA is dependent on Mer1p and on the enhancer element. Since the enhancer element is necessary and sufficient for Mer1p-activated splicing *in vivo* (Spingola & Ares, 2000), the dependence of the enhancer for Mer1p function *in vitro* is a good indication that our *in vitro* conditions sufficiently mimic *in vivo* conditions with regards to studying the function of Mer1p.

***Mer1p recruits U1 and U2 snRNA to pre-mRNA that lacks a branchpoint sequence.*** While the majority of experimental analysis on Mer1p functions implicates the U1 snRNP, some data have implicated the U2 snRNP: Mer1p interacts with the U2 protein Prp11p in two-hybrid experiments, Mer1p can rescue splicing defects that are caused by branchpoint sequence mutations, and Mer1p cannot activate splicing without the nonessential U2 snRNP protein Snu17p (Gottschalk et al., 2001; Wang & Rymond, 2003; Spingola et al., 2004; Wang et al., 2005), which is also a subunit of the RES Retention and Splicing complex (Dziembowski et al., 2004; Scherrer & Spingola, 2006). Recognition of the branchpoint sequence is thought to be mostly a function of U2 since the branchpoint sequence forms base pairs with U2 snRNA (Parker et al., 1987), although the branchpoint sequence is also recognized in the context of the commitment complex by two accessory proteins in yeast, Msl5p (the Branchpoint Binding Protein) and Mud2p,

prior to the binding of U2 snRNP (Abovich & Rosbash, 1997; Berglund et al., 1997). When these two accessory factors bind to the commitment complex, they form CC II, and they are displaced when U2 binds (Rutz & Seraphin, 1999). Despite the evidence above implicating U2 function for Mer1p-activated splicing, the Mer1p co-immunoprecipitate does not include U2 snRNA (Spingola & Ares, 2000). Although this result could be explained if binding of U2 to the CC blocked the binding of anti-Mer1p antibody, or if a Mer1p-U2 interaction were not stable enough to withstand the wash steps, we sought additional proof that Mer1p function directly involves the U2 snRNP. Since the RNA affinity chromatography experiments indicated that Mer1p increases the binding of several snRNAs to pre-mRNA, we originally sought to design a pre-mRNA that would only bind U1 snRNA. A pre-mRNA was designed that was truncated prior to the branchpoint sequence (Xho-bt-3X-Act). This shortened transcript includes the 5' exon, the 5' splice site and the enhancer element repeat, but no branchpoint sequence or 3' splice site. Surprisingly, when this RNA was subjected to RNA affinity chromatography, extracts containing Mer1p led not only to increased levels of bound U1, but also U2 (*Figure 4.2A*). There are at least two interpretations of these data: the increased abundance of U2 could simply be the result of increased U1 since U2 cannot bind to pre-mRNA without U1 bound, or Mer1p can directly recruit U2 to commitment complexes. The latter is consistent with the observation that Mer1p interacts with the U2 snRNP protein Prp11p. If the increased binding of U2 were solely the consequence of higher U1 binding, one might also predict increased U6 binding, which was not observed. In either event, Mer1p leads to an increase in the binding of both U1 and U2, but not U6, to a pre-mRNA that lacks a branchpoint sequence.

Without the branchpoint sequence, Mer1p's ability to increase the amount of U2 snRNA bound is slightly reduced (from 2.52 fold to 1.78 fold) indicating that the binding of U2 to pre-mRNA containing a branchpoint sequence is more stable. Interestingly, without the branchpoint sequence, Mer1p does not recruit as much U1 snRNA either. The increase due to Mer1p decreases from 3.6-fold to 1.8-fold when the bps is deleted. This could be because Msl5p and Mud2p cannot bind to this RNA to contribute stability to the commitment complex, or possibly because the binding of U2 affects the stability of U1. The possibility that U2 binding influences the binding of U1 is explored later.

***Recruitment of the U2 snRNP to pre-mRNA by Mer1p does not require ATP.***

Normally, the addition of U2 snRNP to the CC requires ATP hydrolysis (Perriman & Ares, 2000). Although the role of ATP in pre-spliceosome formation is still debatable, recent data suggest that Prp5p must hydrolyze ATP for the stable addition of U2 at the branchpoint sequence (Perriman et al., 2003) and that the ATP-imposing region of a pre-mRNA is immediately upstream of the branchpoint sequence (Newnham & Query, 2001; Xu et al., 2004). Since Xho-bt-3X-Act RNA lacks a branchpoint sequence and its adjacent 5' region, ATP should not be necessary for the addition of the U2 snRNP to commitment complexes that include Mer1p. RNA affinity experiments using Xho-bt-3X-Act RNA confirm this prediction (*Figure 4.2B*). Extracts containing Mer1p lead to an increase in U2 snRNA bound to pre-mRNA that lacks a branchpoint sequence even when ATP has been depleted from the splicing extract by the addition of dextrose (compare lanes 2-3). The addition of dextrose to 0.2mM efficiently depletes any ATP in the extract by providing a substrate for hexokinase in the extracts and inhibits splicing



(see lanes 3-4 of *Figure 4.6*) These data imply that the base pairing between U2 snRNA and the branchpoint sequence is not required for U2 snRNP to be added to commitment complexes that include Mer1p, and that Mer1p could be activating splicing by facilitating the formation of early splicing complexes that form prior to the hydrolysis of ATP but include U1 and U2.

***Loss of the Nam8p U1 snRNP protein abolishes the ability of Mer1p to increase the binding of snRNAs to pre-mRNA.*** Without the integral U1 snRNP protein Nam8p, Mer1p cannot activate splicing *in vivo*, and the inability to efficiently splice Mer1p-dependent introns leads to inviable spores (Nakagawa & Ogawa, 1997, 1999; Spingola & Ares, 2000). Nam8p has three RNA recognition motifs, cross-links to pre-mRNA just downstream of the 5' splice site, is important for splicing introns with weak 5' splice site sequences, and stabilizes commitment complexes on pre-mRNAs lacking caps (Gottschalk et al., 1998; Puig et al., 1999; Zhang & Rosbash, 1999). Nam8p is not essential for viability, and its loss has very little effect on splicing of most introns (Clark et al., 2002), other than those regulated by Mer1p. The loss of other nonessential U1 snRNP proteins does not affect Mer1p-activated splicing, indicating that there is a specific role for Nam8p in Mer1p function. U1 snRNPs prepared from *nam8Δ* cells have increased electrophoretic mobility and are deficient in two essential U1 proteins, Snu71p and Snu56p (Gottschalk et al., 1998). Our hypothesis is that Nam8p contributes stability to the commitment complex only on specific pre-mRNAs that are poorly spliced. We propose that Mer1p activates splicing by stabilizing commitment complexes on inefficiently spliced pre-mRNAs that do not interact strongly with U1 *in vivo*, and

furthermore, that the combined free energies contributed by both Mer1p and Nam8p are required to sufficiently stabilize the commitment complex for further assembly into an active spliceosome. In the absence of Nam8p, commitment complexes are too unstable on Mer1p-dependent introns for Mer1p to sufficiently stabilize the commitment complex to promote rapid assembly into an active enzyme. To begin testing this hypothesis, we have asked whether Mer1p can increase the binding of snRNAs to immobilized pre-mRNA in the absence of Nam8p. The results from this RNA affinity assay are displayed in *Figure 4.3*. Without Nam8p the levels of bound U1 and U2 are significantly reduced, and there is no difference in bound snRNA levels with or without Mer1p. This suggests that Nam8p can stabilize the binding of U1 to pre-mRNAs, and that this stabilization is necessary for Mer1p to function. Moreover, since the amount of U2 bound is also much lower with the *nam8Δ* extracts, the recruitment of U2 by Mer1p is also directly or indirectly dependent on Nam8p.

***Disrupting base pairs between U1 snRNA and pre-mRNA does not block the ability of Mer1p to increase the binding of U1 or U2 snRNPs.*** To determine whether the loss of Mer1p-enhanced binding of U1 and U2 snRNA to pre-mRNA with *nam8Δ* extracts was specifically due to the loss of Nam8p or a general feature of weakened U1 snRNPs or commitment complexes, the RNA affinity assay was repeated using extracts with a truncated U1 snRNA lacking the nucleotides that form base pairs to the 5' splice site. Previous experiments have shown that base pairing between U1 snRNA and pre-mRNA is not essential for the formation of CC, but that the CC is much less stable without these interactions (Du & Rosbash, 2001). In order to disrupt the 5' splice site-U1

snRNA base pairing interaction, extracts were treated with a synthetic DNA oligonucleotide that is complementary to the 5' end of U1 snRNA, which base pairs to the 5' splice site, and leads to its RNase H-mediated degradation. Remarkably, treated extracts containing Mer1p retained the ability to increase the binding of U1 snRNP or U2 snRNPs to pre-mRNA (*Figure 4.4*). When Mer1p is present, the levels of truncated U1 bound to pre-mRNA are slightly reduced compared to intact U1 snRNA - about a twofold increase of truncated U1 versus a 3.5-fold increase of full-length U1 by Mer1p. Levels of bound U2 are also increased by Mer1p when the base pairing between U1 and the intron is disrupted, although the values are reduced to less than twofold. However, the truncation of U1 snRNA was not complete in this experiment, and it is possible that the recruitment of U2 by Mer1p measured in this experiment is a function of the residual full-length U1. We conclude that Mer1p can increase/stabilize the binding of U1 to pre-mRNA without the base pairing interaction between U1 and the 5' splice site.

***Mer1p accelerates the formation of commitment complexes.*** We propose that Mer1p increases the equilibrium constant ( $K_{eq}$ ) for one or more early-forming splicing complexes; in particular, the commitment complex and pre-spliceosome. The  $K_{eq}$  is determined by the ratio of association to dissociation rate constants ( $k_a/k_d$ ). Hence, Mer1p could affect the equilibrium constant for an early splicing complex by either increasing its rate of formation (increasing  $k_a$ ) or decreasing its rate of dissociation ( $k_d$ ), or some combination of each. To test whether Mer1p affects the rate of formation of commitment complexes, we measured the binding of U1 to pre-mRNA over time using the RNA affinity assay. The results are presented in *Figure 4.5*. The data from samples

containing or lacking Mer1p were first normalized as in *Figure 4.1*. Then the data were normalized to the maximum amount of U1 bound and plotted over time. When Mer1p is present, greater amounts of U1 bind more rapidly to the pre-mRNA. From minutes 10-20, a significant increase in the amount of bound U1 is apparent when Mer1p is present. We conclude from these analyses that Mer1p can increase the equilibrium constant of the commitment complex at least in part by increasing the association rate constant for U1 binding to pre-mRNA.

***Mer1p commits pre-mRNA to splicing prior to ATP hydrolysis.*** Commitment assays have been used to demonstrate that a particular splicing factor or regulator can stabilize the binding of snRNPs to pre-mRNA to elicit splicing. In a typical commitment assay, radiolabeled pre-mRNA is first incubated with splicing regulator protein to allow the regulator to bind to the pre-mRNA. Then splicing extracts are added in conjunction with excess unlabeled pre-mRNA, and the amount of splicing is measured by PAGE. In the absence of splicing activator protein, labeled and unlabeled pre-mRNAs compete for the same limiting splicing factors in the extract, and the vast excess of unlabeled pre-mRNA out-competes the radiolabeled pre-mRNA for these factors. Fewer molecules of radiolabeled mRNA get spliced than if no competitor was added. However, if the splicing activator protein has bound to the pre-mRNA prior to the addition of competitor and splicing extract, it gives these pre-mRNAs an advantage over the competitor pre-mRNAs for binding snRNPs and splicing. The radiolabeled mRNA with bound regulator is preferentially chased into active splicing complexes over competitor RNA. This assay has been used to show that SR proteins are limiting splicing factors that commit pre-

mRNAs to splicing (Fu, 1993), and that pre-mRNAs are committed to splicing when U1 snRNP binds, prior to the binding of U2 (Legrain et al., 1988; Seraphin & Rosbash, 1989). We have adopted a similar strategy to determine if Mer1p can stabilize the binding of U1 and commit the pre-mRNA to splicing prior to U2 function. First, extracts are depleted of ATP by adding dextrose, which provides a plentiful substrate for hexokinase in the extracts. Treatment with dextrose inhibits splicing (See lanes 3-4 of *Figure 4.6*) but does not affect the formation of commitment complexes, which do not require ATP (Legrain et al., 1988) nor the ability of Mer1p to recruit U1 and U2 to the pre-mRNA (see *Figure 4.2B*). After ATP depletion, the extracts are added to radiolabeled 3X-Act pre-mRNA. As indicated by the RNA affinity experiments, U1 and U2 will bind to the pre-mRNA at this stage, but further assembly into an active spliceosome is stalled without ATP. After a short incubation, a second extract is added along with ATP and excess unlabeled pre-mRNA to chase the reaction to completion. After PAGE analysis, splicing is analyzed by measuring the relative amount of lariat product and lariat-second exon intermediate that form. By this method, Mer1p provides about a twofold activation of splicing (see *Figure 4.6*). Without dextrose treatment, very little difference in the amount of lariats formed can be seen with extracts that contain or lack Mer1p (lanes 5-6 of *Figure 4.6*). However, with dextrose treatment of the first extract, about twice as many lariats form after addition of a second functional extract and competitor RNA if Mer1p is included in the first extract (lanes 1-2). These results suggest that Mer1p commits pre-mRNA to splicing by stabilizing early splicing complexes prior to the hydrolysis of ATP and base pairing of U2 snRNP to the branchpoint sequence.

***Inactivation of the U2 snRNP reduces the ability of Mer1p to increase U1 snRNA binding.*** To analyze only the binding of U1 snRNA to pre-mRNA, we treated extracts with an anti-sense oligo that base pairs to the branchpoint interaction sequence of U2 to inactivate U2 in extracts and then performed RNA affinity chromatography. Surprisingly, the inactivation of U2 reproducibly led to decreased recruitment of U1 snRNA by Mer1p. In *Figure 4.7*, the amount of intact U2 is reduced by 80% (see lanes 1-4) by anti-sense treatment, and Mer1p only leads to a 1.4 fold increase in U1 snRNA bound to pre-mRNA as opposed to 2-3 fold when U2 is intact (compare lanes 6-8). The levels of total U1 in the extract are unchanged by treatment with the U2 anti-sense oligo (lanes 1-4). These results suggest that Mer1p's ability to recruit or stabilize the binding of U1 relies on U2. Additionally, *Figure 3* shows the recruitment of U2 is dependent on a U1 snRNP that includes Nam8p. These observations suggest that Mer1p must interact with U1, U2 and pre-mRNA simultaneously to activate splicing.

#### **4.4 Discussion**

We have developed assays that measure Mer1p's effect on the splicing process *in vitro*. While the traditional *in vitro* splicing assay with radiolabeled 3X-Act RNA and splicing extracts does not routinely show reproducible differences due to Mer1p, the RNA affinity assay results in highly reproducible differences in snRNP binding due to Mer1p. With the RNA affinity and commitment assays we demonstrate that Mer1p affects the binding of snRNPs to pre-mRNAs and commits or stabilizes early splicing complexes *in vitro*. Although the addition of highly purified recombinant Mer1p to

splicing extracts has advantages over extracts produced from cells expressing Mer1p, recombinant Mer1p produced in bacteria is very difficult to keep soluble. Nonetheless, with extracts that contain endogenously produced Mer1p and the RNA affinity and commitment assays, Mer1p activity *in vitro* faithfully reproduces its *in vivo* activity. Both *in vivo* and *in vitro* Mer1p function relies on Nam8p and on the presence of the intronic enhancer element.

The differences in snRNA binding and splicing commitment between extracts that contain or lack Mer1p are likely to be directly due to Mer1p and not a downstream effect of Mer1p, e.g., the production of a another splicing factor whose mRNA requires Mer1p for splicing. There are only a few genes for splicing factors that contain an intron, and none of these has the signature Mer1p enhancer element or is regulated by Mer1p. Of all the currently annotated introns in the Ares Lab Yeast Intron Database (<http://metarray.ucsc.edu/cgi-bin/intron/yirIntrondb>), only three genes contain a Mer1p-activatable intron, and each of these genes is directly involved in meiosis, not splicing. Therefore it is unlikely that extracts containing endogenously produced Mer1p also contain an additional splicing factor whose expression or activity relies on Mer1p and is responsible for the activities that we are attributing to Mer1p in the above experiments. In corroboration of this, the effects on splicing and spliceosome assembly that we are attributing to Mer1p rely on a pre-mRNA substrate that contains an intact enhancer element. We have already shown that Mer1p specifically binds to RNA containing this element, and it seems unlikely that Mer1p would activate the splicing of an unknown splicing factor that also binds to the enhancer element of our pre-mRNA substrate. For these reasons, we believe the effects on splicing in our assays are directly due to Mer1p.

A mechanism for Mer1p regulated splicing is beginning to emerge from these studies in which Mer1p activates splicing by overcoming a rate-limiting step in the formation of active spliceosomes. In the case of a Mer1p-regulated intron, the rate-limiting step in the splicing process is most likely the formation of a splicing complex that includes pre-mRNA, Mer1p, U1, and U2. Mer1p activates spliceosome assembly through a combination of increasing the rate of formation of early splicing complexes and by stabilizing these complexes sufficiently for continued assembly into an active spliceosome. Time course analysis of snRNAs binding to pre-mRNA indicates that Mer1p increases the velocity at which the first snRNP, U1, binds to the pre-mRNA. In addition to increasing the rate of binding, Mer1p stabilizes the binding of U1 and U2 to the pre-mRNA as indicated by commitment assays. Without Mer1p, the rate of binding of snRNPs and the stability of the complexes that do form are too low for efficient spliceosome assembly and splicing. In order to activate splicing, Mer1p must simultaneously interact with the intronic enhancer element, U1 snRNP, and U2 snRNP. Disrupting anyone of these interactions by deleting the enhancer element, deleting Nam8p, or inactivating U2 has the effect of preventing any increased snRNA binding by Mer1p, suggesting that all three interactions must occur simultaneously in order for Mer1p to activate splicing.

Splicing activation may be most readily accomplished during the initiation stages of spliceosome assembly. It would be more economical to regulate splicing prior to the expenditure of ATP. Detailed mechanisms of splicing activators are scarce with the exception of Mer1p, the *Drosophila* Transformer protein, and the mammalian TIA-1 protein. Transformer is expressed only in female flies, and it forms a complex with the



Transformer 2 protein. This complex binds specifically to an exonic purine-rich enhancer found on at least two pre-mRNAs, *Doublesex* and *Fruitless*. When bound to these enhancers, specific members of the SR protein family of splicing regulators are recruited and the formation of commitment complexes is enhanced (Tian & Maniatis, 1992, 1993; Lynch & Maniatis, 1996). In the case of *Dsx* pre-mRNA, the regulatory complex helps select a female-specific 3' splice site (Baker, 1989), whereas with *Fru* pre-mRNA the complex helps select a female-specific 5' splice site (Ryner et al., 1996; Heinrichs et al., 1998). Although yeast lack obvious members of the SR family (Mud2p is the closest homolog of an SR protein), Mer1p, like Transformer, regulates splicing in the initial stages of spliceosome assembly and activates a meiosis-specific 5' splice site.

TIA-1 is an animal splicing factor that is homologous to the yeast Nam8p (Del Gatto-Konczak et al., 2000; Forch et al., 2000). However, unlike Nam8p, TIA-1 is not an integral U1 snRNP protein, but rather an accessory U1 protein. TIA-1 binds to a U-rich region of introns just downstream of the 5' splice site (Forch et al., 2000), and co-immunoprecipitates the U1-C protein of the U1 snRNP (Forch et al., 2002). These observations suggest that TIA-1 may regulate splicing by binding adjacently to a 5' splice site and recruiting U1 snRNP to the 5' splice site by a protein interaction with U1-C. Mer1p seems to function in a similar manner to TIA-1, but recruits and stabilizes both U1 and U2.

In contrast, negative splicing regulators are known to function at various stages of spliceosome assembly and splicing. Several, like the *Drosophila* PSI or yeast Rpl30p function early in the assembly of spliceosomes (Siebel et al., 1992; Siebel et al., 1994; Vilardell & Warner, 1994) by binding the U1 snRNP to a pseudo-5' splice site or by

preventing the binding of U2, respectively. Others like the *Drosophila* Sex lethal protein have been shown to function after the first step of splicing and regulate the second step and 3' splice site selection (Lallena et al., 2002). There are too few mechanistic details and studies on splicing activators at this date to conclude that their function is limited to either the initial stages of spliceosome assembly or the second transesterification reaction.

#### 4.5 Materials and Methods

**Plasmids and yeast strains.** The following yeast strains were used for extract preparations: *KH46* (*MATa*, *leu2-3, 112*, *ura3-52*, *trp1-1*, *his3-1*, *lys2D*, *ade2-101*, *cup1D::ura3-52*), and *nam8Δ* (*MATa*, *ura3-52*, *leu2-3,112*, *trp1*, *lys2D*, *his3-1*, *ade2-101*, *cup1D::ura3-52*, *nam8::HIS3*). These strains either carried a constitutive *MER1* expression plasmid, pRS426FLAGMER1, or empty pRS426 vector. Plasmid pT73XAct has three copies of the *MER1* enhancer element (ACACCCTT) beginning at nucleotide 25 of the actin intron and was constructed by site-directed mutagenesis of pT7Act.

**Splicing extracts and RNA transcription.** Splicing extracts were prepared from *KH46*, or *nam8Δ* by the liquid nitrogen crushing procedure (Umen & Guthrie, 1995). pT7 plasmids were linearized by digestion with Hind III, Xho I, or Cla I. Run off transcripts from Hind III digested plasmids include the 5' exon, intron, and 3' exon; transcripts from Xho I digested plasmids include the 5' exon and approximately the first 70 nt of the intron and lack a branchpoint sequence; transcripts from Cla I digested plasmids lack the 3' splice site, but contain the branchpoint sequence. Radiolabeled RNAs for *in vitro* splicing reactions and commitment assays were transcribed with T7

RNA polymerase to a specific activity of approximately 6000 cpm/fmol. Transcription of biotinylated RNA was performed in 50 ml reactions with 2 mM ATP, GTP, UTP, 1.7 mM UTP, 0.3 mM biotinylated UTP (biotin-16-UTP, Roche), 40 mM Tris pH 7.9, 12 mM MgCl<sub>2</sub>, 2 mM spermidine, 20 mM DTT, 1mM NaCl, 100 units of T7 RNA polymerase, and 1 unit of RNasin. Transcripts were PAGE purified.

***RNA affinity chromatography and primer extension analysis.*** For a 50 ml reaction, 20 ml of a 50% slurry of streptavidin-agarose beads (Invitrogen) were used. Beads were blocked three times with 5 volumes of Blocking Buffer (Binding Buffer with 0.05% NP-40 and 2 mg/ml Heparin) and washed three times with 5 volumes of Binding Buffer (5 mM Tris pH 7.5, 0.5 mM EDTA, 1 M NaCl) before the addition of biotinylated RNA. 0.2 mg of biotinylated RNA (transcribed from Cla I or Xho I digested plasmids) was bound to the 20 ml beads in 100 ml of Binding buffer for 1 hour with gentle mixing at 4° C. The beads were then washed twice with 5 volumes of Buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 M EDTA, 20% glycerol) to remove unbound biotinylated RNA. 10 ml splicing extract, 10 ml 5X splicing buffer, 1 ml 100 mM ATP and 9 ml water were added to the bed of beads. The mixture was incubated for 20 mins at 23° C, except during the time-course experiments, where aliquots of 50 ml were collected over time, followed by three washes with 10 volumes of Washing Buffer (20 mM HEPES-KOH pH 7.8, 150 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5 mM DTT, 15% glycerol, 0.05% NP-40 and 2 mg/ml Heparin). RNA was recovered by adding 200 ml of Recovery Buffer (0.1 M Tris pH 8.8, 10 mM EDTA, 1% SDS, 0.15 M NaCl, 0.3 M NaOAc and 3 mM MgCl<sub>2</sub>), incubating for 10 mins at 65°C, followed by a phenol/chloroform extraction and ethanol

precipitation. For ATP depletion, 0.2 mM dextrose was added to the reaction prior to addition to the beads and incubated 10 mins at 37°C. For snRNA depletion experiments, extracts were pre-incubated with U1-KO oligo (5' TCTTAAGGTAAGTAT) to a final concentration of 1 mg/ml or with 0.5 mg/ml U2-KO oligo (5' CAGATACTACACTTG) for 10 mins at 37°C, and then added to the reaction.

Reverse transcriptions were performed according to the Superscript III protocol. After reverse transcription, PAGE was performed as described before (Spingola & Ares, 2000). Primer sequences for reverse transcription are available upon request.

In order to calculate any changes of bound snRNA when Mer1p is present, the data were normalized to the actin pre-mRNA band, which serves to indicate the relative amount of recovery from the beads. We took the ratio of each snRNA to actin, and then divided ratios from samples with Mer1p by ratios from samples lacking Mer1p to obtain the fold of increased binding by Mer1p.

***Commitment Assays.*** *In vitro* splicing commitment assays were performed in two steps or reactions. The first reaction contained 100 fmol of radiolabeled pre-mRNA (from run-off transcription of Hind III digested plasmid) and 4 ml of splicing extracts containing or lacking Mer1p in a final volume of 10 ml. ATP was depleted from the extracts used in the first step by pre-incubation with 0.2 mM dextrose for 15 min at 37°C prior to addition of pre-mRNA. After incubation at 25°C for 20 mins with the first extract, 10 ml of new extract were added with 20 mM ATP and a 400-fold molar excess of unlabeled competitor actin pre-mRNA. Reactions were then incubated for additional 30 min at 16° C and terminated by adding 200 ml of stop buffer (0.3 M NaOAc, 0.2%

SDS, 1 mM EDTA and 10 mg/ml proteinase K). PAGE analysis and phosphorimaging were conducted to determine the extent of splicing.

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#### 4.8 Figure Legends

**Figure 4.1** Mer1p increases the amount of snRNAs bound to pre-mRNAs that contain the Mer1p-enhancer element. A) Primer extension analysis of U1, U2, and U6 snRNAs bound to immobilized pre-mRNA (bt-3X-Act) with and without Mer1p. The amount of Actin pre-mRNA recovered from the beads was quantitated by primer extension and used to normalize each lane by taking the ratio of snRNA band to actin band. Dividing the ratio of snRNA/actin with Mer1p by snRNA/actin without Mer1p provides the fold increase of snRNA binding by Mer1p. Lanes 1-2 are primer extension on extracts to show that each extract has an equal amount of each snRNA. Lane 5 represents control beads that lack pre-mRNA and shows the background level of snRNAs



that bind to beads nonspecifically. The data in the tables below each gel include the fold-increase of bound snRNAs by Mer1p for the displayed gel and the averages with standard deviations of three or more replicate experiments. **B)** Mer1p's ability to increase the binding of snRNAs to pre-mRNA is specific to pre-mRNA that contains the enhancer element. Primer extension analysis of snRNAs bound to immobilized actin pre-mRNA lacking the Mer1p-enhancer element. In B a different actin primer was used that generates a longer product than in A.

**Figure 4.2** Mer1p influences the binding of U1 and U2 snRNA to pre-mRNA lacking a branchpoint sequence without ATP hydrolysis. **A)** The RNA affinity assay and analysis of bound snRNAs was carried out as in Figure 1A, except that a pre-mRNA lacking a branchpoint sequence was used. Lane 5 is a beads only control, and lanes 1-2 are controls showing equal amounts of each snRNA in the two extracts. The fold increases in bound snRNAs from this gel and the averages of three or more independent experiments are reported in the tables below each gel. **B)** Primer extension analysis of snRNAs bound to a pre-mRNA lacking a bps when ATP has been depleted by addition of dextrose.

**Figure 4.3** Recruitment of snRNAs by Mer1p is compromised by the deletion of U1 snRNP protein Nam8p. Splicing extracts from *nam8Δ* cells either expressing or not expressing Mer1p were prepared and used in RNA affinity assays. Lane 3 is a beads only control, and lanes 1-2 are controls showing equal amounts of each snRNA in the two extracts. Lanes were normalized, and lanes 4 and 5 were compared to calculate the fold

increase in bound RNA when Mer1p is present. The fold increases in bound snRNAs from this gel and the averages of three or more independent experiments are reported in the table below the gel.

**Figure 4.4** Disruption of the U1-5' splice site base pairing interaction does not disable Mer1p function. Splicing extracts were pre-incubated with an anti-sense oligo against the 5' end of the U1 snRNA before their addition to immobilized pre-mRNA. RNA recruitment levels were analyzed by primer extension (lanes 3 and 4). As a control, the RNA affinity assay was also performed as described in Figure 1A (lanes 5 and 6) without anti-sense treatment. Lane 7 and 8 are beads only control, and lanes 1-2 are controls showing equal amounts of each snRNA in the two extracts. U1\* represents cDNA from truncated U1 snRNA. The fold increases in bound snRNAs from this gel and the averages of three or more independent experiments are reported in the table below the gel.

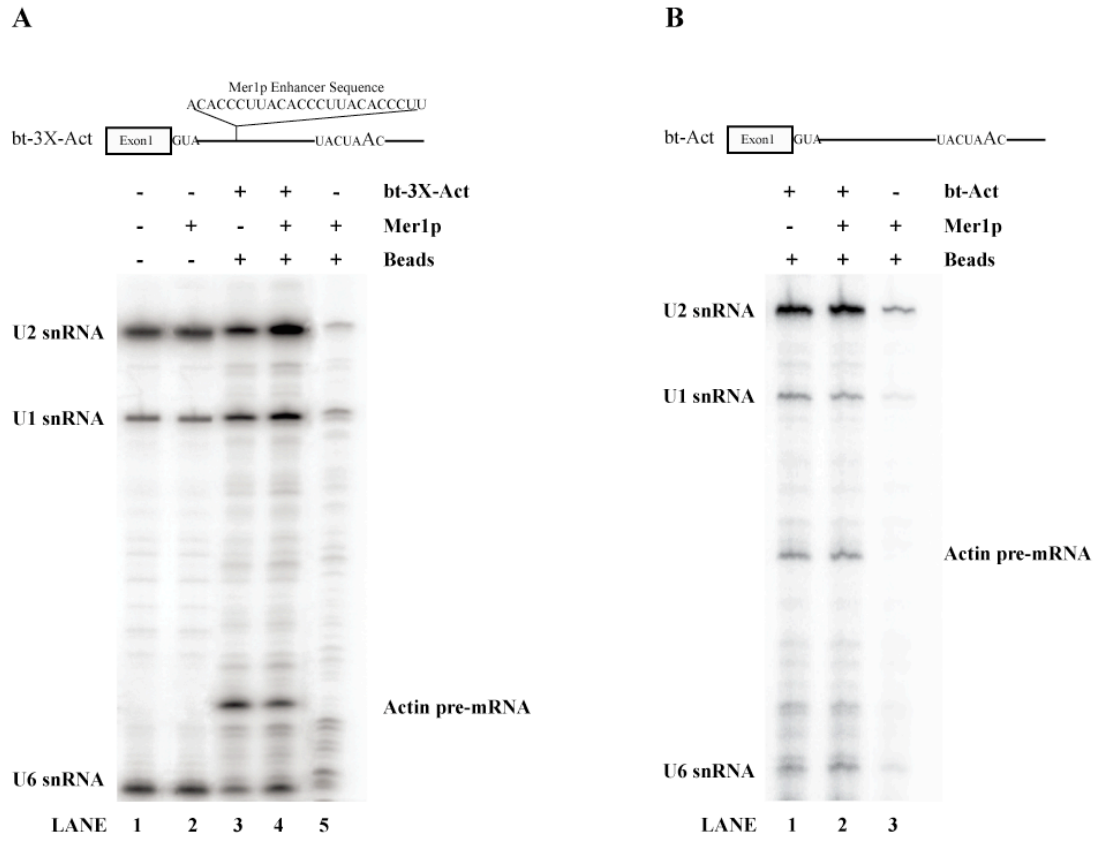
**Figure 4.5** Time-course analysis of U1 snRNP recruitment to pre-mRNA by Mer1p. After various incubation periods, bound U1 snRNA was eluted from the pre-mRNA and analyzed by primer extension (left). The data were normalized to the highest amount of U1 recovered and plotted as the maximum fraction of U1 bound versus time (right). The averages of two experiments with error bars representing standard error are shown on the graph.

**Figure 4.6** Mer1p commits pre-mRNA to splicing prior to ATP hydrolysis. In the first step of the commitment assay ATP was depleted to block the progression of the assembling spliceosome after U1 binding. In the second step, ATP was added along with competitor pre-mRNA to chase the reaction to completion. Splicing products were then analyzed by PAGE. Lariat formation was measured as the percentage of total radioactivity in the both the lariat product and lariat-exon two intermediate bands. For controls, reactions were performed without a second step incubation (lanes 3 and 4) to indicate that ATP depletion blocks splicing, or without ATP depletion (lanes 5 and 6). Pre-mRNA, lariat product, and lariat-second exon intermediate are indicated by the objects to the left of the autoradiograph. The fold increase in lariat formation from this gel and the averages of three or more independent experiments are reported below the gel.

**Figure 4.7** U2 snRNP plays an active role in the recruitment of U1 snRNP by Mer1p. The RNA affinity chromatography assay was performed as described before except that splicing extracts were incubated with an anti-sense oligo (U2-KO) against the bps interaction sequence of U2 snRNA before their addition to immobilized pre-mRNA. RNAs bound to immobilized pre-mRNA were analyzed by primer extension (lanes 6-9). As controls, RNA affinity chromatography was also performed without oligo treatment (lanes 8-9). In lanes 3-4, U2 anti-sense oligo leads to approximately an 80% reduction in the amount of full length U2 snRNA in the extracts but has no effect on the amounts of U1 in the extracts. U2\* represents cDNAs from truncated U2 snRNA. The fold

increases in bound snRNAs from this gel and the averages of three or more independent experiments are reported in the table below the gels.

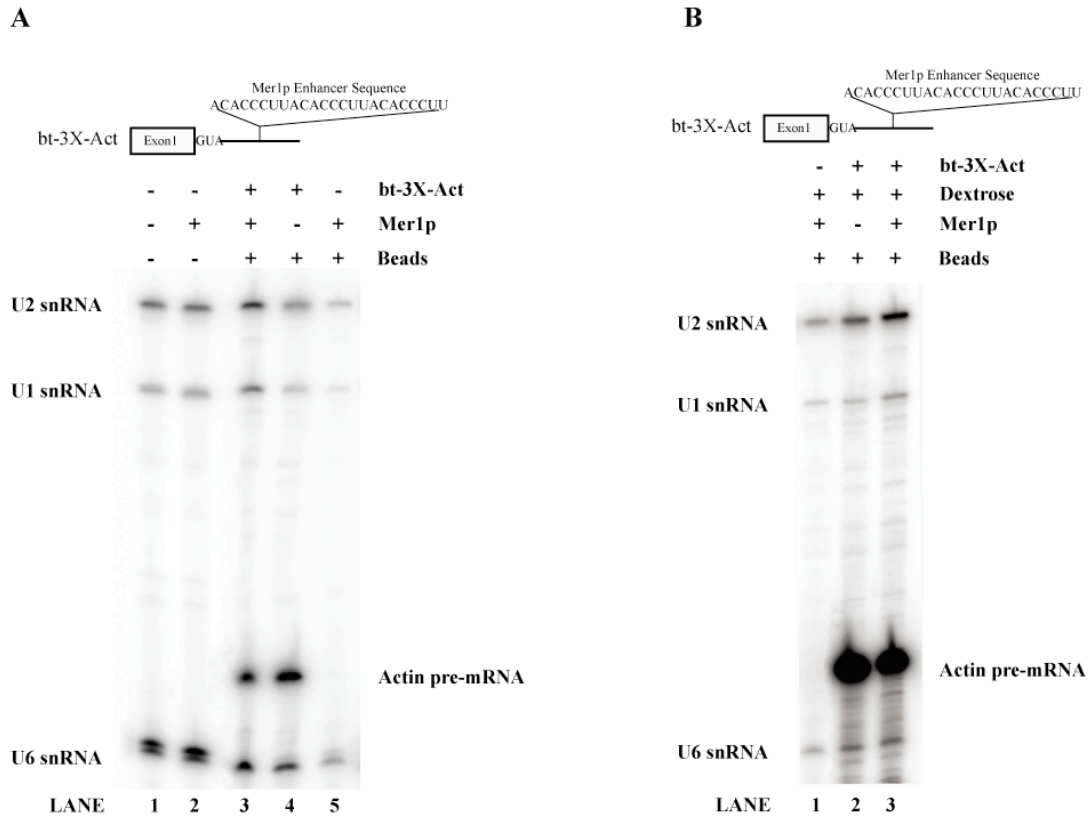
**Figure 4.1**



snRNA	Fold Increase +/- Mer1p	Average
U1 snRNA	4.85	3.52 +/- 1.66
U2 snRNA	2.88	2.52 +/- 0.23
U6 snRNA	1.69	1.70 +/- 0.01

snRNA	Fold Increase +/- Mer1p	Average
U1 snRNA	0.99	1.01 +/- 0.02
U2 snRNA	0.99	1.02 +/- 0.03
U6 snRNA	0.99	0.99 +/- 0.01

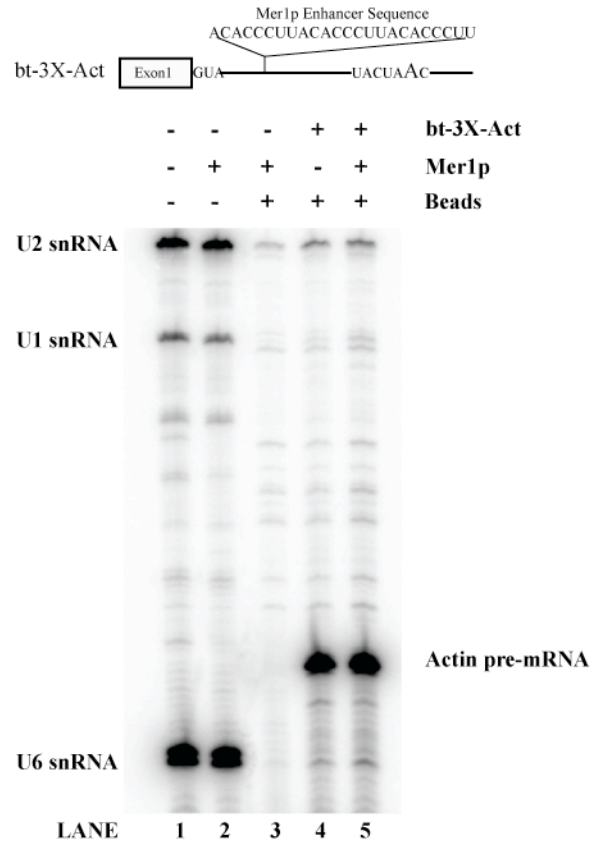
**Figure 4.2**



snRNA	Fold Increase +/- Mer1p	Average
U1 snRNA	1.91	1.82 +/- 0.43
U2 snRNA	1.80	1.78 +/- 0.54
U6 snRNA	0.99	0.99 +/- 0.01

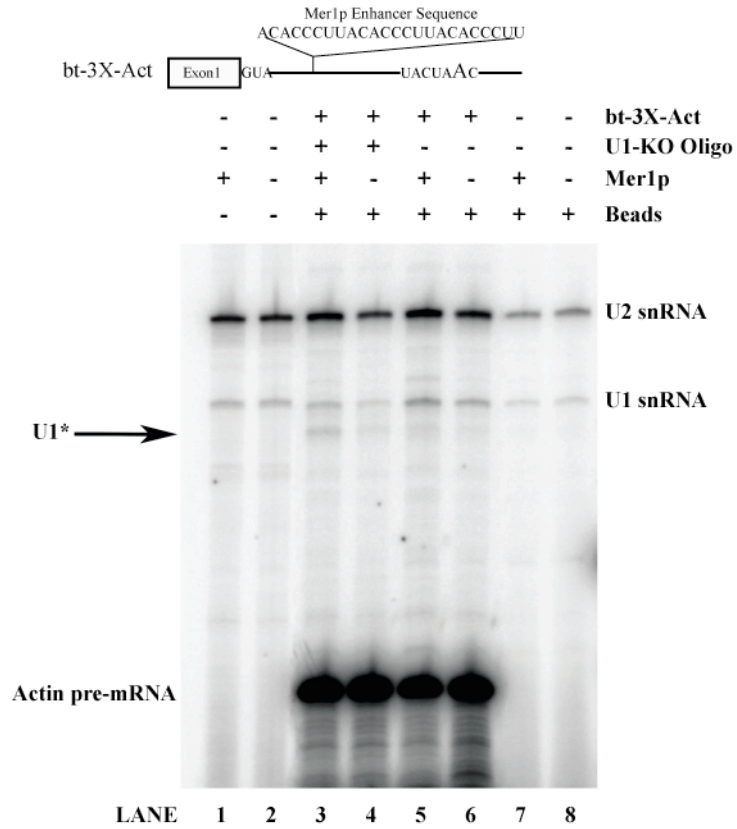
snRNA	Fold Increase +/- Mer1p	Average
U1 snRNA	1.99	1.91 +/- 0.39
U2 snRNA	1.89	1.80 +/- 0.22
U6 snRNA	1.01	1.01 +/- 0.01

**Figure 4.3**



snRNA	Fold Increase +/- Mer1p	Average
U1 snRNA	0.92	0.92 +/- 0.01
U2 snRNA	1.00	1.01 +/- 0.01
U6 snRNA	0.99	0.97 +/- 0.02

**Figure 4.4**

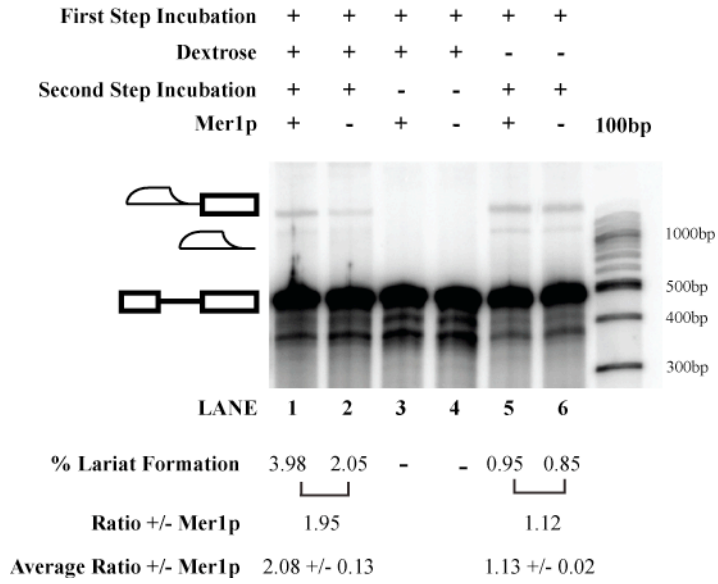


snRNA	Fold Increase +/- Mer1p	Average
U1* snRNA + U1KO	2.39	2.02 +/- 0.52
U2 snRNA + U1KO	2.32	1.82 +/- 0.57
U1 snRNA	1.99	3.52 +/- 1.66
U2 snRNA	1.84	2.52 +/- 0.23

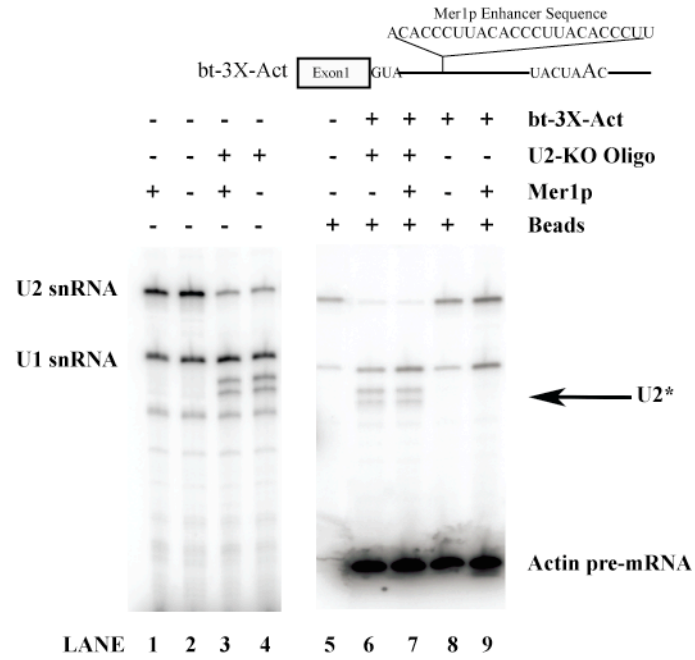




**Figure 4.6**



**Figure 4.7**



snRNA	Fold Increase +/- Mer1p	Average
U1 snRNA + U2KO	1.32	1.41 +/- 0.10
U1 snRNA	2.68	3.52 +/- 1.66
U2 snRNA	1.95	2.52 +/- 0.23

*CHAPTER V*

**REGULATION OF THE U2  
snRNP ASSEMBLY BY Mer1p**

## 5.1 Introduction

The human U2 snRNP was initially purified as a 12S particle containing the U2 snRNA, the seven Sm proteins common to all snRNPs, and two additional specific U2 snRNP proteins called U2-A' and U2-B'' (Polycarpou-Schwarz, Gunderson et al. 1996), whose yeast homologues were identified as the Lea1p protein and Yib9 protein (or Msl1p) respectively. Both proteins were required for the formation of the pre-spliceosome and presented a high degree of similarity with their human homologues, suggesting a conserved function of the U2 snRNPs specific proteins (Caspary and Seraphin 1998; Tang, Abovich et al. 1996). Posterior's experiments performed under low salt conditions revealed that the U2 snRNP was indeed a 17S particle containing all the 12S particle elements and ten additional U2-specific proteins (Will and Luhrmann 2001). These new U2-specific proteins were classified as components of one big splicing factor, named Splicing Factor 3 (SF3). Further characterization of the splicing factor SF3 revealed that it was composed of two subunits, SF3a and SF3b, each one containing several specific proteins (*Table 5.1*) (Brosi, Hauri et al. 1993).

As occurred with U2-A' and U2-B'', proteins of the SF3a and SF3b subunits were also evolutionarily conserved, and together with the U2 snRNA, were required for the formation of the pre-spliceosome, and for the formation of the mature 17S particle during its biogenesis (both subunits bind to the 12S U2 snRNP particle forming the mature 17S) (Dziembowski, Ventura et al. 2004; Wang, He et al. 2005). Indeed, the depletion of SF3a and/or SF3b abolished the formation of the 17S U2 particle, blocking the progression of the splicing reaction. SF3a and SF3b enter in the spliceosome assembly at the same time as the U2 snRNA and remain associated with the spliceosome until the splicing reaction

is completed (reviewed in Kramer 1996 and Will and Luhrmann 1997). *In vitro*, the formation of the mature U2 snRNP occurs in a stepwise fashion, which is initiated by the association of the SF3b to the 5' half of the U2 snRNA from the 12S particle. This association results in the formation of a 15S particle, which is required for the association of the SF3a to the 3' region of the U2 snRNA (where also the Sm proteins, and U2-A' and U2-B'' proteins reside) (Kramer, Ferfoggia et al. 2005).

Although the role of SF3 subunits is not totally understood, it has been reported that multiple regions of SF3a and SF3b are in close proximity with the pre-mRNA, suggesting that the SF3 complex could be anchoring the U2 snRNP to the pre-mRNA. Additional support for this function comes from the finding that the p14a protein (a component of the SF3b subunit) binds to the branchpoint sequence during the spliceosome assembly (Will and Luhrmann 1997; Gozani, Feld et al. 1996; Gozani, Potashkin et al. 1998). Interestingly, the proposed yeast homologue of P14p is Snu17p.

SF3a is constituted of three proteins Prp9p, Prp11p, and Prp21p. All of them are required for the formation of the mature 17S particle, and they influence the structure of the U2 snRNP in addition to regulating the accessibility of the U2 snRNA to the branchpoint region (Kramer, Ferfoggia et al. 2005). Prp9p, Prp11p, and Prp21p interact with one another, and they cross-link to the pre-mRNA, upstream of the branchpoint sequence during the pre-spliceosome formation (Wang, He et al. 2005). Blocking the binding of any of these splicing factors to the pre-mRNA prevents the association of U2 snRNP to the pre-mRNA indicating that only fully assembled SF3a associated with the U2 snRNP are functional in the splicing reaction (Rain, Tartakoff et al. 1996; Wiest, O'Day et al. 1996). Experimental evidence indicates that during the biogenesis of U2

snRNP, the SF3a heterotrimer is formed in the cytoplasm and then is targeted to the nucleus, independent of the U2 snRNP core (U2 snRNA; Sm proteins; U2-A' and U2-B'') and SF3b. Once in the nucleus, SF3a is associated with the U2 snRNP in Cajal Bodies where the U2 snRNA is processed. Finally, in addition to its role during splicing, the human SF3a subunits contain characteristic structural motifs (most of which are conserved) that are found in proteins involved in the regulation of transcription and chromatin remodeling (Kramer, Ferfaglia et al. 2005).

The proteins (*Table 5.1*) and function of the SF3b subunit are also well studied in the yeast *Saccharomyces cerevisiae* (reviewed in Wang, He et al. 2005). SF3b subunit is required for the commitment complex/pre-spliceosome transition as part of the U2 snRNP. Additionally, novel interactions have been described between proteins of the SF3b subunit (Cus1p) and components of the splicing machinery, such as the NineTeen Complex (NTC), which is involved in the addition and stabilization of the tri-snRNP to the pre-mRNA, and proper formation of the active spliceosome (Wang, He et al. 2005). Moreover, several proteins of the SF3b are required for the proper progression of the splicing reaction. For example, the deletion of Rcp10 affects the stability of SF3b, compromising the formation of the pre-spliceosome. Rcp1p is also essential for pre-mRNA splicing as well as Rsd3p. In the case of Rsd3p, which initially was classified as an auxiliary splicing factor, it has been demonstrated to be associated with other SF3b proteins, and its deletion causes the paralysis of the splicing reaction after the formation of commitment complex (Wang and Rymond 2003). Rsd3p is also associated with Yra1p, which is an RNA export factor, linking the U2 snRNP with RNA trafficking, and therefore the splicing reaction with other intracellular events.

<b>Human</b>	<b><i>S. cerevisiae</i></b>
<i>Core snRNP proteins</i>	
SmB/B	Smb1
SmD1	Smd1
SmD2	Smd2
SmD3	Smd3
SmE1	Sme1
SmF1	Smf1
SmG1	Smx2
LSM2	Lsm2
LSM3	Lsm3
LSM4	Lsm4
LSM5	Lsm5
LSM6	Lsm6
LSM7	Lsm7
LSM8	Lsm8
<i>U2 snRNP specific proteins</i>	
U2-A'	Lea1
U2-B''	Msl1
SF3a60	Prp9
SF3a66	Prp11
SF3a120	Prp21
SF3b49	Hsh49
SF3b145	Cus1
SF3b130	Rse1
SF3b155	Hsh155
SF3b14a-p14	Snu17
SF3b14b	Rds3
SF3b10	?
SF3b125	?

**Table 5.1** Proteins of the U2 snRNP particle in yeast and in humans (SGD).



In addition to the U2 snRNP-specific proteins required for the proper progression of the splicing reaction, the U2 snRNA also plays an important role during the splicing reaction. For example, it forms base pairs with the branchpoint sequence of the pre-mRNA and it is required in the formation of the active spliceosome. However, before the mature U2 snRNA can be assembled into the U2 snRNP, it needs to undergo a large number of post-transcriptional modifications, including addition of a 5' cap, internal 2'-O-methylation and pseudouridylation modifications (Massenet, Motorin et al. 1999). All these modifications, which are common to all five snRNAs, are often clustered in regions important for pre-mRNA splicing, indicating their importance in the splicing reaction (Zhao and Yu 2004; Yu et al., 2005). Interestingly, the U2 snRNA is the most extensively modified snRNA compared to the other snRNAs. In fact, more than 10% of its total nucleotides (approximately 189 nucleotides) are either 2'-O-methylated or pseudouridylated. In yeast, the process of pseudouridylation modification can be catalyzed by two different mechanisms (RNA-independent mechanism and RNA-guided mechanism) (Ma, Yang et al. 2005). Furthermore, the specific location of a given uridine within the U2 snRNA determines whether it is modified by an RNA-dependent or RNA-independent mechanism, but more important is that the uridines, located in the branchpoint sequence and its adjacent 3' region, are usually converted to pseudouridines following transcription. All these modifications are important requirements for the proper conformation of the U2 particle and for the binding of U2 snRNA to the pre-mRNA.

In previous chapters, we have observed that defects in the integrity of the U2 snRNP were compromising Mer1p activity. For example, in the absence of the non-essential U2 snRNP Snu17p, Mer1p fails to activate the splicing of pre-mRNAs. In

addition, when the U2 essential protein Prp11p was truncated to abolish its interaction with the U2 particle, Mer1p could not activate the splicing of Mer1p-dependent introns. Moreover, previous studies with Bud13p, a protein associated with the Retention and Splicing complex (RES) that interacts with Snu17p and Prp11p (Dziembowski, Ventura et al. 2004; Krogan, Cagney et al. 2006;), have shown that it was able to modulate the splicing activity of Mer1p for some of the Mer1p-dependent introns (Scherrer and Spingola 2006). Finally, disruption of the U2 snRNA affected the efficiency of Mer1p to recruit U1 snRNP (*Chapter IV*), suggesting that the U2 snRNP particle, together with U1 snRNP and the enhancer sequence, was required for Mer1p-function.

In this chapter, we focus our study in the effect of U2 snRNP over Mer1p activity by analyzing the recruitment of the snRNAs in the absence of Snu17p or Bud13p. In addition, we measure the recruitment of U2 snRNA by Mer1p over time and compare the relative levels of U1 snRNA over time.

The results show that while *snu17Δ* affects Mer1p function to enhance the recruitment of different snRNP to a pre-mRNA containing Mer1p-enhancer element, *bud13Δ* partially reduces Mer1p activity. In addition, a time course experiment reveals that the U2 snRNA is recruited practically at the same time as the U1 snRNA, suggesting that both U1 and U2 snRNPs could be associated simultaneously to the pre-mRNA when Mer1p is present. Finally, the requirement of U2 snRNP for Mer1p enhancement activity is also analyzed by commitment assay using U2 antisense-oligo, leading to the conclusion that in the absence of the U2 snRNP, Mer1p fails to stabilize the commitment complex.

## 5.2 Materials and Methods

**Yeast strains.** *snu17Δ* and *bud13Δ* gene deletion strains were created from strain *BY4741* (MAT  $\alpha$  his3- $\Delta$ 1 leu2- $\Delta$ 0 met15- $\Delta$ 0 ura3- $\Delta$ 0) (Invitrogen). Splicing extracts from the strains were performed as described in *Chapter IV*.

**Commitment Assays.** Commitment Assays were performed as described in *Chapter IV*, but for U2 snRNA depletion, extracts were pre-incubated with U2-KO oligo (5' CAGATACTACACTTG) to a final concentration of 0.5  $\mu$ g/ $\mu$ l for 10 mins at 37°C, and then added to the reaction.

*In vitro* transcription reaction, RNA affinity chromatography assays and primer extension analyses were done essentially as described in *Chapter IV*, with the above modification.

### 5.3 Results

*Loss of Snu17p abolishes the ability of Mer1p to increase the binding of snRNAs to pre-mRNA.* Snu17p (*IST3*) is a non-essential, but conserved evolutionary U2 snRNP protein with an RNA recognition motif (RRM) whose proposed mammalian homolog (P14p) is involved in recognition and binding to the conserved adenosine of the branchpoint sequence (Gottschalk, Bartels et al. 2001). Gottschalk et al., showed that during the *in vitro* splicing reaction Snu17p was able to co-precipitate with pre-mRNA and with lariat intron-exon 2 intermediate, indicating a direct binding between Snu17p and the pre-mRNA. Moreover, in yeast the U2 snRNA was specifically co-immunoprecipitated with Snu17p and in humans P14p interacts directly with the SF3b protein SF3b155 (Will, Schneider et al. 2001). *In vitro*, the absence of Snu17p stalled the splicing reaction before the first transesterification reaction, (Gottschalk, Bartels et al. 2001) and also it caused the formation of a spliceosome complex with a migration pattern different than in a wild type strain. Indeed, the absence of Snu17p caused the accumulation of a particle containing the pre-mRNA/penta-snRNP complex. When analyses of this unusual spliceosome complex were performed, they revealed normal levels for U2, U5 and U6 snRNP. Surprisingly, the levels of U1 and U4 snRNP were higher than normal, suggesting that deletion of Snu17p affected the splicing reaction before the release of U1 snRNP and after the binding of the tri-snRNP to the pre-mRNA (Gottschalk, Bartels et al. 2001). In addition to the formation of this unusual spliceosome complex, *snu17Δ* mutants also exhibited a growth defect on a fermentable carbon source and a unipolar budding phenotype (Ni and Snyder 2001).

Protein analyses using TAP tagged Rcp10p (a component of the SF3b particle)

revealed that Snu17p was also able to associate with Bud31p, forming a new subunit of SF3b, denominated p17. The two proteins showed similar levels of recovery at increasing salt concentrations, indicating a stable association among them. Little is known about the role of Bud31p and its influences over Snu17p function. Moreover, Bud31p is capable of interacting with different splicing factors such as Prp19p (part of the NTC complex) or the U2 protein Prp11p, and its deletion causes splicing defects (Masciadri, Areces et al. 2004). Surprisingly, *bud31* diploid mutants exhibit random budding pattern, instead of a unipolar pattern like *snu17* mutants.

In addition to forming a complex with Bud31p, Snu17p also interacts with several other splicing factors (*Table 5.2*) including components of the SF3b subunit Rds3p, Rse1p and Cus1p; components of the commitment complex, Mud2p; components of the NTC complex Prp19p; and non-splicing factors, including the transcription factor Tbp1p. Finally, Snu17p has also been associated with two other proteins, Bud13p and Pml1p, in the RES complex. The RES complex was characterized as a requirement for proper splicing *in vivo* as well as nuclear pre-mRNA retention (Dziembowski, Ventura et al. 2004). Since Snu17p was able to interact with two complexes (SF3b and the RES complex), it was proposed to have a dual function/state. It remains unknown when, with which regions, or how Snu17p is interacting with its different complexes, and in which situations Snu17p is part of the U2 snRNP complex or part of other complexes.

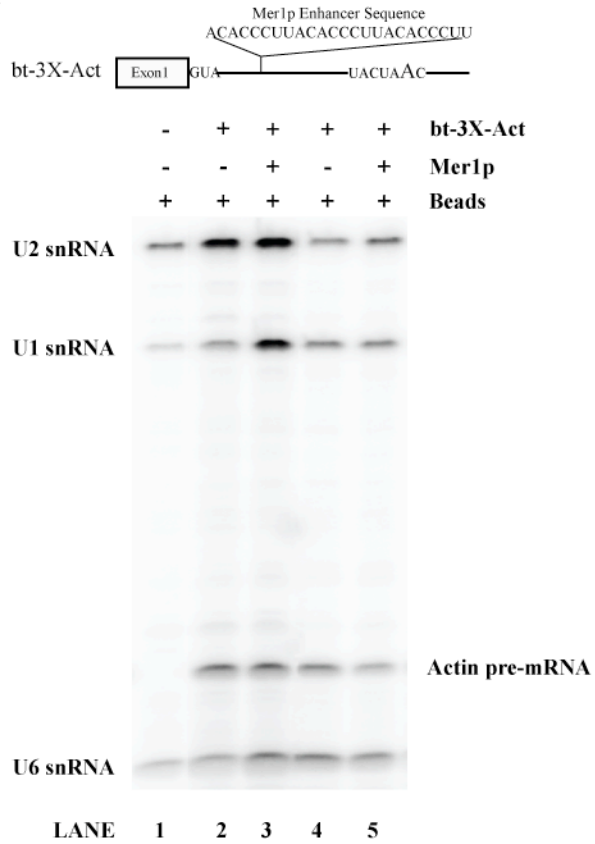
<b>Snu17p interactions</b>	
<b>Protein</b>	<b>Function</b>
Sah1p	Hydrolase
Cpr1p	Cytoplasmic isomerase
Bud13p	Subunit of the RES complex
Pgm2p	Phosphoglucomutase
Ded81p	Cytosolic asparaginyl-tRNA synthetase
RRS1	Cytoplasmic arginyl-tRNA synthetase
Ssz1p	Hsp70 protein
Car2p	Transaminase (OTase)
Cef1p	Essential splicing factor
Prp19p	Splicing factor
Rds3p	Splicing factor
Gts1p	Many cellular events; cell cycle and sporulation
Spt15p	Transcription factor

**Table 5.2** Snu17p interactions. The deletion of Snu17p can affect many different cellular processes, including splicing, transcription and sporulation (SGD).

*In vivo*, the deletion of Snu17p caused a reduction in splicing levels of pre-mRNA including those regulated by Mer1p, suggesting that Snu17p could be required for the recruitment of the snRNPs to the pre-mRNA. Our hypothesis is that as occurred with Nam8p, Snu17p is required for the proper recruitment of U1 and U2 snRNP by Mer1p to the pre-mRNA. Using RNA chromatography, we tested whether Mer1p was able to increase the binding of U1 and U2 snRNAs to immobilize pre-mRNA in the absence of Snu17p. The results from this RNA affinity assay are displayed in *Figure 5.1\_A*. Without Snu17p, the levels of bound U2 snRNAs are significantly reduced, and there is no difference in bound snRNA levels with or without Mer1p (lanes 4 & 5). As occurred in *nam8Δ*, in the absence of Snu17p, Mer1p cannot stabilize the binding of U2 to pre-mRNA. Surprisingly, the amount of U1 bound to the pre-mRNA is also much lower with

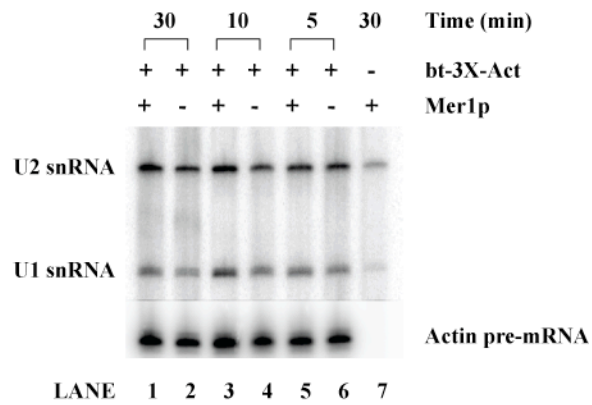
the *snu17Δ* extracts (lane 5), indicating that the recruitment of U1 by Mer1p is dependent on the U2 snRNP protein Snu17p, and supporting the hypothesis that Mer1p must interact properly with U1, U2 and the intronic enhancer sequence in order to activate the splicing of Mer1p-dependent introns. Additionally, we looked at the recruitment of U1 and U2 in *snu17Δ* splicing extracts at earlier stages during the splicing reaction to measure the true effect over U1 snRNA (*Figure 5.1\_B*). Since *snu17Δ* only affects the spliceosome assembly after the binding of U1 to the pre-mRNA (Gottschalk, Bartels et al. 2001), the effects observed previously at 20 minutes could be reflecting the pre-spliceosome stage, in which case U1 was already bound to the pre-mRNA and therefore its levels were already too high to see any difference with or without Mer1p. We measured the RNA bound at 5 min, 10 min and 30 min (*Figure 5.1\_B*). In all the time points, in the absence of Snu17p, Mer1p fails to enhance the recruitment of snRNP at any time during the splicing reaction, suggesting that in order for Mer1p to stabilize the formation of commitment complex, the U2 snRNP protein Snu17p is required. We conclude that the defect caused by *snu17Δ* is a consequence, at least partially, of the destabilization of the complex, even in the presence of Mer1p.

**A**



snRNAs	Fold Increase with Mer1p	Std. Dev.
U1 snRNA	1.03	0.2
U2 snRNA	1.09	0.2
U6 snRNA	0.97	0.1

**B**





**Figure 5.1** Snu17p is required for the recruitment of snRNAs by Mer1p. **A)** Splicing extracts from *snu17Δ* cells either expressing or not expressing Mer1p were prepared and used in RNA affinity assays. Lane 1 is a beads only control, and lanes 2-3 are controls of RNA affinity assay performed in the presence of Snu17p. Lanes were normalized, and lanes 4 and 5 were compared to calculate the fold increase in bound RNA when Mer1p is present. The fold increases in bound snRNAs from the averages of three independent experiments are reported in the table below the gel. **B)** Time course analysis of U1 and U2 snRNP recruitment to pre-mRNA by Mer1p in the absence of Snu17p. Lane 7 is a beads only control.

***Bud13p modulates Mer1p-splicing activity in a different manner than Snu17p or Nam8p.*** Bud13p is a 30.5 KD basic protein required for bud site selection in yeast. Its deletion results in a unipolar budding phenotype (Ni and Snyder 2001). *BUD13* has also been characterized in many other organisms as a splicing factor (Bud13p mutants cause splicing defects). Bud13p also interacts with the SF3b subunit Hsh155p, whose human homologue SF3b155 interacts with P14p (suggested mammalian homologue of Snu17p), and with several splicing factors (*table 5.3*) (Ito, Chiba et al. 2001). Additionally, Bud13p and Snu17p proteins interact *in vivo* by the yeast two-hybrid assay (Uetz, Giot et al. 2000), and both proteins have been localized throughout the nucleus, and expressed at different phases during the cell cycle. Curiously, *snu17Δ/snu17Δ* and *bud13Δ/bud13Δ* exhibit the same phenotype of elongated cell shapes. Finally, *bud 13* mutants are synthetically lethal with *clf1* mutants. Clf1p plays an important role during the spliceosome assembly, acting as support between pre-mRNA and spliceosome machinery (Wang and Rymond 2003). Therefore, Bud13p is not only involved in bud site selection, but it also participates in other cellular events such as the regulation of the splicing reaction.

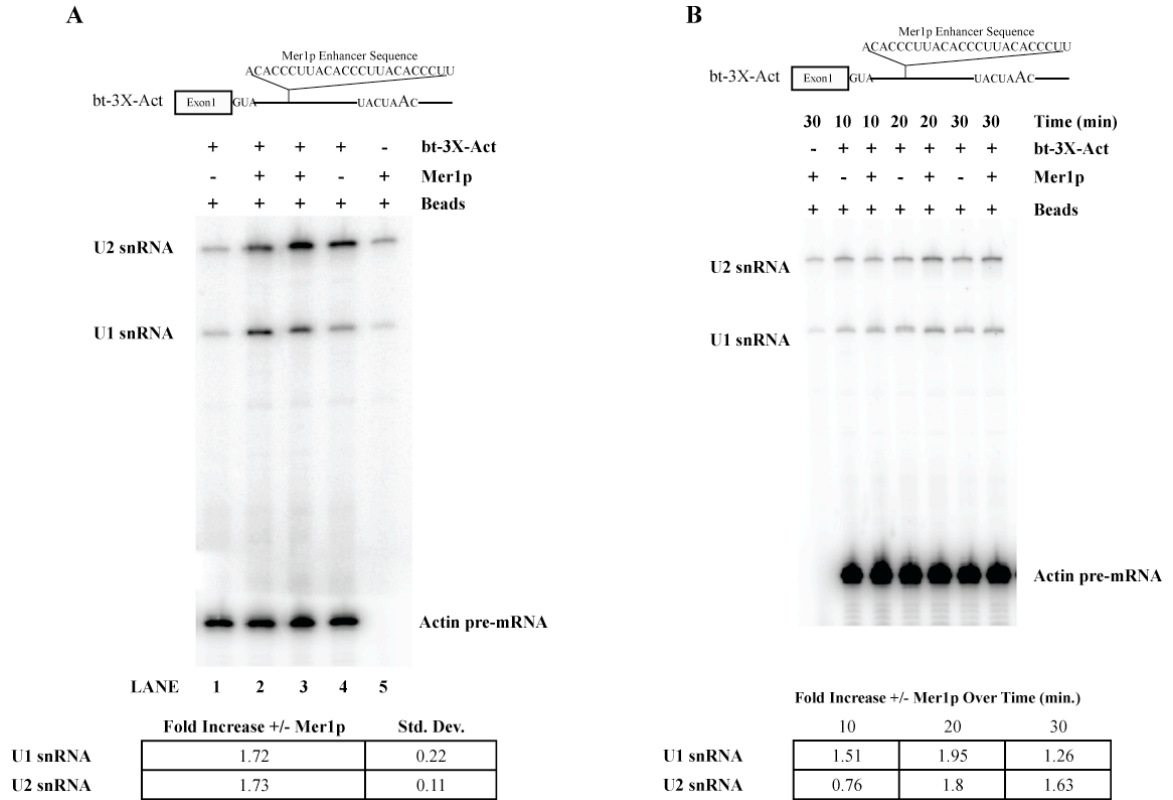
### Bud13p Interactions

Protein	Function
Kip3p	Kinesin-related motor protein involved in mitotic spindle positioning
Clu1p	eIF3 component
Ist3p	Splicing factor
Pml1p	Subunit of the RES complex
Cef1p	Splicing factor; associated with Prp19p and the spliceosome
Cwc2p	Splicing; interacts with Prp19p
Prp19p	Splicing factor
Prp11p	Subunit of the SF3a splicing factor complex
Cus1p	Required for assembly of U2 snRNP into the spliceosome

**Table 5.3** Bud13p interactions (SGD).

Bud13p, together with Snu17p and Pml1p, was also identified in a complex involved in Retention and Splicing of pre-mRNA (RES). Purification of the RES complex did not show association with snRNA, even though previous results indicated a specific association between Snu17p and the U2 snRNA. Moreover, Pml1p, which was previously identified in a complex containing all five snRNPs (Stevens, Ryan et al. 2002), seems to not be involved in the splicing reaction when is forming part of the RES complex. While commitment complex formation and mobility of spliceosome migration were affected in a *snu17Δ* or *bud13Δ* strain, *pml1Δ* did not affect the migration of the spliceosome or commitment complex formation (Dziembowski, Ventura et al. 2004), and therefore, the hypothesis of dual function/stage of some of the RES proteins could explain these results. However, it is unclear when or how the RES complex is formed; although its function remains largely unknown, it has been proposed to be required for efficient splicing of introns with weak 5' splice sites by preventing the export of pre-mRNA rather than interacting with the splicing machinery. *In vitro*, the association of

RES with pre-mRNA is weaker than other splicing factors (Dziembowski, Ventura et al. 2004). Additionally, Bud13p modulates the splicing efficiency of some Mer1p-dependent introns such as *AMA1*, which contains a conserved 5' splice site (Scherrer and Spingola 2006). Nonetheless, since Bud13p is not part of the U2 particle, but still interacts with some of the U2 snRNP proteins, we hypothesize that in contrast to what was happening with *snu17Δ* extract, its deletion will cause only moderate effects, or none, over Mer1p activity (maybe as a consequence of its interaction with Snu17p and other splicing factors). Using RNA chromatography assay, the recruitment of U1 and U2 snRNAs to immobilize pre-mRNA by Mer1p was measured in the absence of Bud13p. Results from the RNA chromatography assay are displayed in *Figure 5.2\_A*. In the absence of Bud13p, Mer1p still was able to enhance the recruitment of U1 and U2 snRNAs to the pre-mRNA, and even though the snRNA levels recruited by Mer1p in a *bud13Δ* background were lower than in a wild type strain, they were much higher than the levels observed in a *nam8Δ* strain or in a *snu17Δ*. The result indicated that the activity of Mer1p was not totally dependent on Bud13p. Since Bud13p has been classified as a splicing factor, and interacts with many other splicing factors including Snu17p (which is required for Mer1p activity), its deletion could cause a destabilization of the spliceosomal complex or malfunction of Snu17p, rather than cause a defect in Mer1p activity.



**Figure 5.2** Recruitment of U1 and U2 snRNAs in a *bud13Δ* strain. **A)** Splicing extracts from *bud13Δ* cells either expressing or not expressing Mer1p were prepared and used in RNA affinity assays. Lane 5 is a beads only control, and lanes 3-4 are controls of RNA affinity assay performed in the presence of Bud13p. Lanes were normalized, and lanes 2 and 1 were compared to calculate the fold increase in bound RNA when Mer1p is present. The fold increases in bound snRNAs from the averages of three independent experiments are reported in the table below the gel. **B)** Time course analysis of U1 and U2 snRNP recruitment to pre-mRNA by Mer1p in the absence of Bud13p.

Moreover, since it has been reported that, *in vivo*, for some Mer1p-dependent introns Bud13p was required for Mer1p activity, maybe *bud13Δ* effects could be observed at different stages of the splicing reaction, rather than acting at all stages, as is the case in *snu17Δ* or *nam8Δ* extracts. Time course experiments were performed to analyze the levels of U1 and U2 in a *bud13Δ* strain at different times during the formation of the spliceosome. As it is shown in *Figure 5.2\_B* at early times (10 min) Mer1p slightly enhances the recruitment of U1, but is only after 20 min. when the differences +/- Mer1p are significant. This result indicates that Bud13p as a splicing factor could be required for Mer1p activity at early times of the splicing reaction (during the formation of the commitment complex or pre-spliceosome), but it does not support the *in vivo* observation since Bud13p deletion cannot prevent completely the association of U1 and U2 snRNAs (as it does the deletion of Nam8p or Snu17p, see *Figure 4.3* and *Figure 5.1*). Since Bud13p is also a subunit of the RES complex, it was proposed that the RES complex could play an important role in Mer1p activity regulation, and therefore Mer1p could have different mode of actions, depending on its interaction with the splicing machinery or with the RES complex.

***Intact U2 snRNP is required for Mer1p activity in vitro.*** In *Chapter IV*, we observed that disruption of U2 snRNA structure by using an anti-sense oligo prevented almost completely the recruitment of U1 to the pre-mRNA (*Figure 4.7*). While this result implies that Mer1p must interact directly or indirectly with U1, U2 and pre-mRNA simultaneously to activate splicing, this does not prove the necessity of U2 for Mer1p to fully stabilize the binding of U1 to pre-mRNA. Therefore a commitment assay using a

primary extract that has U2 inactivated by an anti-sense oligo was performed (*Figure 5.3*). The results from the commitment assay indicate that intact U2 is necessary for Mer1p to commit pre-mRNA to splicing. After adding an active secondary extract and competitor RNA to the commitment reaction, samples with Mer1p show no more splicing than reactions lacking Mer1p (lanes 1 and 2). Without intact U2 in the first incubation, Mer1p provides no competitive advantage to splicing in the commitment assay, presumably because Mer1p must recruit U1 and intact U2 during the first incubation. Without intact U2, U1 is insufficiently recruited to the pre-mRNA to provide any advantage in this assay, suggesting that Mer1p commits pre-mRNA to the splicing process by simultaneously recruiting and/or stabilizing the binding of U1 and U2. When ATP is depleted, Mer1p can still recruit or stabilize the binding of U1 and U2 during the first incubation and provide a competitive advantage in assembling into an active spliceosome when the remaining components are added along with an unlabeled competitor. However, if U2 is damaged or destroyed, Mer1p cannot sufficiently stabilize the binding of U1 during the first incubation and provides no advantage to the radiolabeled pre-mRNA when a second fully active extract is added in conjunction with unlabeled competitor RNA.

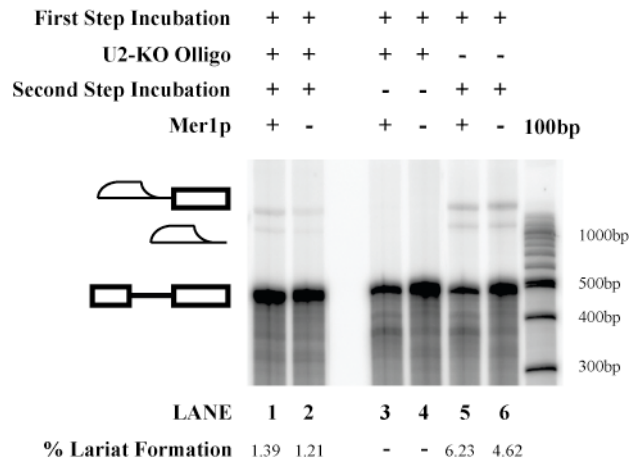
***In the presence of Mer1p, U2 snRNP is recruited to similar levels as U1 snRNP.***

The current model for spliceosome assembly proposes that U2 snRNP is recruited to the pre-mRNA after the binding of U1 snRNP (Tardiff and Rosbash 2006). In addition, it has also been proposed that the spliceosome could be pre-assembled before its interaction with the pre-mRNA (Stevens, Ryan et al. 2002). Therefore, based on the idea that the

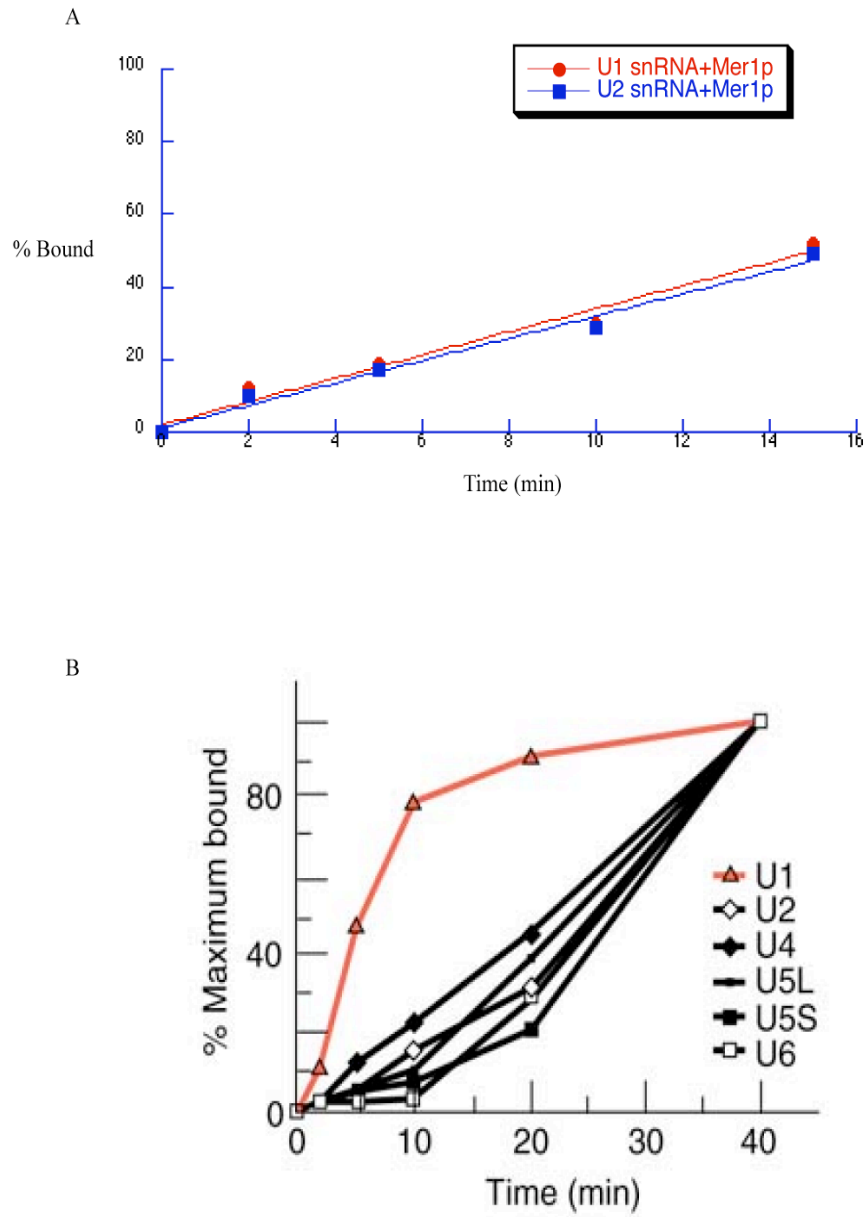
different snRNPs could be interacting prior to their association with the pre-mRNA, and that U2 snRNP is required for Mer1p function, we propose that Mer1p could be recruiting jointly U1 and U2 snRNAs to the pre-mRNA. Time course experiments were performed to measure the recruitment of U2 snRNA in the presence of Mer1p. Then the U2 snRNA levels were plotted against the levels of U1 snRNA recruited when Mer1p was expressed (*Figure 5.4*). Surprisingly, in the presence of Mer1p, the amounts of U1 and U2 bound to the pre-mRNA over different time points are very similar, versus previous models of snRNA recruitment, where under “normal condition” (no splicing regulator is required for proper splicing of the intron) U1 snRNA is first recruited and then U2 snRNA (*Figure 5.4\_B*) (Ruby and Abelson 1988).

In conclusion, our data support a model for the spliceosome assembly where in the presence of a splicing enhancer regulator, such as Mer1p, U1 and U2 could be recruited simultaneously to the pre-mRNA. While the current model supports assembly of the spliceosome in a stepwise fashion, this has only been tested in a few introns, of which neither were poorly spliced or required enhancer elements for their splicing.





**Figure 5.3** Requirement of U2 snRNP for Mer1p to activate splicing. The commitment assay was performed by using the anti-sense U2 oligo to block spliceosome assembly during the first step incubation. For the second step incubation, extract with intact U2 and competitor pre-mRNA were added and splicing was measured by PAGE. Lanes 5-6 are controls indicating that the anti-sense oligo blocks splicing, and lanes 7-8 are controls showing that splicing occurs in the absence of anti-sense oligo. Lanes 1-4 show that Mer1p cannot commit pre-mRNA to splicing if intact U2 is not available during the first incubation.



**Figure 5.4** Amount of U1 and U2 snRNA recruited at early times in the presence of Mer1p. The values are expressed as percent of each snRNA bound at 15 min. Panel B represents previous observations of how the different snRNAs are recruited to the pre-mRNA (Ruby and Abelson 1988).

## 5.4 Discussion

Previous observations concluded that, *in vivo*, Mer1p required the snRNP specific proteins Nam8p and Snu17p for the splicing activation of pre-mRNA containing the Mer1p enhancer element (Spingola and Ares 2000; Spingola, Armisen et al. 2004). Moreover, *in vitro*, *nam8Δ* blocked the ability of Mer1p to enhance the recruitment of snRNAs to the pre-mRNA. In addition, *in vivo*, Bud13p was a requirement for the splicing activation of some Mer1p-dependent introns (Scherrer and Spingola 2006). In this chapter, we analyzed the *in vitro* effects of Snu17p and Bud13p over Mer1p function to enhance the binding of U1 and U2 snRNAs by measuring the levels of RNA recruited when either protein was deleted. While the deletion of Snu17p caused the same severe defects in Mer1p activity as *nam8Δ* extracts, *bud13Δ* extracts had only a moderate effect over the recruitment of snRNAs. Indeed, some defect in the snRNAs recruitment in a *bud13Δ* strain was only observed at early stages of the reaction, suggesting that it could be affecting the stabilization of the commitment complex or the transition between commitment complex and pre-spliceosome. Since the *in vitro* results for Bud13p deletion do not reflect the *in vivo* observations, we propose that the ability of Mer1p to recruit the snRNPs is linked to its ability to interact properly with them, and since Bud13p is not a component of any of the snRNPs, its deletion does not affect the recruitment of snRNP by Mer1p.

Additionally, and in order to support the hypothesis of the U2 requirement for Mer1p, a commitment assay was performed using extracts that had U2 inactivated by an anti-sense oligo. The inactivation of the U2 affected the ability of Mer1p to stabilize the

formation of the commitment complex, indicating that intact U2 snRNP is required for Mer1p activity.

Finally, and confirming the previous observations that U2 snRNA is required for recruitment of U1 snRNA, the depletion of the U2 snRNP protein Snu17p also affects the recruitment of the U1 snRNP, suggesting Mer1p, and maybe other splicing enhancer regulators modulate the assembly of the spliceosome in a different manner, rather than in a stepwise fashion. Indeed, when the recruitment of U1 and U2 snRNA is compared at early stages of the splicing reaction there is no clear difference in the association of the snRNA to the pre-mRNA, supporting the hypothesis that Mer1p could bring together U1 and U2 snRNPs to the pre-mRNA.

In conclusion, the ability of Mer1p to enhance the recruitment and stabilization of snRNPs to the pre-mRNA resides in its ability to interact properly and simultaneously with specific components of the U1 snRNP and U2 snRNP particles.

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*CHAPTER VI*

**SUMMARY and FUTURE  
DIRECTION**

In this work we have examined the molecular mechanisms by which Mer1p activates the splicing of pre-mRNA containing the Mer1p enhancer sequence to better understand how Mer1p and other enhancer regulators (for which mechanisms of action remain unknown) regulate the splicing reaction. *In vivo*, the splicing efficiency of the meiosis-specific genes *AMA1*, *MER2* and *MER3* is significantly increased and detectable in the presence of Mer1p (Spingola and Ares 2000). *In vitro* these pre-mRNAs splice poorly and any attempt to study increases in the splicing efficiency by Mer1p using traditional *in vitro* assays have failed (Marc Spingola, personal communication). Therefore, one of the main goals of this study was to develop an assay that could measure Mer1p's effects *in vitro* and at the same time reflect the *in vivo* observations. For this purpose an RNA chromatography assay, modified to study Mer1p function, was developed. Briefly, a modified biotinylated pre-mRNA was immobilized to streptavidin-agarose beads, and incubated with splicing extracts, with or without Mer1p. After extensive washes, RNAs bound to the pre-mRNA were recovered and analyzed by primer extension. Using this powerful technique, the *in vitro* effects of Mer1p on the recruitment of snRNAs to the pre-mRNA were analyzed and a new model for Mer1p activity was proposed.

As presented in *chapter IV*, Mer1p was able to enhance the binding of snRNAs, and therefore the snRNPs, to pre-mRNA containing the Mer1p-enhancer sequence. Indeed, the levels observed in the presence of Mer1p were increased about three fold for U1 snRNA and increased two fold for U2 and U6 snRNAs. Furthermore, in order to confirm that the *in vitro* assays reflected the *in vivo* effects of Mer1p, the same experiments were conducted, using a pre-mRNA lacking the Mer1p-enhancer sequence



or using a splicing extract from a *nam8Δ* strain. As occurred *in vivo*, the removal of the enhancer sequence or the absence of Nam8p from the extract prevented Mer1p activity *in vitro*, confirming that our *in vitro* system was mimicking what occurs *in vivo*.

In addition, previous results suggested that Mer1p could be acting during the early stages of the spliceosome assembly, influencing the formation of commitment complexes I and II (Spingola and Ares 2000). In order to focus our studies to the early stages of the splicing reaction, the branchpoint sequence and its adjacent 3' region were eliminated from the pre-mRNA. While the branchpoint sequence is required for the addition of U2 snRNP to the pre-mRNA (Liao, Colot et al. 1992), and its adjacent 3' region is required for the ATP activity during the spliceosome assembly (Newnham and Query 2001), neither region is required for the association of U1 snRNP. Therefore, the deletion of these regions should prevent the binding of U2 (no pre-spliceosome will be formed) but U1 should still be able to bind and form the commitment complexes I and II. Surprisingly, the deletion of these regions not only allowed Mer1p to enhance the recruitment of U1 (as expected) but also U2 recruitment was enhanced. When the experiments were performed in the absence of ATP, Mer1p was still able to enhance the recruitment of U1 and U2 snRNAs, indicating that for the first time, an enhancer regulator was able to bypass (at least partially) the sequence requirement for the spliceosome assembly. This set of experiments concluded that Mer1p activated the splicing by recruiting directly the snRNPs to the pre-mRNA and that the recruitment of U2 snRNP was ATP independent as well as branchpoint region independent. Previous work on enhancer elements has been done with the *Drosophila* Transformer protein and the mammalian TIA-1 protein and providing evidence that other enhancer factors could

also recruit directly the snRNPs to the pre-mRNA (Tian and Maniatis 1993; Lynch and Maniatis 1996; Forch, Puig et al. 2002).

While the fold increase observed for U1 could be a direct effect of the Mer1p activity, the fold increase observed for U2 and U6 could be an indirect effect due to an increase in the recruitment of U1. According to the most current model for spliceosome assembly, U1 is first recruited followed by U2 and then U6 (Tardiff and Rosbash 2006). Interestingly, while the model proposes an order of assembly of the different snRNPs, it does not prove that U1 recruits U2 to the pre-mRNA, and U2 recruits the tri-snRNPs. It only indicates that in order for U2 to be recruited, first U1 needs to be bound to the pre-mRNA. Therefore, in order to analyze any possible direct effect that Mer1p could have on the recruitment of U2 and U6 snRNAs, different conditions were tested using the RNA chromatography assay. First, it was attempted to block the binding of U1 by eliminating the base pairs between the U1 snRNA and the 5' splice site of the pre-mRNA (an important interaction for U1 snRNP binding stability) (Cheng and Abelson 1987). Disruption of this interaction did not prevent the recruitment of the truncated U1 snRNA nor the increase in the recruitment of U2 snRNA to the pre-mRNA, indicating that the initial base pairing in the formation of the commitment complex was not a requirement for Mer1p activity. Moreover, if U2 is recruited after U1, any alteration of U2 should not affect the recruitment of U1. Subsequently, it was tested whether Mer1p was still able to enhance the recruitment of U1 when U2 was depleted from the splicing extract. Surprisingly, and contrary to expectations, when the U2 particle was disrupted, U1 recruitment was severely affected, indicating that the binding of U1 was U2-dependent and, therefore, in the presence of Mer1p, U1 and U2 did not follow the current stepwise

assembly model. Indeed, the actual proposed model has only been verified for a few introns and none of them required any enhancer element for their splicing efficiency. Additionally, it has been shown that it is possible to purify an active pre-assembled spliceosome containing all five snRNPs (Stevens, Ryan et al. 2002). Finally, experimental evidence indicates that the assembly of the spliceosome can be intron specific, and for some introns this process occurs co-transcriptionally while for other introns is a post-transcriptional event (Tardiff, Lacadie et al. 2006; Lacadie and Rosbash 2005). Therefore, taking into consideration all of these observations, there is not a clear and defined mechanism of spliceosome assembly for all pre-mRNAs. The spliceosome is a very dynamic particle and it is possible that its mode of action/assembly is dependent on intron characteristics in addition to splicing factors.

The results obtained with the RNA chromatography assay do not distinguish between a kinetic effect and/or stabilization effect on the snRNPs recruited by Mer1p. To evaluate any possible kinetic effect that Mer1p could have over the assembly of the snRNPs, a time course experiment was performed to measure the levels of U1 snRNA recruited over time. When the levels of U1 snRNA were plotted, with and without Mer1p, a clear difference in the kinetic association of U1 snRNA recruitment was observed. Mer1p accelerated the binding of U1 to the pre-mRNA and, therefore, accelerated the formation of the commitment complex. Although the kinetic experiment indicates that Mer1p increases the  $K_{eq}$  of the commitment complex, it does not address the possibility that Mer1p could also have a stabilizing effect on the complexes formed, such as the commitment complex I and II. To address this possibility commitment assays were performed, where the progression of the splicing reaction was blocked by depleting

the ATP from the splicing extracts. ATP depletion does not prevent Mer1p activity but it is an important requirement for the progression of the spliceosome assembly. A proper pre-spliceosome is not formed in the absence of ATP, blocking the splicing reaction before the first catalytic step (Cheng and Abelson 1987; Liao, Colot et al. 1992; Perriman, Barta et al. 2003). The results obtained from the commitment assays indicated that Mer1p was indeed stabilizing the formation of the commitment complex prior to the ATP hydrolysis and, therefore, the effect of Mer1p over the snRNPs was a combination of accelerating the recruitment of the snRNPs to the pre-mRNA and stabilizing the complexes formed. Using the commitment assay, it was also confirmed that without the U2 snRNP, Mer1p was no longer able to stabilize these early splicing complexes.

During the progression of this work new splicing factors were identified to be required for the *in vivo* Mer1p activity (the U2 snRNP proteins Prp11p and Snu17p and the RES subunit Bud13p) (Spingola, Armisen et al. 2004; Scherrer and Spingola 2006). Since Snu17p and Bud13p were non-essential splicing factors, splicing extracts from *snu17Δ* and *bud13Δ* were used in the RNA chromatography assay to test for their ability to enhance the recruitment of the snRNAs. Surprisingly, only *snu17Δ* extract reproduced the same effects observed as with the *nam8Δ* splicing extract or pre-mRNA lacking Mer1p-enhancer sequence: a non-enhancement effect. However, the *bud13Δ* splicing extract did not produce the same defects as *snu17Δ* or *nam8Δ* strains. While *snu17Δ* affects all Mer1p-dependent introns *in vivo*, *bud13Δ* only affects specific Mer1p-dependent introns, so maybe in this particular pre-mRNA the defects were not the ones expected (maybe due to the pre-mRNA structure), and therefore further experimentation using a more specific pre-mRNA would be required to determine if deletion of bud13p

blocks the recruitment of snRNAs. Moreover, there is the possibility that *bud13Δ* affects Snu17p function in an intron-specific manner. Finally, the *in vivo* effects of Bud13p could be linked to its association with the RES complex and, therefore, the *in vitro* results would suggest two different modes of regulation of Mer1p activity, one mediated by the RES complex and the other mediated by the snRNP factors. Since Snu17p also forms a complex with another splicing factor, Bud31p, maybe *bud31Δ* mimics the *bud13Δ* effect, in which case it would support the hypothesis of an indirect effect of Bud13p on Mer1p activity. Thus, additional work is required to address the Bud13p effect on Mer1p function.

Additionally, there are several splicing factors that remain to be tested for their ability to interact with Mer1p and/or regulate its activity, and those could bring some new perspectives on how Mer1p enhances each one of its targets and how Mer1p is regulated.

Interestingly, *nam8Δ* causes the loss of two splicing factors *Snu56p* and *Snu71p* from the U1 snRNP (Gottschalk, Tang et al. 1998; Zhang and Rosbash 1999) and these two factors interact via two-hybrid with Mer1p (Spingola, Armisen et al. 2004). Since Mer1p requires Nam8p and, at the same time, Mer1p interacts with Snu71p and Snu56p, gel mobility shift assays were used to determine, whether the presence of Mer1p was sufficient to rescue the loss these factors from the U1 particle in a *nam8Δ* strain. The results indicate that without Nam8p, Mer1p could not stabilize the binding of Snu71p and Snu56p to the U1 snRNP (data not shown). Since Nam8p is required for Mer1p activity and its loss produces the disassociation of Snu56p and Snu71p from the U1 particle, one hypothesis is that the loss of interaction between Mer1p with Snu56p and Snu71p in a *nam8Δ* background is sufficient to block Mer1p function. In the absence of Snu56p and

Snu71p, Mer1p could be no longer interact properly with the U1 particle and, therefore, it would fail to recruit U1 into a proper conformation to the pre-mRNA. It would be interesting to determine if Snu56p and Snu71p play a role in the activity of the Mer1p and if both factors are required for Mer1p function.

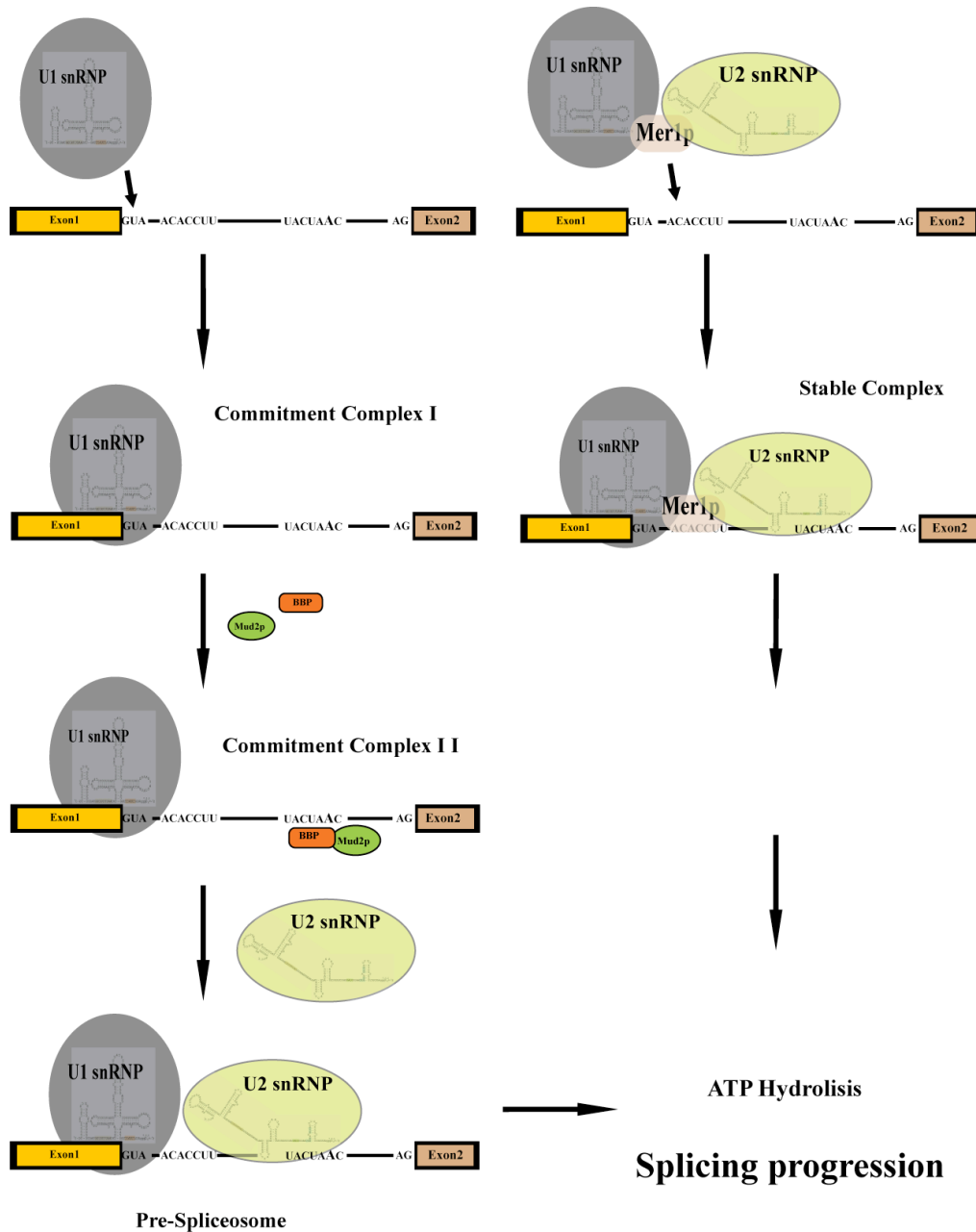
Finally, the recruitment of U1 and U2 in the presence of Mer1p was measured and compared to the actual model proposed for the assembly of the snRNPs. If the assembly of the snRNPs in the presence of Mer1p followed the classic stepwise assembly model, then the recruitment of U1 snRNA should differ from the recruitment of U2 snRNA, especially at early stages where the differences are more prominent (between commitment complex formation and pre-spliceosome) (Ruby and Abelson 1988). Surprisingly, instead of observing clear differences between U1 and U2 snRNA binding, both snRNPs appeared to be recruited to the pre-mRNA at the same rate and at the same time, even at early stages of the splicing reaction, suggesting that in the presence of Mer1p the recruitment of at least U1 and U2 snRNPs do not follow the stepwise assembly pathway. Moreover, these results, in combination with the previous finding that U2 snRNA was required for Mer1p to recruit U1 snRNA, support a different model of snRNPs recruitment to the pre-mRNA in the presence of the enhancer regulator Mer1p (*Figure 6.1*).

While in the absence of Mer1p, U1 is first recruited to form the commitment complex and then U2 to form the pre-spliceosome, in the presence of Mer1p U1 and U2 snRNPs could be recruited simultaneously (before their binding to the pre-mRNA), creating an advantageous situation over the stepwise process. This advantageous situation could result in an increase in the assembly rates and, therefore, an increase in splicing

efficiency of the Mer1p-dependent introns that otherwise and, due to their specific characteristics, would be at a disadvantage compared to other efficiently spliced introns.

Since this work is mainly focused on the early stages of the splicing reaction, the possibility remains that Mer1p could also regulate later stage of the splicing reaction. Future work is required in order to address whether Mer1p still plays an important role during the recruitment of the tri-snRNP and/or during the transesterification reactions. In addition, and based on the limited information about enhancer elements, it would be interesting to characterize new splicing regulators that may follow a similar mode of action as Mer1p.

In conclusion, based on the above results that Mer1p must interact with U1 snRNP, U2 snRNP, and the enhancer sequence simultaneously in order to activate the splicing of its targets, a new model of spliceosome assembly emerges for Mer1p-dependent introns. Introns that splice efficiently could recruit the snRNPs in a stepwise fashion, but when introns require the aid of enhancer factors, such as Mer1p, then a pre-assembled model promoted by these enhancer elements could provide an advantage over the classic model, and, as a consequence, an increase in the splicing efficiency of their targets. The model proposed in this work could also be applied to other splicing enhancer elements, not only Mer1p, constituting an alternative methods of splicing regulation.



**Figure 6.1** Model for the regulation of spliceosome assembly by Mer1p. Mer1p could recruit simultaneously U1 and U2, accelerating steps in the spliceosome formation and at the same time stabilizing any weak complexes formed between the pre-mRNA and the snRNPs, creating an advantage in the formation of active spliceosomes, increasing the splicing efficiency of its targets.



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