UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of a Novel Role for the Bur Cyclin Dependent Kinase Complex in pre-mRNA Splicing

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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This Dissertation of Hervé Tiriac is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

For my parents, Cornelia and Stephan, who have made incredible sacrifices for my brother and I.

For my brother, Alexandre, who has been a role model for me in so many ways.

For my wife, Dannielle, who has always been my staunchest supporter and toughest critic.

For my mentor, Tracy, who has always been constructive and positive even in the most difficult times.

For my colleagues who taught me a lot, inside as well as outside the laboratory.

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ABSTRACT OF THE DISSERTATION

Characterization of a Novel Role for the Bur Cyclin Dependent Kinase Complex in pre-mRNA Splicing

by

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Doctor of Philosophy in Biology

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Pre-messenger RNA (pre-mRNA) splicing is carried out by a dynamic ribonucleoprotein machine called the spliceosome. From budding yeast to mammalian cells, the majority of splicing occurs cotranscriptionally. Such spatial and temporal coupling suggest coordinated regulation. To begin to understand this coupled regulation we must first identify factors that play functional roles both in transcription and in splicing, and elucidate their mechanism of action. This motivated a directed genetic screen to identify canonical transcription factors that affect splicing *in vitro*. Here we describe the discovery that the essential *Saccharomyces cerevisiae* cyclin dependent kinase Bur1/2, which has previously been shown to regulate transcription elongation, is necessary for efficient pre-mRNA splicing *in vivo*. Remarkably, the complex also plays a transcription-independent role in splicing that can be

observed *in vitro*. Using *in vitro* spliceosome assembly assays we demonstrate that integrity of the Bur complex is essential for early prespliceosome formation as well as catalytic spliceosome formation and activation. Our data indicates that the Bur complex associates with prespliceosomes and interacts with the pre-mRNA substrate as well as the U1 snRNA. We further show that the Bur1 kinase can phosphorylate known splicing factors both *in vitro* as well as in extracts. Furthermore we show that the kinase likely mediates numerous phosphorylation events leading to phosphorylated protein in prespliceosomes and spliceosomes. Finally with the use of a modular analog sensitive Bur1 kinase we uncover a role for Bur1 activation through phosphorylation in spliceosome formation.

These results provide the first example in budding yeast of a cyclin dependent kinase affecting pre-mRNA splicing and spliceosomal formation through physical interactions with the spliceosome. This highlights the importance of post translational modifications, and the proteins that mediate them, in regulating critical spliceosome rearrangements. Furthermore, our data paint a picture of a model whereby a transcription factor plays a previously uncharacterized and important regulatory role in pre-mRNA splicing, and hint at an intricate coupling mechanism between multiple RNA processes.

Chapter 1: Introduction

Section 1.1: Gene expression and regulation

Francis Crick enunciated the principals underlying gene expression in 1958 in what he called the “central dogma of molecular biology” (Crick 1970). At its core, the central dogma states that information can be transferred from DNA to RNA and from RNA to protein. Indeed, RNA is first transcribed from a DNA template in a process called transcription, and this RNA molecule is then translated into a polypeptide in a process called translation. Since Crick first spelled out the central dogma, many additional molecular processes critical to gene expression have been described. In particular, one area of interest focuses on the generation and maturation of the RNA molecule and is referred to as RNA processing.

The newly transcribed precursor messenger RNA molecule (pre-mRNA) must undergo several processing steps before it becomes a mature mRNA and is ready to be translated (Figure 1.1). First the pre-mRNA is modified on its 5’ end with a 7-methylguanosine (m^7G) cap. Once bound by the cap binding complex this structure, among other things, protects the RNA from degradation and facilitates recognition by the translation machinery. If the pre-

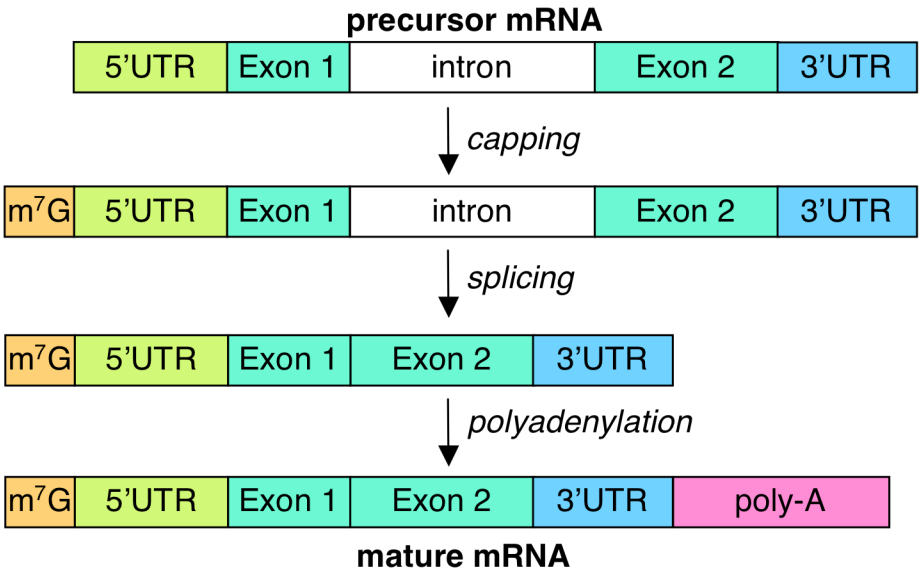


Figure 1.1. RNA processing steps. Depiction of the pre-mRNA maturation steps. The pre-mRNA contains 5' and 3' un-translated regions (UTR). The two protein coding regions (exons) are separated by a non-coding region (intron). The first step in pre-mRNA maturation is capping which adds a 7-methylguanosine residue to the 5' end of the RNA (m^7G). Splicing then removes the non-coding intron and ligates the exons together. Finally the pre-mRNA is polyadenylated on the 3' end to fully mature.

mRNA contains non-coding sequences (introns), then it must undergo pre-mRNA splicing so that the introns can be removed and the coding sequences (exons) can be ligated together. Finally the capped and spliced pre-mRNA is polyadenylated on the 3' end to protect against degradation and to promote the export and translation of the now mature mRNA molecule.

Section 1.2: Pre-mRNA splicing

Pre-mRNA splicing is essential for the viability and diversity of eukaryotic cells. At its core the splicing reaction involves two transesterification reactions (Figure 1.2). In 1985 Brody and Abelson identified a macromolecular complex using glycerol gradients containing a pre-mRNA molecule which underwent the first transesterification reaction (Brody and Abelson 1985). This macromolecular machine could catalyze splicing and was named the spliceosome (reviewed in (Staley and Guthrie 1998; Valadkhan 2005; Ritchie, Schellenberg et al. 2009; Wahl, Will et al. 2009)). The spliceosome, which is composed of five small nuclear RNAs (snRNAs) and hundreds of associated proteins, assembles onto the pre-mRNA molecule in a step-wise manner (Figure 1.3). The U1 small nuclear ribonucleoprotein complex (snRNP), containing the U1 snRNA and its associated protein factors, first recognizes the 5' splice site (SS). Following recognition of the 5' SS, the

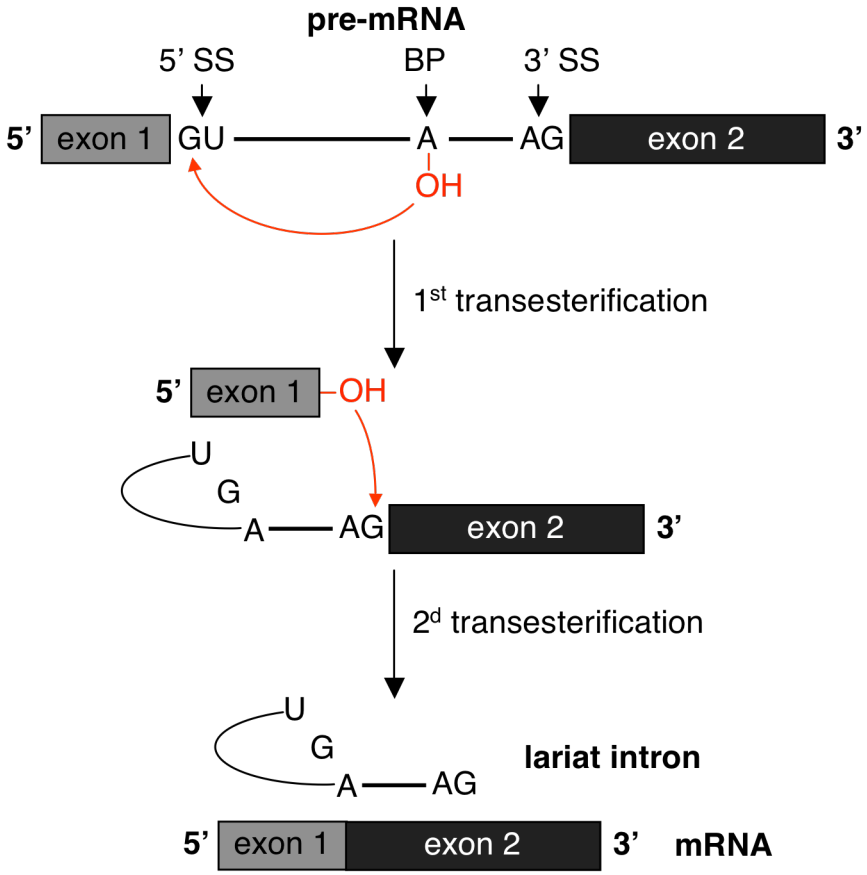


Figure 1.2. Pre-mRNA splicing requires two transesterification reaction. Cartoon diagram of the two transesterification reactions that take place during pre-mRNA splicing. Exon 1 (grey) and exon 2 (black) are represented as rectangles, while the intron is represented by a line. The 5' splice site (5' SS), branch point (BP), and 3' splice site (3' SS) nucleotides are shown within the intron. Nucleophilic attacks are color coded red.

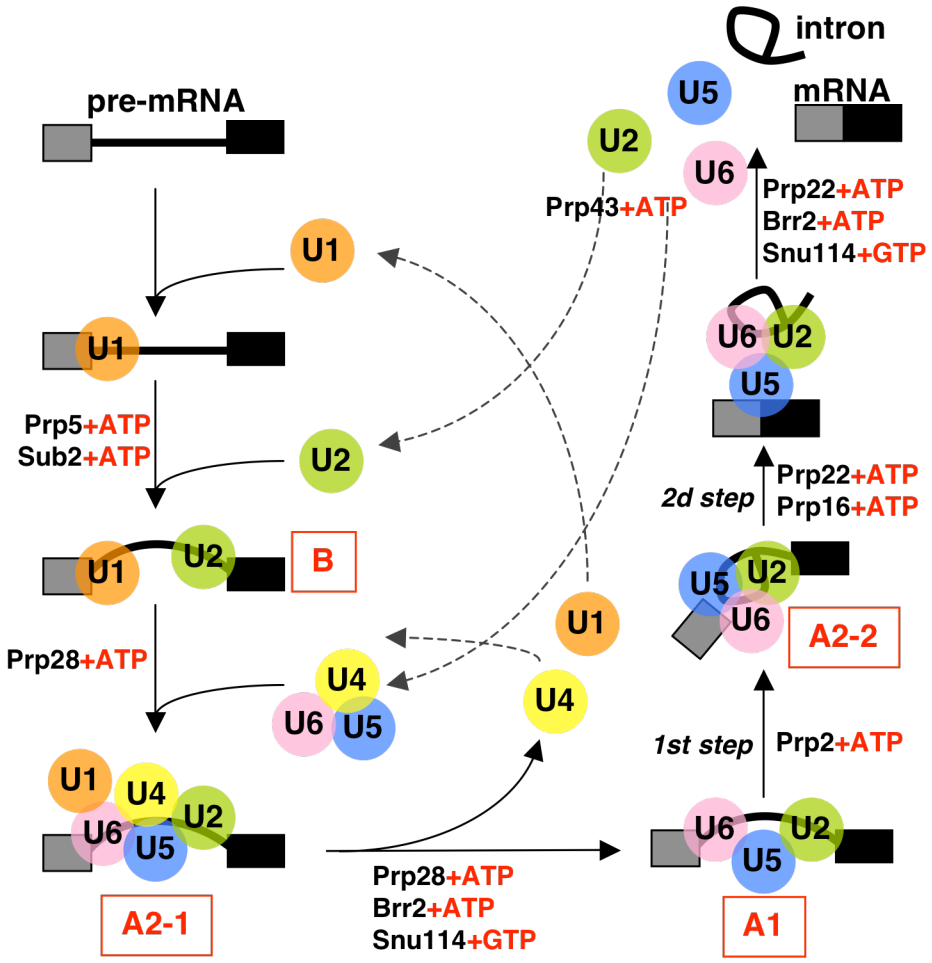


Figure 1.3. Spliceosome assembly. Cartoon depiction of the step-wise assembly of the spliceosome. The five snRNP are represented as colored circles. Spliceosomal complexes are labeled in black boxes. RNA helicases of the DExD/H family are shown at the required NTP hydrolysis step.

U1 snRNA base pairs with the pre-mRNA forming the commitment complex (CC). This first step in spliceosome assembly is ATP independent and serves as a platform for the recruitment of the subsequent snRNPs. The subsequent stable recruitment of the U2 snRNP to the branchpoint (BP) is the first ATP dependent step in spliceosome assembly. This prespliceosome complex containing the U1 and U2 snRNA was first identified using native gel analysis of spliceosome assembly and was called the B complex in *Saccharomyces cerevisiae* (Cheng and Abelson 1987). The remaining three snRNPs are recruited as U5•U4/U6 tri-snRNP complex. The resulting spliceosome (A2-1 complex) is still inactive and must undergo dramatic rearrangements to lead to catalytic activation. The U1 snRNP is first released from the spliceosome and is replaced by the U6 snRNA at the 5' SS. This is followed by the destabilization and subsequent release of the U4 snRNP, which leads to the catalytic activation of the spliceosome (A1 complex). This active spliceosome catalyzes the first and second transesterification reactions (A2-2 complex), which result in the release of the excised intron lariat byproduct and the ligated mRNA.

The dynamic rearrangements of the spliceosome are facilitated and regulated by associated splicing protein factors. One particularly conserved class of splicing factors are RNA helicases of the DExD/H protein family and

use NTP hydrolysis to promote snRNA conformational changes (Figure 1.3). Most of these factors were first identified in early budding yeast temperature sensitive screens looking for RNA processing defects (Hartwell 1967) and later named pre-mRNA processing (Prp) factors because they all cause splicing defects when mutated (Vijayraghavan, Company et al. 1989). While some DExD/H factors act through direct RNA remodeling, others may help remodel and displace proteins bound to snRNAs or to the pre-mRNA. The first ATP dependent step in spliceosome assembly is the stable addition of the U2 snRNA to the BP and is catalyzed by the U2 snRNP factors Prp5 and Sub2. The following addition of the tri-snRNP requires Prp28 and ATP hydrolysis. Subsequent destabilization of the U1 and U4 snRNP and catalytic activation of the spliceosome are facilitated by the Brr2 ATPase and Snu114 GTPase. Finally the conformational rearrangements that catalyze the transesterification reactions are promoted by the DExD/H factors Prp2, Prp16, Prp22 ATPases. The disassembly of the spliceosome and recycling of the snRNPs is regulated by the Prp43 helicases and ATP hydrolysis (Figure 1.3).

Section 1.3: Co-transcriptional spliceosome assembly

Pre-mRNA splicing is closely integrated with other reactions in gene expression. Spliceosome assembly must occurs within close spatial and

temporal proximity to transcription and other RNA processing reactions, suggesting the possibility that these processes might be coupled (Neugebauer 2002; Reed 2003). Indeed recent work in yeast and in mammals suggest that the majority of splicing occurs co-transcriptionally (Neugebauer and Roth 1997; Gornemann, Kotovic et al. 2005; Listerman, Sapra et al. 2006; Alexander, Innocente et al. 2011; Oesterreich, Bieberstein et al. 2011). Co-transcriptional splicing may allow the cell to regulate efficiently two processes that are interdependent. For instance, transcription in the absence of splicing would waste precious resources, thus coupling pre-mRNA synthesis with spliceosome assembly would enable resource conservation and could lead to more efficient RNA processing. Co-transcriptionality arises both from kinetic coupling (for example polymerase pausing) as well as from recruitment of snRNPs to the transcriptional machinery (for example recruitment of snRNP to the CTD of RNA polymerase).

There are several RNA polymerases (Pol) in Eukaryotic cells, but RNA Pol II is responsible for the transcription of all intron containing genes. During early transcription, the nascent pre-mRNA must be capped on its 5' end. Once the m_7G cap has been added to the pre-mRNA, the cap binding complex (CBC) binds to the cap and helps recruit the U1 snRNP cotranscriptionally (Gornemann, Kotovic et al. 2005). In fact this recruitment of U1 is so

ubiquitous that it is recruited to intronless genes as well as intron containing ones (Brody, Neufeld et al. 2011). Once the U1 snRNP is stably associated with the 5' SS, it promotes the following recruitment of the U2 and tri-snRNP. While spliceosome assembly can occur independently of transcription and capping (*in* vivo as well as *in vitro*), the majority of splicing is thought to take place cotranscriptionally (Alexander, Innocente et al. 2011; Oesterreich, Bieberstein et al. 2011).

In addition to the CBC promoting co-transcriptional recruitment of the snRNPs, chromatin associated factors also play a critical role in co-transcriptional spliceosome assembly. For example, recent work in yeast demonstrated that the histone acetyl transferase Gcn5 is important for promoting co-transcriptional recruitment of the U2 snRNA and of the subsequent tri-snRNP (Gunderson and Johnson 2009). Other examples continue to emerge in both mammals and in yeast (Alexander and Beggs 2010; Luco, Allo et al. 2011). There is also mounting evidence for regulation of transcription by proteins implicated in splicing. In higher eukaryotes, the U1 snRNP associated factor CBC, the U2 snRNP factor TAT-SF1 and the second step splicing factor SKIP have been shown to interact with the transcription complex to promote polymerase processitivity (Fong and Zhou 2001; Bres, Gomes et al. 2005; Lenasi, Peterlin et al. 2011). Interestingly these splicing

factors, which act at different steps of spliceosome assembly and activity all have been shown to interact with the same transcription factor, the positive transcription elongation factor b (P-TEFb)

Section 1.4: The Bur transcription elongation complex

The P-TEFb complex is an essential cyclin dependent kinase (CDK) complex identified because of its ability to promotes transcription elongation (Marshall and Price 1995). P-TEFb is composed of two subunits, the cyclin dependent kinase 9 (CDK9) and its associated cyclin (either CycT1, CycT2, or CycK) (reviewed in (Bres, Yoh et al. 2008)). The complex acts to promote productive transcription elongation by phosphorylating the C-terminal domain (CTD) of RNA Pol II on the second serine of the heptapeptide repeat that makes much of the CTD structure. The P-TEFb complex also promotes transcription through phosphorylation of transcription negative regulator DSIF.

The homolog of P-TEFb in the yeast *Saccharomyces cerevisiae* is the Bur1/2 complex (Bartkowiak, Liu et al. 2011). The Bur complex was identified in a screen for factors that could bypass the upstream activating sequence requirement (BUR) (Yao, Neiman et al. 2000). The Bur1 CDK assembles with its cyclin subunit, Bur2, to form the Bur complex. In the absence of Bur2, the

kinase activity of Bur1 is extremely reduced (Yao, Neiman et al. 2000; Keogh, Podolny et al. 2003), and the kinase activity of Bur1 is essential for viability. Bur1 is activated via phosphorylation by the CDK activating kinase 1 (Cak1) on threonine 240 within its T-loop domain (Yao and Prelich 2002).

The Bur complex is one of four CDKs that phosphorylate the RNA pol II heptapeptide repeat $YS_2PTS_5PS_7$ (Murray, Udupa et al. 2001). Serine 5 phosphorylation is important for transcription initiation and promoter clearance while serine 2 phosphorylation promotes transcription elongation. The Bur complex is responsible for serine 2 phosphorylation in the 5' end of the gene, while the Ctk CDK complex phosphorylates the CTD of Pol II towards the 3' end of the gene (Qiu, Hu et al. 2009). Furthermore *in vitro*, Bur1 can phosphorylate serine 5 of the CTD yet this phosphorylation is performed by Kin28 *in vivo* (Murray, Udupa et al. 2001; Keogh, Podolny et al. 2003).

The Bur complex interacts with other factors to promote transcription elongation. The Bur complex phosphorylates the CTD of Spt5, which along with Spt4 forms a platform for recruitment of factors involved in chromatin modification (Liu, Warfield et al. 2009; Zhou, Kuo et al. 2009). The ubiquitin-conjugating enzyme Rad6 is also phosphorylated by Bur1, which leads to the histone H2B mono-ubiquitination and promotes transcription (Wood, Schneider

et al. 2005). Through the phosphorylations of both Spt5 and Rad6, the Bur complex promotes the recruitment of downstream chromatin modifying factors which facilitate elongation by promoting transcriptional open state of chromatin (Wood and Shilatifard 2006).

Section 1.5: Specific aims

The coupling of reactions such as transcription and pre-mRNA splicing imply a high level of inter-regulation between these molecular processes. While in higher eukaryotes some splicing factors have been shown to directly affect transcription regulation (such as Tat-SF1, SKIP, and CBC), independently of their roles in splicing, there have been limited examples of transcription factors shown to directly impact spliceosome assembly independently of their roles in transcription.

When this work was initiated, the goal was to identify splicing regulators within the transcription machinery using the genetically and biochemically tractable system offered by the yeast *Saccharomyces cerevisiae*. As we started our investigations we discovered a peculiar splicing defect phenotype when the cells harbored a knockout of the cyclin *BUR2*. The Bur complex offered an interesting candidate for a splicing regulator in light of its role in

early transcription elongation, as well as its ability to interact with a diverse array of substrates. Furthermore its mammalian homolog, the P-TEFb complex, had been shown to interact with splicing factors, albeit to affect transcription not splicing.

We first characterized pre-mRNA splicing in Bur mutants *in vivo*, but quickly decided to take a more biochemical approach and shifted focus to an *in vitro* system which allowed us to uncouple splicing and transcription. These *in vitro* assays were paramount in our studies of spliceosome assembly dynamics in the presence of a mutant Bur complex.

Once the splicing phenotypes of the Bur mutants were clear, we characterized the interactions between Bur and the spliceosome. We uncovered an interaction between the Bur complex and the U1 snRNP as well as the prespliceosome. These interactions between the splicing machinery and a CDK led to our second specific aim: to assay spliceosome phosphorylation state and to discover potential substrates of Bur kinase activity. To accomplish this we assayed phosphorylation of two potential substrates of the Bur complex: the U2 snRNP factor Cus2, the homolog of mammalian Tat-SF1, and the U1 snRNP factor CBC. While we found that Cus2 was phosphorylated, this phosphorylation event was not unique to Bur's

activity. We then took a broad approach and tested phosphorylation of the spliceosome in the presence of normal Bur activity versus reduced Bur activity. To our surprise many spliceosomes undergo phosphorylation, some of which seemed to be Bur-dependent. Finally we tested a modular analog sensitive Bur1 kinase and uncovered a role for Bur1 activation in promoting spliceosomal formation.

Our cumulative results show that the Bur complex acts to regulate early spliceosome assembly independently from its role in transcription. Furthermore the kinase activity of the Bur complex is important for splicing regulation. This is the first reported role for a CDK in regulating pre-mRNA splicing in yeast through a phosphorylation event.

Chapter 2: The Bur complex plays a transcription independent role in pre-mRNA splicing regulation

Section 2.1: Background

Precursor messenger RNA splicing is an essential reaction in eukaryotic gene expression. Splicing signals along the pre-mRNA are recognized by five small nuclear ribonucleoproteins (snRNP) complexes (U1, U2, U4, U5 and U6), which assemble onto the pre-mRNA to form the spliceosome, a large dynamic macromolecular complex (Wahl, Will et al. 2009). First using *in vitro*, and more recently *in vivo* techniques, it has been shown that the spliceosome assembles onto the pre-mRNA in a stepwise manner (Hoskins, Friedman et al.; Cheng and Abelson 1987). These data are consistent with a model in which, first, the U1 snRNP recognizes and base pairs with the 5' splice site (SS) forming the commitment complex. This is quickly followed by the ATP dependent recruitment of the U2 snRNP to the branchpoint forming the prespliceosome (B complex). Next, the addition of the tri-snRNP (U4/U6,U5) forms the pre-catalytic spliceosome (A2-1 complex). Dynamic rearrangements allow for catalytic activation of the spliceosome after the removal of the U1 and U4 snRNP (A1 complex). Finally the spliceosome is able to catalyze the two transesterification reactions leading to the formation

of the mRNA and excision of the intron lariat (A2-2 complex).

Mounting evidence in mammals as in yeast suggest that most splicing happens cotranscriptionally (Neugebauer 2002; Reed 2003; Kornblihtt 2007; Alexander and Beggs 2010; Alexander, Innocente et al. 2010; Luco, Allo et al. 2011; Oesterreich, Bieberstein et al. 2011), and that the close spatial and temporal proximity between these reactions has important regulatory consequences. Transcription and splicing are kinetically coupled (Alexander, Innocente et al. 2010; Oesterreich, Bieberstein et al. 2011); transcription factors that affect polymerase processivity and pausing have a direct effect on cotranscriptional splicing and can affect alternative splicing regulation. Interestingly, chromatin modifying factors have been shown to directly affect spliceosome assembly cotranscriptionally (Gunderson and Johnson 2009; Luco, Allo et al. 2011).

There is also surprising evidence that splicing factors can stimulate transcription independently of their roles in splicing by interacting directly with components of the transcription machinery. The mammalian Tat stimulatory factor 1 (TAT-SF1), a U2 snRNP factor, has been shown to enhance transcription through interactions with the transcription machinery (Fong and Zhou 2001). Subsequent studies also demonstrated that the Ski-interacting

protein (SKIP), a second step splicing factor, functions as a transcriptional coactivator necessary for induced gene expression (Bres, Gomes et al. 2005; Bres, Yoshida et al. 2009). More recently, it has been shown in mammals that the U1 snRNP associated cap binding complex (CBC) was shown to promote transcriptional activity (Lenasi, Peterlin et al. 2011). These reports suggest the "splicing factors" may have dual roles in RNA processing and RNA synthesis and raise the possibility that single proteins, by playing dual roles, may coordinate transcription with splicing. While these examples illustrate that splicing factors may influence transcription independently of their roles in splicing, less is known about transcription independent roles of transcription factors in regulating spliceosome assembly or catalysis. Intriguingly, while these three splicing factors (TAT-SF1, SKIP, CBC) play distinct roles in pre-mRNA splicing in mammals, they all interact with a common transcription elongation factor, the positive transcription elongation factor b (P-TEFb), to affect transcription.

The mammalian complex P-TEFb is composed of cyclin dependent kinase 9 (CDK9) and one of three cyclin (Cyc) subunits (CycT1, CycT2, CycK) (Bres, Yoh et al. 2008). P-TEFb acts early in transcription by phosphorylating the C-terminal domain of RNA polymerase II (CTD of RNA-Pol II) as well as the CTD of the DSIF subunit Spt5. These phosphorylation events act to

releases the polymerase from promoter proximal pausing and lead to productive transcription elongation (Cho, Schroeder et al. 2010). There is evidence that, in mammals P-TEFb's role in transcription can indirectly affect splicing. Phosphorylation of the CTD of RNA-Pol II by P-TEFb leads to the recruitment of SR splicing factors to the CTD which in turn promote early cotranscriptional spliceosome assembly (Millhouse and Manley 2005; Lenasi and Barboric 2010). It has been shown that increased P-TEFb kinase activity, by repression of its specific negative regulator, can promote alternative splicing of specific genes via increased CTD phosphorylation (Barboric, Lenasi et al. 2009). While P-TEFb interacts with the above described splicing factors, it has not been shown to have a transcription independent role in regulating splicing.

The Saccharomyces cerevisiae homolog of P-TEFb is the Bur complex (Yao, Neiman et al. 2000; Murray, Udupa et al. 2001; Bartkowiak, Liu et al. 2011). It is composed of the CDK Bur1 and the cyclin Bur2 and serves parallel transcription elongation functions as P-TEFb. In light of the role for P-TEFb in alternative splicing, we sought to determine if the P-TEFb homolog Bur1/2 was required for constitutive splicing in yeast. Indeed we discovered that deletion of *BUR2* and mutation of the essential *BUR1* gene resulted in splicing defects *in vivo.* Surprisingly *BUR2* deletion and a *BUR1* kinase

mutant abrogated *in vitro* splicing suggesting a transcription independent role for the Bur complex in pre-mRNA splicing. Additionally, the Bur complex affects early spliceosome formation, likely through interactions with the U1 snRNP. Furthermore both Bur1 and Bur2 associate with the prespliceosome complexes. These findings demonstrate a novel role for a transcription factor in regulation of pre-mRNA splicing and spliceosomal formation.

Section 2.2: Results

Bur1/2 complex plays a role in pre-mRNA splicing *in vivo*.

To test if the Bur complex is involved in pre-mRNA splicing regulation we tested a strain deleted of *BUR2* to assess splicing defects of the *U3* genes using radiolabeled primer extension (Figure 2.1). The U3 snoRNA is a RNA-Pol II transcript and is encoded by two genes *U3A* and *U3B*, both of which contain an intron. Although the introns differ slightly in length, both genes encode the same mRNA (Myslinski, Segault et al. 1990). Deletion of *BUR2* led to the accumulation of both U3A and U3B pre-mRNAs (lane 3). For comparison the intron accumulation when a core component of the U2 snRNP, Msl1, was deleted is shown in lane 2. The pre-mRNA accumulation, while only a fraction of total transcript is characteristic of an *in vivo* splicing defect observed when core splicing factors are absent (Noble and Guthrie 1996).

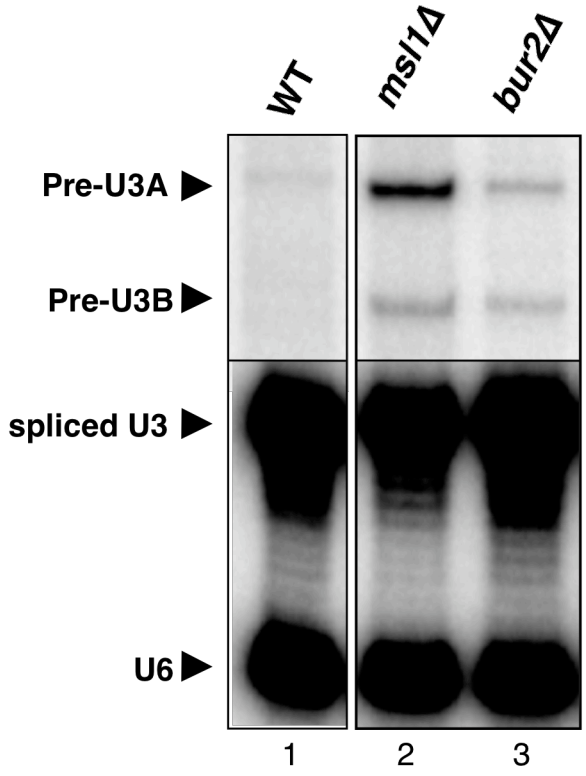


Figure 2.1. Deletion of the gene encoding *BUR2* leads to an *in vivo* splicing defect. RNA was isolated from the indicated cells, and equal amounts were subjected to primer extension of the U3A, U3B, and U6 RNA using the primers indicated in table 5.3. Pre-U3A and pre-U3B differ in intron size but yield a mature mRNA of the same size. U6 RNA is a Pol III transcript that is not spliced and is used here as an internal loading control. These samples represent a single gel in which the top and bottom parts of the gel are shown.

This assay indicates that Bur2 could be a regulator of splicing, but also highlights the robustness of splicing in cells.

In order to evaluate whether the Bur complex was involved in splicing of other transcripts and to increase the sensitivity of detection of splicing products, low cycle PCR was used to analyze *ACT1* splicing (Figure 2.2). We were particularly interested in analyzing splicing of full length pre-mRNA, so cDNA was generated from a primer located in the 3' UTR of *ACT1*, just downstream of the predicted polyadenylation site as determined by sequence analysis (Figure 2.2 A). This also allows us to rule out that intron retention was due to incomplete synthesis of the pre-mRNA due to altered elongation in the *BUR1/2* mutants. Using low cycle PCR we detected a significant increase in pre-mRNA accumulation as a fraction of total *ACT1* RNA for *bur2Δ* (Figure 2.2 B). The Bur1 kinase is essential for survival (Yao, Neiman et al. 2000), therefore to analyze the role of Bur1 in splicing, a mutation in Bur1 that prevents its activation by the Cak1 kinase and which displays reduced kinase activity *in vitro* (Yao and Prelich 2002), Bur1-T240A was assayed for accumulation of unspliced *ACT1* pre-mRNA. While slightly less pronounced than the accumulation observed in a *BUR2* knockout, the *bur1-T240A* strain did show significant accumulation of unspliced RNA. For positive controls, deletion of the splicing factors *MSL1* and *LEA1* were analyzed, and these also

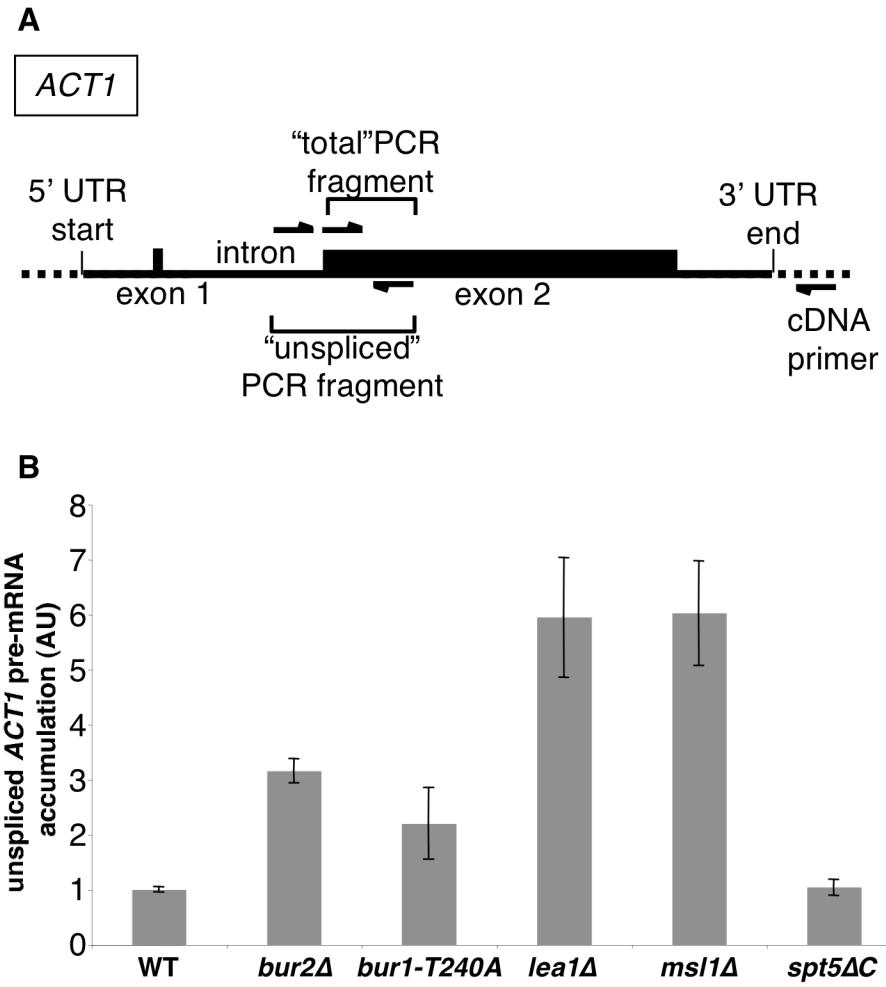


Figure 2.2. Bur1/2 complex mutations lead to *in vivo* splicing defects. A. Schematic of the *ACT1* gene with the location of the cDNA primer. The PCR primers used to quantitative the unspliced actin are indicated. The total *ACT1* RNA is determined using primers within the second exon. Amplicons for the PCR are shown as brackets. **B.** SYBR-green quantitation of the amount of unspliced *ACT1* pre-mRNA isolated from triplicate low cycle PCR assays from the indicated strains. Unspliced product accumulation is shown as a fraction of total *ACT1* RNA. Data for *spt5ΔC* is the average of two experiments. Error bars indicate standard error of the mean.

show intron retention. Since Bur1 has been shown to phosphorylate the transcription elongation factor Spt5 in yeast (Liu, Warfield et al. 2009), and since Spt5 has been implicated in splicing (Lindstrom, Squazzo et al. 2003), we assayed splicing in strain in which the C-terminal domain of Spt5 (target of Bur1 activity) was deleted. No splicing defect was detected in the *SPT5* mutant (Figure 2.2 B). These results suggest that the Bur complex influences pre-mRNA splicing in a manner that is distinct from its Spt5-dependent role in transcription elongation. In order to determine if the effect on splicing of the *BUR1/2* mutants are only observed *in vivo*, during co-transcriptional splicing, we assayed splicing and spliceosome assembly *in vitro*.

Functional Bur1/2 complex is required for pre-mRNA splicing *in vitro*.

The *in vivo* results above led us to test Bur1/2 mutants in an *in vitro* assay that allowed us to uncouple transcription from splicing and test whether Bur plays a direct role in spliceosome assembly. Denaturing gel analysis of *ACT1* pre-mRNA splicing revealed that *bur1-T240A* and *bur2Δ* extracts did not form splicing intermediates or products thus indicating a lack of activated spliceosomes in these extracts (Figure 2.3 A, lanes 3 and 4 vs. 2). Indeed when spliceosome formation was analyzed on a native gel, which allows us to separate splicing complexes, we observe no spliceosome formation in *bur2Δ* (Figure 2.3 B, lane 4). Using this native gel system, first characterized by

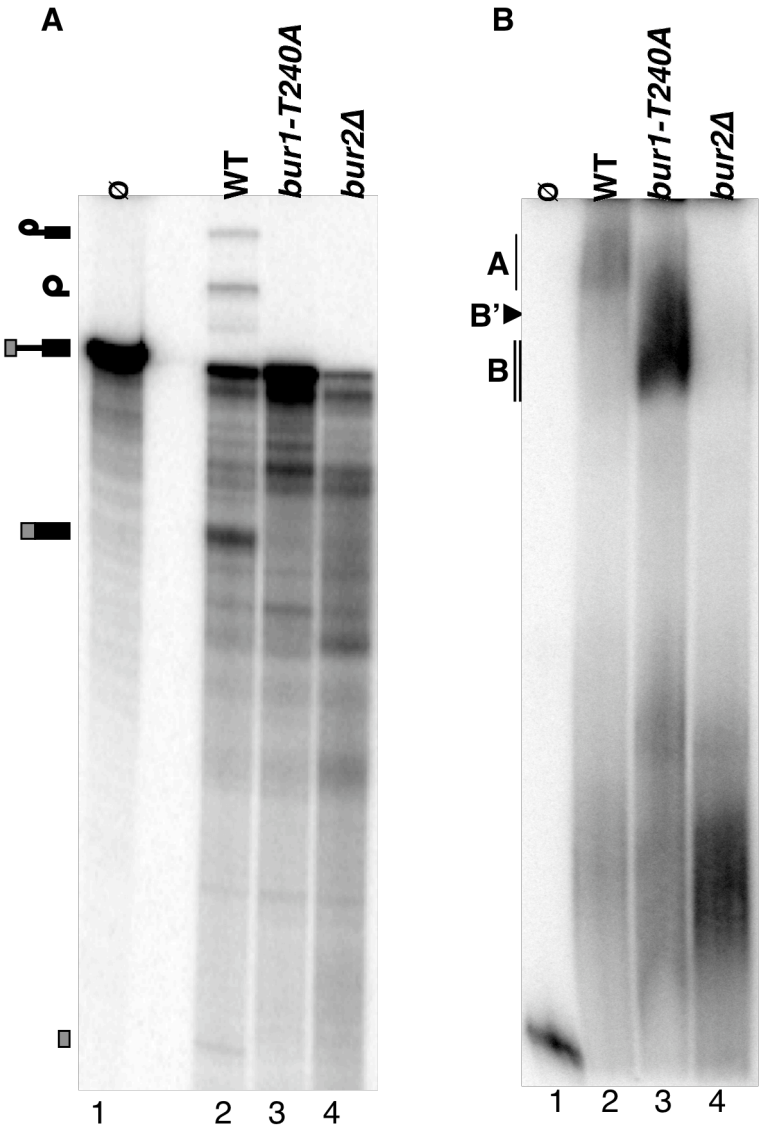


Figure 2.3. Bur1/2 complex mutations lead to *in vitro* splicing defects. A. *In vitro* splicing reactions using *in vitro* synthesized, radiolabeled *ACT1* pre-mRNA as a substrate were run on a denaturing gel allowing separation of splicing intermediates. Radiolabeled pre-messenger RNA is shown in lane 1. Lanes 2-4 show splicing of the pre-mRNA in presence of WT, *bur1-T240A*, and *bur2Δ* splicing extracts. **B.** Same reactions as part A run on a native gel allowing for the separation of A and B complexes. The previously described A and B complexes are indicated, as is the B' complex described in the text.

Cheng and Abelson (Cheng and Abelson 1987), the prespliceosomes (B complex) can be separated from the spliceosomes (A complexes). These later A complexes can be further separated into pre-catalytic spliceosome (A2-1 complex) and catalytic spliceosome (A1 and A2-2 complexes) with the use of EDTA to chelate magnesium ions important for rearrangements of the catalytic spliceosome (Cheng and Abelson 1987). Intriguingly we do observe formation of B complexes with the *bur1-T240A* extracts, but this extract does not form functional A complexes. Furthermore a complex with aberrant mobility between B and A complexes (B') forms (Figure 2.3 B, lane 3). This B' complex could stem from accumulation of B complex leading to a mobility shift of the B complex or the B' complex could represent an aberrant transitional spliceosome complex.

To determine whether the loss of splicing activity was due to a loss of Bur1/2 function we sought to reconstitute splicing activity with addition of functional Bur1/2 proteins. Unfortunately, attempts to generate recombinantly active Bur1/2 complex were unsuccessful, so we set out to reconstitute splicing activity in the *BUR1/2* mutant extracts with addition of micrococcal nuclease (MN) treated extract (Figure 2.4 A). MN treatment degrades spliceosomal RNAs, but leaves the proteins in the extract intact. While the *in vitro* splicing reactions usually contain 40% extract (Figure 2.3), these

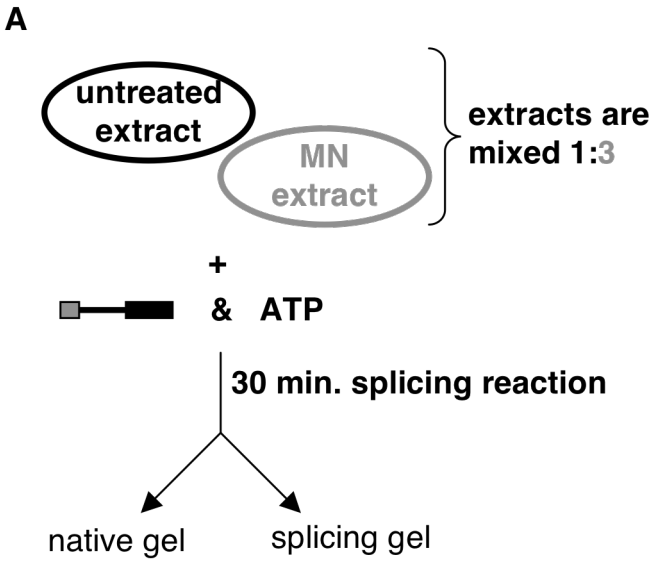


Figure 2.4. Reconstitution of splicing in Bur1/2 mutant splicing extracts. **A.** Schematic of the order of addition of splicing extracts (black), MN-treated extract (grey), pre-mRNA, and ATP. **B.** *In vitro* splicing reactions run on a denaturing gel. Radiolabeled pre-messenger RNA is shown in lane 1. Lanes 2-4 contain splicing reactions with 10% v/v splicing extract generated from cells of the indicated genotype. Lanes 5-13 are reconstitution reactions with 10% v/v splicing extract and 30% v/v MN-treated extract. Lanes 14-16 show splicing for the MN-treated extracts alone (30% v/v). The top panel shows an overexposuse of the top of the gel. **B.** The same reactions as those in part A run on a native gel, which allows for the separation of A and B complexes.

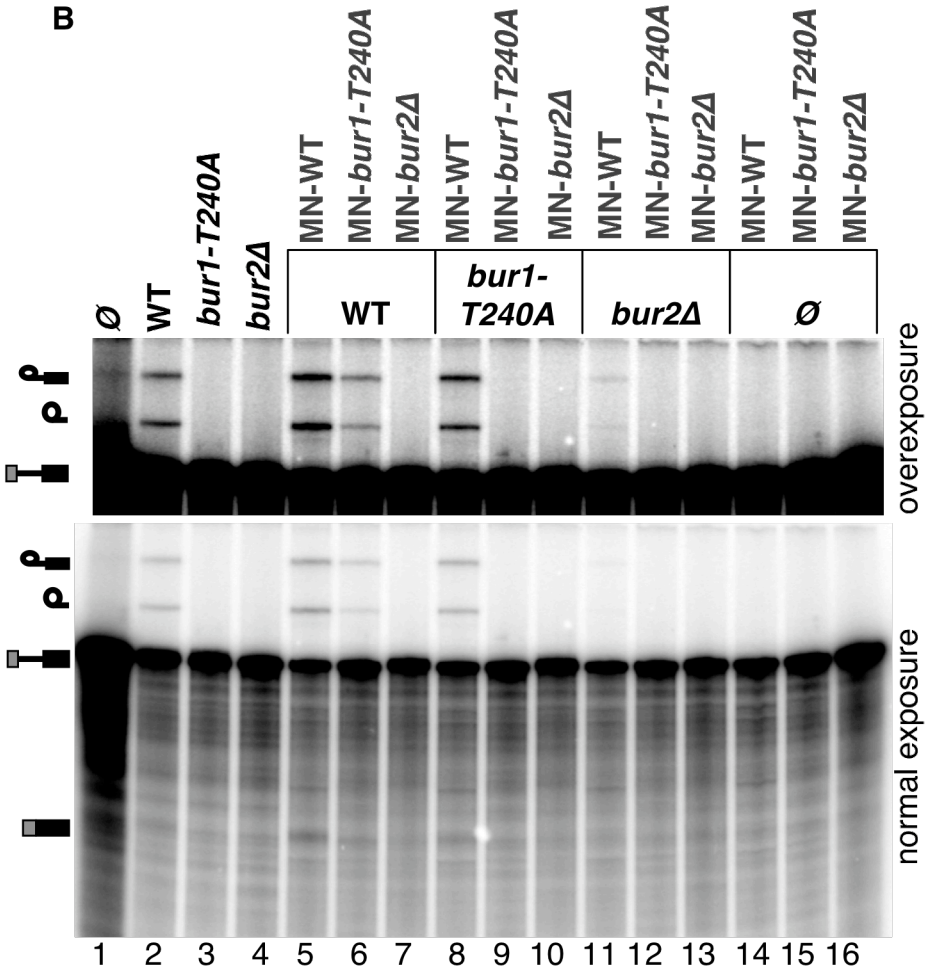


Figure 2.4. Reconstitution of splicing in Bur1/2 mutant splicing extracts, continued.

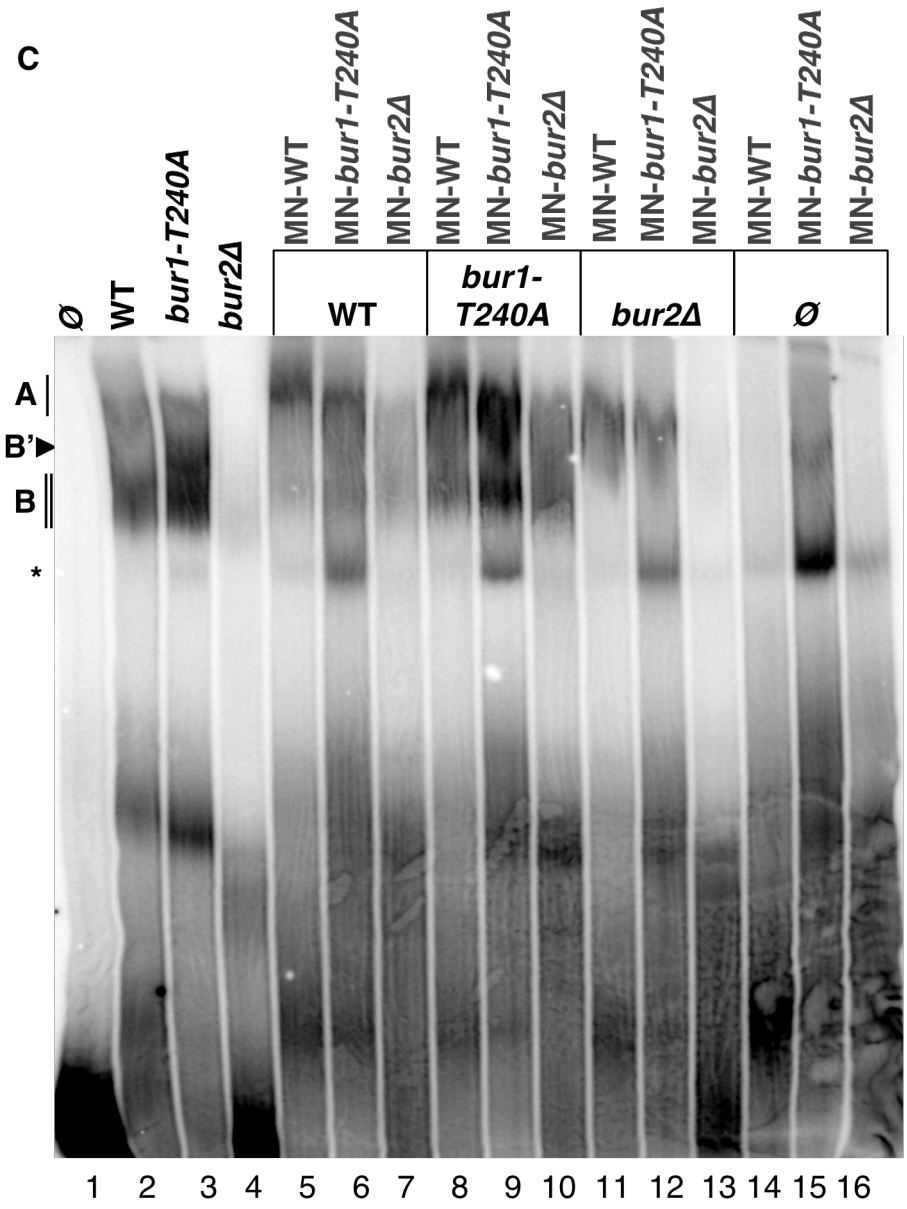


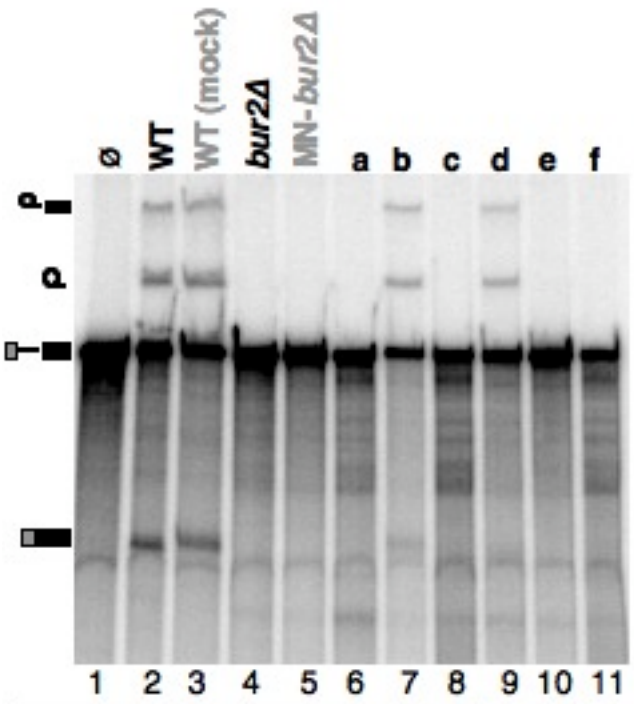
Figure 2.4. Reconstitution of splicing in Bur1/2 mutant splicing extracts, continued.

reactions are comprised of 10% extract and 30% MN-treated extract (Figure 2.4). As expected, the MN-treated extracts by themselves show no splicing or spliceosome formation (Figure 2.4 B & C, Lanes 14-16), however we hypothesized that a MN-treated WT extract, containing functional Bur complex, would reconstitute splicing in the mutant extracts. Addition of MN-WT to WT extract stimulated splicing and spliceosome formation, most likely due to an increase of splicing factors available to form snRNPs (Figure 2.4 B & C, lane 5 vs. 2). Similarly, addition of MN-WT extract reconstituted the activity of the *bur1-T240A* extract. This was accompanied by a decrease in the blocked "B" complex and formation of A complexes (Figure 2.4 B & C, lane 8 vs. 3). Notably, when MN-treated *bur1-T240A* was added to the *bur1-T240A* extract we observe an increase in the aberrant B' complex but no formation of splicing intermediates or products (Figure 2.4 C, lane 9 vs. 3). Interestingly, splicing was only partially reconstituted in the *bur2Δ* extract upon addition of MN-WT (Figure 2.4 B, darker exposure, lane 11). The native gel analysis of the *bur2Δ* reconstitution revealed formation of A complex (Figure 2.4 C, lane 11 vs. 4). The efficiency of *bur2Δ* splicing reconstitution was low even though there is three fold more MN-treated WT extract to *bur2Δ* extract. Indeed the *bur2Δ* extract, either non-treated or MN-treated possesses a dominant negative effect on splicing and spliceosome formation when added to a WT extract (Figure 2.4 B & C, lane 7 vs. 5). This dominant negative effect also

prevented efficient complementation between the two mutant Bur extracts (Figure 2.4 B & C, lanes 10 &12). Taken together these data reveal an important role for the Bur complex in early spliceosome formation, independent from its role in transcription. Similar to the *in vivo* splicing assay, we observe a stronger phenotype with deletion of *BUR2* as compared to a *BUR1* mutant that affects its catalytic activity, indicating that complex integrity is essential for Bur's role in splicing. Furthermore, since MN-treated *bur2Δ* extracts inhibit splicing, we speculate that an extract containing Bur1 alone allows formation of an aberrant complex with splicing factors, which can inhibit prespliceosome formation.

Extracts lacking Bur2 dominantly inhibit splicing *in vitro.*

Reconstitution of splicing in the *bur2Δ* extract caused a dominant negative effect inhibition of WT splicing activity. To further investigate this effect we performed an order of addition experiment in which WT and *bur2Δ* extract, with and without MN-treatment, were mixed in different orders (Figure 2.5). First, an untreated splicing extract was pre-incubated with the *ACT1* pre-mRNA at 30˚C in the absence of ATP to allow for early ATP-independent complex formation. After 10 minutes, MN or mock treated extract was added to the reaction along with ATP to allow for splicing (orders *a* and *d*). Alternatively, the extract addition was reversed so that the pre-mRNA was first



order of addition	10min pre-incubations with pre-mRNA	add-back with ATP and buffer
a	*bur2Δ*	WT (mock)
b	WT (mock)	*bur2Δ*
c	WT (mock) & *bur2Δ*	Ø
d	WT	MN-*bur2Δ*
e	MN-*bur2Δ*	WT
f	MN-*bur2Δ* & WT	Ø

Figure 2.5. Lack of Bur2 cyclin leads to a dominant negative effect on pre-mRNA splicing. Denaturing gel showing splicing for 40% v/v WT, mock WT, *bur2Δ*, and MN-*bur2Δ*, first individually (lanes 1-5), then in the orders of addition listed in the table (where each extract is 20% v/v, so that total extract is 40% v/v) (lanes 6-11). In the first five lanes, the extract indicated were pre-incubated with pre-mRNA for 10 min at 30˚C, then ATP was added for 30 min splicing reaction.

incubated with mock treated or MN-treated extract, presumably to allow the proteins to interact with the pre-mRNA. The untreated extract was added at a later time along with ATP (orders *b* and *e*). Finally, for the third set of reactions, the pre-mRNA was incubated with a mixture of WT and mutant extract (+/- MN treatment) and the ATP was added after 10 min (orders *c* and *f*). In all cases, splicing was allowed to proceed for 30 min. For comparison, untreated and mock-treated WT extract is analyzed (lanes 2 & 3) as were *bur2Δ* and MN-treated *bur2Δ* extracts (lanes 4 & 5). Splicing was only observed when the WT (untreated or mock) extract was pre-incubated first with the pre-mRNA before *bur2Δ* extract (+/- MN treatment) was added (lanes 7 & 9, respectively). A pre-incubation of the pre-mRNA with the *bur2Δ* extracts abrogated splicing (lane 6 & 10) as did pre-incubation with both extracts (lane 8 & 11). These results suggest a fundamental change in the complex formation in the *bur2Δ* extract which lead to this inhibition of splicing. Furthermore, the dominant negative effect of the *bur2Δ* extract was observed when the extract was MN treated suggesting that this effect is not mediated through snRNA interactions (lanes 10-11). These results further suggest that in the absence of Bur2, the interactions between Bur1 and splicing proteins block splicing. It is also possible that without Bur2 mediated kinase activity, an unphosphorylated protein is having “rogue” interactions with other factors or the pre-mRNA. Whichever is the case, this block occurs early in spliceosome

formation, possibly at the U1 snRNP recruitment, since no B complex (Figures 2.3 and 2.4) and no splicing intermediates were produced in the *bur2Δ* dominant negative reactions.

Activation of the Bur1 kinase is necessary for progression through the splicing pathway.

Since a mutant that inhibits activation of Bur1 kinase activity Bur1-T240A, prevents functional spliceosome assembly beyond B complexes it appears that this activity is required for early spliceosome. To further investigate this, we compared WT and mutant extracts for their abilities to form complexes over time. As we observed previously, no RNA intermediates or products form in the mutant extract (Figure 2.6 A). When complex formation was analyzed over time we observed that both WT and *bur1-T240A* extracts form B complexes at early time points (Figure 2.6 B). With the WT extract, B complex shifts quickly in the upper A complexes, while in the *bur1-T240A* extract, B complexes accumulate over time. Addition of EDTA slows down catalytic activation of the spliceosome and reveals the A sub-complexes (Cheng and Abelson 1987) (Figure 2.6 B, lanes 13-14). Comparing the 40min time point for *bur1-T240A* with the EDTA control suggests that there may be a partial formation of pre-catalytic A2-1 complex in the *BUR1* mutant extract (Figure 2.6 B, lane 12 vs. 14), although if it does form, it does so very inefficiently. Again, the most prominent band corresponds to the B complex

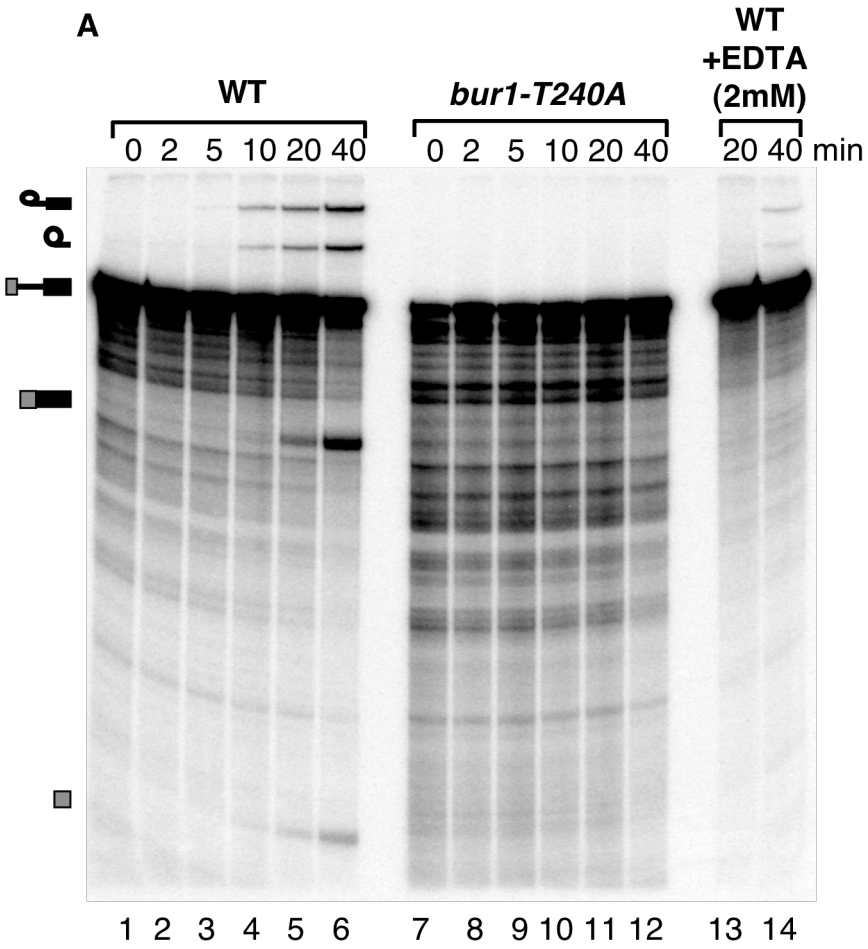


Figure 2.6. The T240A Bur1 mutant causes accumulation of prespliceosomal complexes *in vitro*. A. Denaturing gel showing the RNA products of *in vitro* splicing over a 40 min time course. Lanes 13-14 are similar reactions as lanes 5-6 with the addition of 2mM EDTA, which slows the formation of catalytic active spliceosomes thus leading to overall slower splicing kinetics. **B.** Same reactions as part A run on a native gel. Addition of EDTA in lanes 13-14 allows for visualization of A1 and A2 spliceosomal complexes. The complexes that form, A (A1, A2-1, A2-2), B, and B' are indicated.

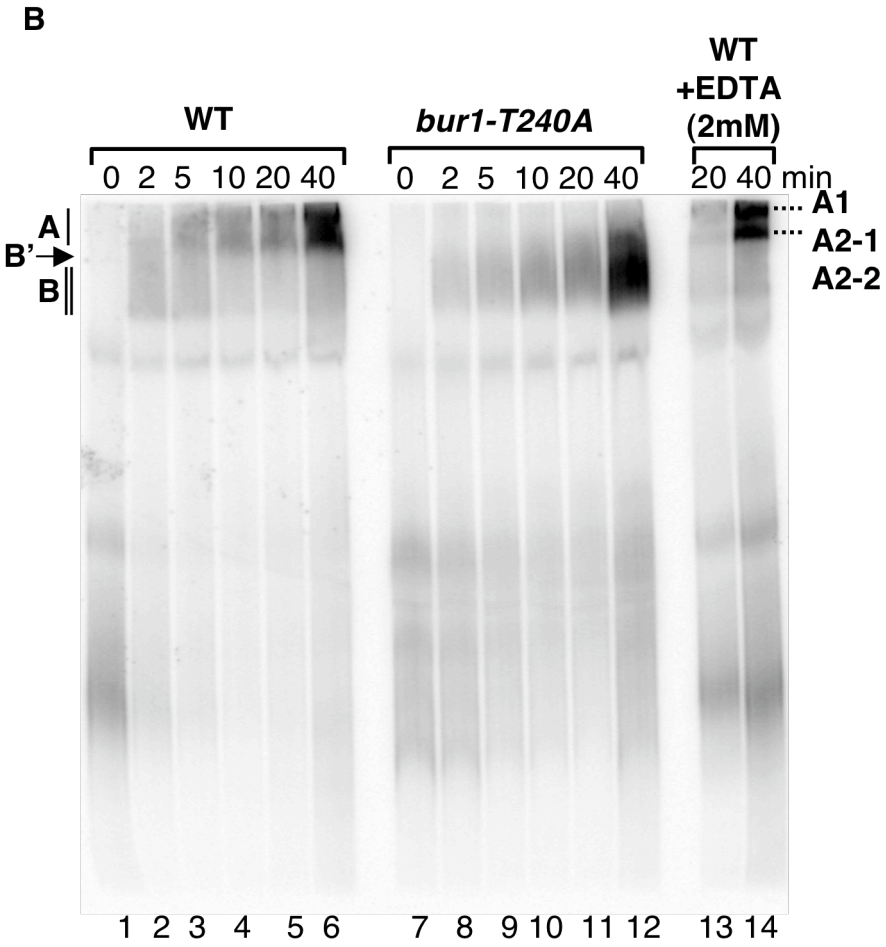


Figure 2.6. The T240A Bur1 mutant causes accumulation of prespliceosomal complexes *in vitro*, continued.

and B' complex that runs between the B and A complexes. This analysis indicates an important role for the Bur1 kinase in regulating the transition from prespliceosomes to catalytically active spliceosomes.

The Bur1 kinase associates with the pre-mRNA substrate and U1 snRNA during splicing *in vitro*.

Since our analysis indicated a direct role for the Bur complex in pre-mRNA splicing, we next investigated whether the Bur1 kinase can associate with the pre-mRNA or the spliceosome in the context of the *in vitro* reaction. To assess interactions between Bur1 and the spliceosomal machinery we used extracts with either untagged *BUR1* or ectopically expressed Bur1-HA or Bur1-T240A-HA (Figure 2.7). Following an *in vitro* splicing reaction using these extracts we immunoprecipitated the HA tagged proteins and ran the associated *ACT1* RNA on a denaturing gel (Figure 2.8 A). Bur1-HA strongly enriched the pre-mRNA substrate (5 fold), and to a much lower extent the 1st step splicing intermediate (Figure 2.8 A & B). Bur1-T240A-HA showed reduced enrichment of the pre-mRNA (~3 fold) but no enrichment of intermediates. Although we observe a lower level of Bur1-T240A-HA protein in the extracts (Figure 2.7, lane 7 vs. 4), the lack of enrichment of the splicing intermediates in the mutant Bur1 extract relative to WT suggests that kinase activation is necessary for progression of splicing.

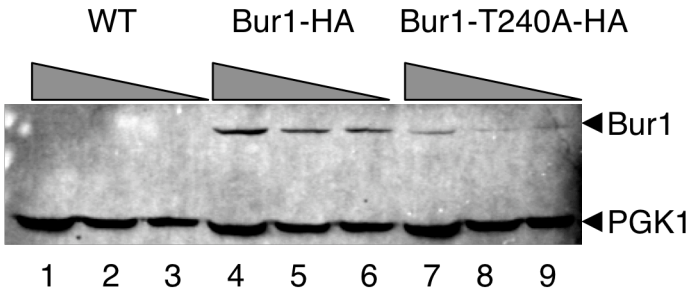


Figure 2.7. Expression of HA tagged Bur1 kinase in splicing extracts. Splicing extracts were derived from WT strains expressing *BUR1-HA* on a pRS315 plasmid (lanes 4-6), *bur1-T240A-HA* on a pRS315 plasmid (lanes 7-9), or no plasmid (lanes 1-3). 1μl of each extract was loaded in lanes 1, 4 and 7, and 2-fold serial dilutions were loaded in lanes 2-3, 5-6, and 8-9. HA-tagged protein was assayed by immunoblotting. PGK1 serves as a loading control.

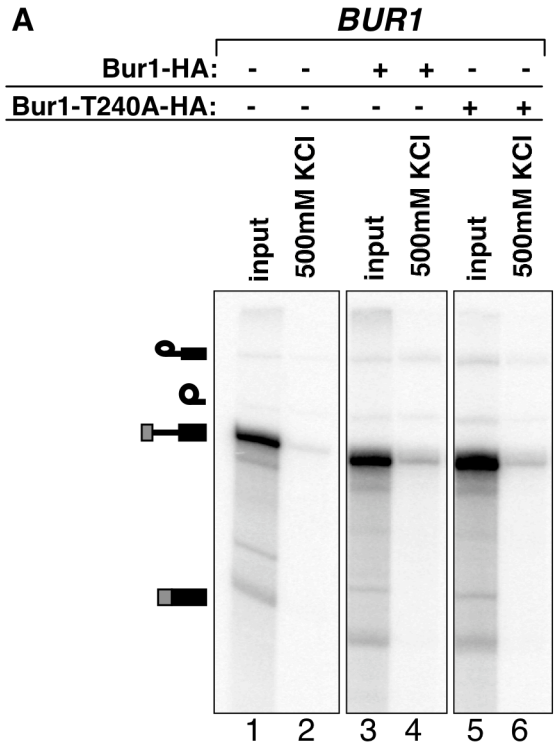


Figure 2.8. The Bur1 kinase associates with pre-mRNA during *in vitro* splicing. A. Representative denaturing gel showing labeled *ACT1* splicing intermediates immunoprecipitated by the indicated splicing extract. Input (25% loaded) and 500mM KCl wash IP are shown side by side for each extract. The indicated plasmids (Bur1-HA, Bur1-T240A-HA or no plasmid) were transformed into WT splicing competent cells, and these transformants were immunoprecipitated with anti-HA conjugated beads (Thermo Scientific). **B.** Enrichment quantitation for each *ACT1* RNA intermediate in part A for the two tagged splicing extracts. IP Data is normalized to the respective input and subsequently normalized to the untagged control IP/In. The mRNA is not present in the immunoprecipitation samples therefore it is not quantitated.

Figure 2.8. The Bur1 kinase associates with pre-mRNA during *in vitro* splicing, continued.

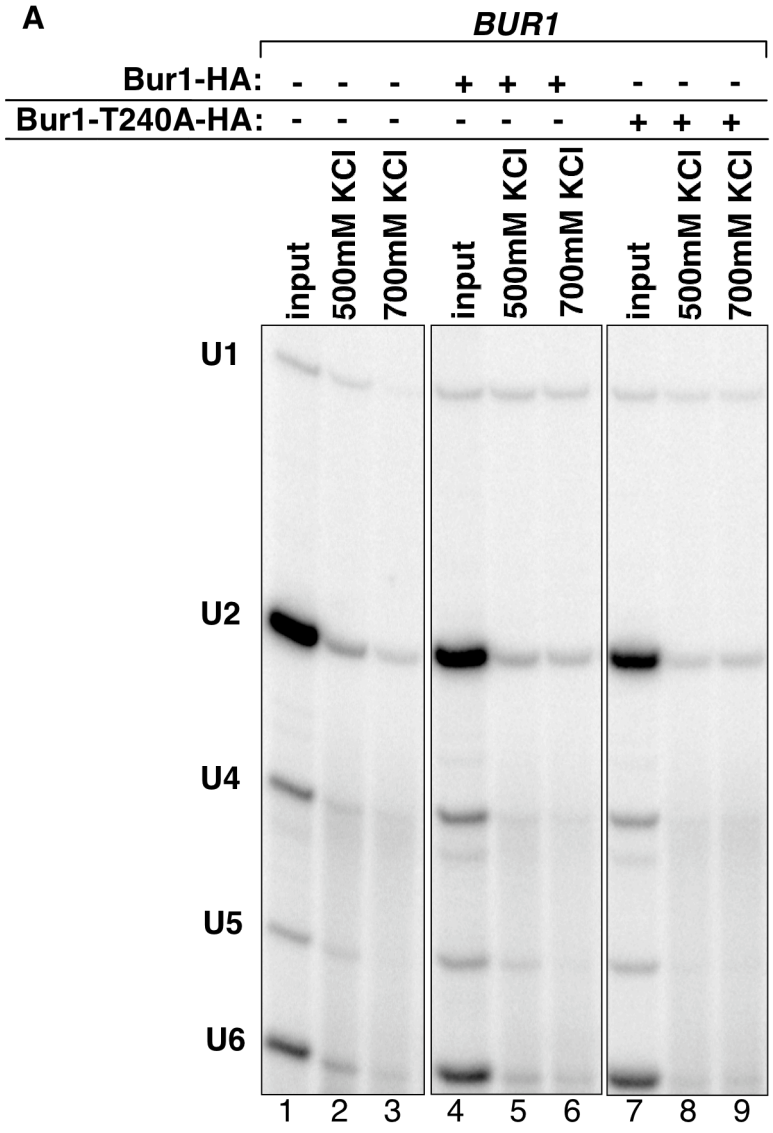


Figure 2.9. The Bur1 kinase associates with U1 snRNA during *in vitro* splicing. A. Representative primer extension showing labeled snRNAs immunoprecipitated by indicated splicing extract. Input (25% loaded), 500mM and 700mM KCl wash IP are shown side by side for each extract. WT splicing competent cells were transformed with the indicated plasmids (Bur1-HA, Bur1-T240A-HA or no plasmid) and subjected to 20 min *in vitro* splicing reaction. Lanes 1-3 represent input and IP for an untagged WT extract (negative control), while lane 4-6 and 7-9 show input and IP for the Bur1-HA and Bur1-T240A-HA extracts respectively. **B.** Quantitation of immunoprecipitated snRNAs is represented as a ratio of immunoprecipitated RNA relative to the input. Error bars represent S.E.M. (n=5).

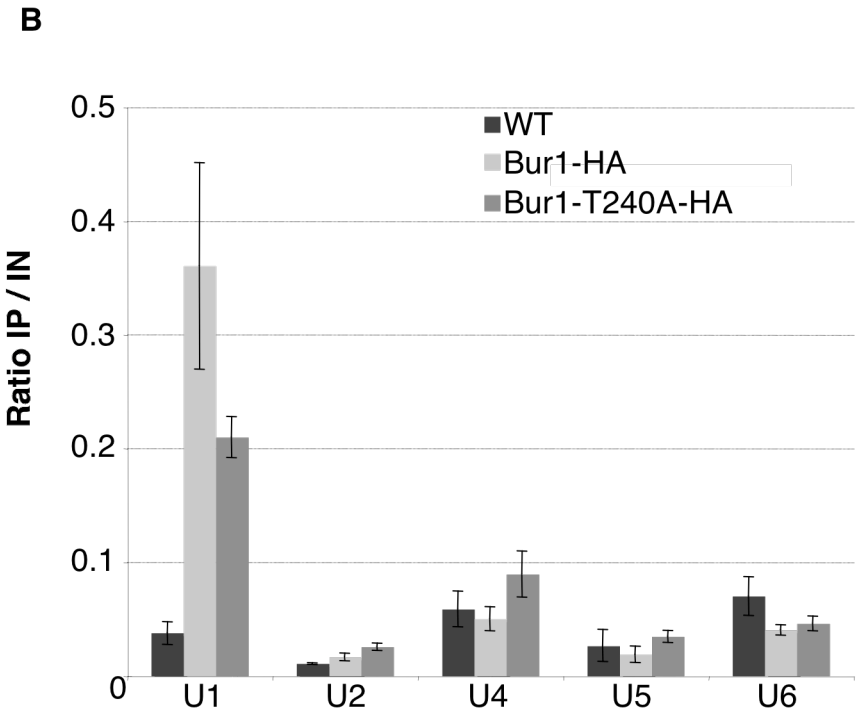


Figure 2.9. The Bur1 kinase associates with U1 snRNA during *in vitro* splicing, continued.

We next analyzed interactions between the tagged *BUR1* extracts and the five spliceosomal snRNAs by immunoprecipitation of the HA-tagged proteins and performing primer extension on the precipitated RNAs (Figure 2.9 A & B). We observe a stable and enriched interaction between the U1 snRNA and both tagged Bur1 proteins. Even though the mutant protein expression levels are lower, association of U1 snRNA with this protein is high, indicating that in the absence of kinase activation the Bur1 protein interacts strongly with the U1 snRNP. Furthermore, preliminary experiments in the absence of the pre-mRNA substrate also show similar levels of association between Bur1 and the U1 snRNA (data not shown). Finally, in these experiments, we cannot rule out that there is a slight enrichment of the U2 snRNA compared to the untagged control. However this interaction is weak at best. Taken together these data reinforce a role for Bur1 in early spliceosomal formation and suggest that Bur1 interacts with both the U1 snRNP and the pre-mRNA substrate.

The Bur complex associates with prespliceosomes in native gels.

To determine whether the Bur complex interacts with specific splicing complexes, we analyzed whether Bur1 and Bur2 co-migrates with spliceosomal complexes on a native gel, which would also be expected to allow detection of transient interactions. We immunoprobed a native gel in

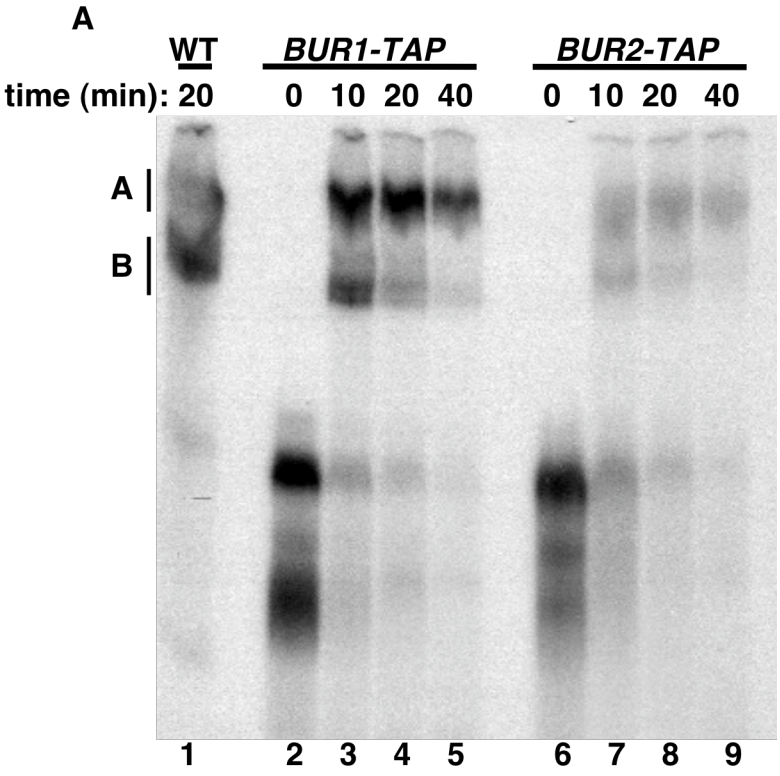


Figure 2.10. Bur1/2 complex localizes with prespliceosomal complexes *in vitro*. A. Time course of an *in vitro* splicing reaction run on a native gel showing formation of B and A spliceosomal complexes. **B.** Anti-TAP immunoblot of native gel in part A shows mobility of tagged Bur complex. The asterisk (*) denotes a non-specific interaction in the *BUR2-TAP* extract at all time points.

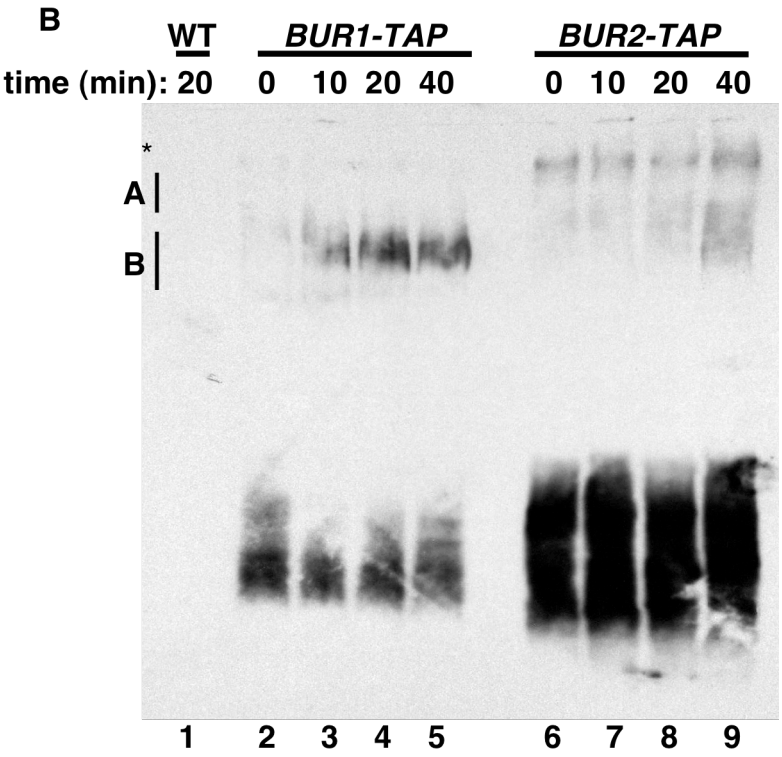


Figure 2.10. Bur1/2 complex localizes with prespliceosomal complexes *in vitro*, continued.

which splicing extracts containing tagged Bur1/2 proteins were allowed to form splicing complexes over 40 minutes (Figure 2.10 A & B). These results show that both Bur1-TAP and Bur2-TAP co-migrate with the B complex, consistent with an association between the Bur complex and prespliceosomes. Furthermore this association increases overtime as the B complex levels decrease and A complex levels increase. These data are consistent with our observed interactions with the U1 snRNA and the pre-mRNA and the requirement of the Bur complex and its kinase activity in early steps of spliceosome assembly.

Section 2.3: Discussion

In this study we provide evidence of a novel role for the Bur complex in the regulation of early spliceosome formation. We first show that mutations altering the function of the Bur complex lead to splicing defects *in vivo* for the *U3* and *ACT1* genes. These results reveal a stronger phenotype when the cyclin Bur2 is absent than when the kinase alone is mutated, suggesting that integrity of the Bur complex is important for pre-mRNA splicing regulation. Furthermore, since Bur2 is necessary for Bur1 kinase activity and regulation (Yao, Neiman et al. 2000), and the *BUR1* mutant *bur1-T240A* also confers an *in vivo* splicing defect (albeit not as strong as *bur2Δ*) we conclude that

missregulation of the kinase activity is important as complex integrity to promote splicing *in vivo*. These observations were confirmed by *in vitro* splicing analysis. Both *bur2Δ* and *bur1-T240A* possess striking *in vitro* splicing defects. No spliceosome formation was observed when Bur2 is absent from the extract, and significant prespliceosome formation is observed when the kinase Bur1 is missregulated. While a comparison of the severity of the *in vivo* vs. *in vitro* phenotypes highlights the robust nature of *in vivo* splicing compared to its *in vitro* counterpart, it does allow us to dissect the role of the Bur complex in early spliceosome dynamics.

Indeed *in vitro* analyses indicate that both Bur1 and Bur2 comigrate with the prespliceosome complexes. Moreover, Bur1 associates most strongly with the U1 snRNA and the pre-mRNA substrate, further implicating this complex in regulation of early spliceosome formation. Through these observations we demonstrate that the role of Bur in splicing is separate from its role in transcription elongation.

Interestingly these data reveal that an extract devoid of the cyclin Bur2 in which Bur1is present without its cyclin, has a dominant negative effect on splicing and spliceosome formation. We hypothesize that the lack of the cyclin allows the Bur1 kinase to form a non-functional complex that is deleterious to

splicing. In order for spliceosomes to form in the presence of extract lacking Bur2, the pre-mRNA must first bind to active spliceosomes. Only then can the complement of proteins found in the *bur2Δ* extract support splicing. This data reinforces the idea that integrity of the Bur complex is paramount for normal splicing regulation (Figure 2.11). We cannot rule out the possibility that in the absence of some Bur2-dependent phosphorylation event(s), some aberrant complexes that may or may not contain Bur1 are present to inhibit splicing.

We propose that the Bur complex interacts transiently with the U1 snRNP to facilitate its recruitment to the pre-mRNA. The activation of Bur1 through Cak1 dependent phosphorylation is then required for the catalytic activation of the spliceosome. Furthermore the Bur complex could be phosphorylating specific splicing factors to promote these spliceosomal events. Although there is little know about the role of specific phosphorylation events in splicing, a handful of reports suggest that this post-translational modification plays an important role in modulating spliceosomal rearrangements (reviewed in McKay). Through these transient interactions, the Bur complex could be promoting early formation and activation of the spliceosome.

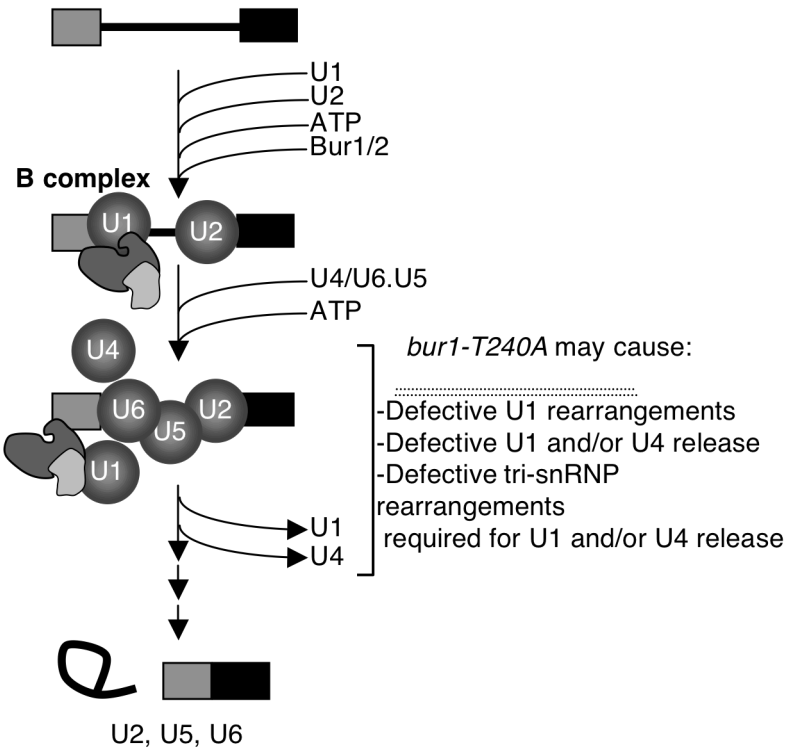


Figure 2.11. Model of Bur's role in pre-mRNA splicing. Diagram showing Bur associating with U1 snRNP and the role of the Bur complex in recruitment of U1 to the pre-mRNA to promote B complex formation. Advancing past the B complex requires the activation of the Bur complex and possibly its kinase activity as suggested by our data with the *bur1-T240A* splicing extract.

Prior studies in mammalian systems have shown a link between P-TEFb and known splicing factors TAT-SF1 (yeast Cus2), SKIP (yeast Prp45), and the CBC. Our analysis of the Bur complex and Cus2 fail to demonstrate any interaction (McKay and Johnson 2011). The yeast homolog of SKIP, Prp45 is essential for viability and has been reported to affect recruitment of second step factor Prp22 (Albers, Diment et al. 2003). However, two-hybrid experiments using Prp45 as bait did not uncover an interaction with the Bur complex (Albers, Diment et al. 2003). Furthermore, Prp45 is important for the 2nd catalytic step of splicing, which is a later step in spliceosome dynamics than our data suggest Bur acts at.

Recent reports have uncovered an interaction between P-TEFb and the Cap Binding Complex (CBC) which affects transcriptional activity (Lenasi, Peterlin et al. 2011). The CBC is recruited to the pre-mRNA cotranscriptionally and promotes the binding of the U1 snRNP to the 5' splice site (Gornemann, Kotovic et al. 2005). Furthermore CBC has been implicating in post-U1 rearrangements such as U2 snRNA recruitment and may also help in the recruitment of U6 at the 5'SS (Bragulat, Meyer et al. 2010). These observations make CBC a good candidate for interacting with the Bur complex to regulate splicing. Future experiments will test whether the Bur complex

interacts with CBC to promote pre-mRNA splicing. Nonetheless experiments are ongoing to identify splicing factors targeted by Bur kinase activity.

Recent studies have shown that most splicing occurs cotranscriptionally (Alexander, Innocente et al. 2010; Oesterreich, Bieberstein et al. 2011). In this study we have shown that a canonical transcription factor acts as in splicing to promote early spliceosome formation. These studies raise the intriguing possibility that these dual activities of the Bur complex allow it to couple splicing with transcription. The Bur complex activity is important early in transcription to promote productive elongation, thus Bur is in close spatial and temporal proximity to early splicing signals such as the 5' cap and the newly-synthesized 5'SS. If the Bur complex can recognize those signals, possibly through interaction with the CBC, it can then respond by promoting spliceosome formation cotranscriptionally. Similarly, since the Bur complex mediates productive elongation it can register when the timing and signals are appropriate for pre-mRNA recognition. This regulation of transcription and splicing by the Bur complex could ensure that commitment of the RNA polymerase to transcription is accompanied by spliceosome assembly, and conversely that spliceosome assembly does not occur on non-functional or aborted transcripts. In sum, as we continue to uncover the mechanistic details

underlying spliceosome regulation, our results reveal the potential for a network of coupled and coordinated nuclear events.

This chapter is currently being prepared for publication of the material. Tiriac, Hervé; Hu, Pan; Johnson, Tracy L. The dissertation author was the primary investigator and author of this material.

Chapter 3: Kinase activity of the Bur complex is important for pre-mRNA splicing

Section 3.1: Background

Regulated gene expression is crucial for every function carried out by the cell, from growth and proliferation to its ability to respond to environmental cues. Eukaryotic genes must be transcribed into pre-messenger RNA (pre-mRNA) molecules, which are then processed and modified through 5' end capping, editing, splicing and polyadenylation. These mature messenger RNAs (mRNA) are then viable substrates for translation into polypeptides.

Pre-mRNA splicing is a key target of regulation in eukaryotic cells. The splicing mechanism involves two trans-esterification reactions that are catalyzed and coordinated by the spliceosome. Five small nuclear RNAs (snRNAs) combine with proteins to form small nuclear ribonucleo-protein complexes (snRNPs) which come together to form the spliceosome. Both snRNA and proteins of the spliceosome are highly conserved from the yeast *Saccharomyces cerevisiae* to metazoans (Ruby and Abelson 1991; Sharp 1994; Staley and Guthrie 1998; Valadkhan 2005).

Intricate and dynamic rearrangements of the spliceosome result in splicing of the pre-mRNA. Spliceosome formation occurs in different steps: first the U1 snRNP binds to the pre-mRNA at the 5' splice site (SS) forming the commitment complex. Secondly the U2 snRNP is added to the branchpoint (BP) which forms the prespliceosome (B complex). Then the U5•U4/U6 tri-snRNP bind to the prespliceosome and forms the pre-catalytic spliceosome (A complex). Extensive snRNA base pairing rearrangements lead to the removal of the U1 and U4 snRNPs and formation of the catalytic spliceosome (A complex) (Staley and Guthrie 1998; Wahl, Will et al. 2009).

Spliceosomal rearrangements which ultimately lead to the trans-esterification reactions are a direct result of snRNA conformational changes. Many studies have shown that post-translational protein modifications (PTM) play an important regulatory role in the rearrangements of the spliceosome and untimely impacts assembly of the spliceosome (Wahl, Will et al. 2009; McKay and Johnson 2010). Protein phosphorylation is a common PTM present in most regulatory pathways to alter protein conformation and protein-protein interactions (Cohen 2000). This PMT has been shown to activate and inactivate protein function, change recruitment and localization, as well act as a signaling marker. In cells, phosphorylation is mediated by protein kinases,

and their activity is essential for cellular viability (Dissmeyer and Schnittger 2011).

Splicing extracts derived from human cell lines have provided evidence for four protein kinases that phosphorylate splicing factors: serine/arginine protein kinase 1 and 2 (SRPK1/2), Prp4 kinase and the Clk/Sty kinase (Wahl, Will et al. 2009). Phosphorylation events performed by these kinases have been shown to directly regulate splicing events. One example of such regulation is the phosphorylation of hPrp28 by the kinase SRPK2 (Mathew, Hartmuth et al. 2008). This phosphorylation event is necessary for the U5•U4/U6 tri-snRNP addition to the prespliceosome to form the pre-catalytic spliceosome. Interestingly while the kinase activity of SRPK2 was known, its substrate(s) eluded researchers until recently. In addition to protein phosphorylation, splicing factor dephosphorylation by phosphatases also plays an important role regulating the second catalytic step of pre-mRNA splicing (Shi, Reddy et al. 2006). The mammalian Sap155 splicing factor (yeast Hsh155) is phosphorylated prior to the first catalytic step, and dephosphorylated prior to the second catalytic step (Wang, Chua et al. 1998). In budding yeast the spliceosome does not contain any SR proteins, which are a major source of protein phosphorylation in the mammalian spliceosome, yet there have been interesting reports of phosphorylation-dependent regulating of

splicing. For example, the splicing factor Aar2 interacts with Prp8 in the precursor U5 snRNP, and must be replaced by Brr2 prior to the addition of U5 to the spliceosome (Gottschalk, Kastner et al. 2001). The interactions between Prp8 and Aar2 and possibly even the recruitement of Brr2 are all mediated by phosphorylation of Aar2 (Weber, Cristao et al. 2011). Upon phosphorylation of Aar2 by a yet unidentified kinase, Aar2 conformationaly rearranges disrupting its interaction with Prp8, which is then free to bind to Brr2. This example highlights the importance of protein phosphorylation in mediating rearrangements of the spliceosome through modulation of protein-protein interactions.

While protein phosphorylation is important for spliceosomal dynamics, identifying these events within the spliceosome has been challenging. Phosphorylation marks are reversible by phosphatase activity, and due to their ability to regulate cellular function, these phosphorylation and dephosphorylation events are tightly regulated (Dissmeyer and Schnittger 2011). With the use of mass spectroscopy analysis, a slew of potential phosphorylation events can be uncovered, yet finding the specific protein kinase responsible for a specific phosphorylation event can be difficult and requires directed analysis of kinases.

In the yeast *Saccharomyces cerevisiae*, the Bur cyclin dependent kinase (CDK) complex plays a central role in transcription regulation. *BUR1* was first identified in a screen for mutations that bypass the upstream activating sequence (Prelich and Winston 1993). *BUR1* encodes a cyclin dependent kinase, and forms a complex with the cyclin *BUR2*. While deletion of the *BUR1* gene is lethal, knockout of *BUR2* is viable and displays the same phenotypes as a *BUR1* mutant with reduced kinase activity, furthermore Bur1/Bur2 association is required for *in vitro* kinase activity (Yao, Neiman et al. 2000). Strong sequence and functional homology suggest that the Bur complex is the homologue of P-TEFb in budding yeast (Bartkowiak, Liu et al. 2011). Bur phosphorylates the Rpb1-CTD of RNA Pol II both *in vitro* (Murray, Udupa et al. 2001) and *in vivo* (Qiu, Hu et al. 2009) regulating transcription elongation. The Bur complex also promotes transcription elongation through phosphorylation of DSIF subunit Spt5 (Zhou, Kuo et al. 2009) as well as phosphorylation of the histone H2B ubiquitin ligase Rad6 (Wood, Schneider et al. 2005). Based upon these known substrates, the Bur1 kinase seems to prefer serine substrates flanked by hydrophobic residues, but no specific kinase motif appears to be used (Table 3.1). Despite the obvious diversity in Bur substrates, no substrates other than transcription factors have been identified. Interestingly some mammalian splicing factors have been shown to interact with the homolog of the Bur complex. These are the U2 snRNP factor

Table 3.1. List of known Bur substrates

Substrate	phosphorylation site	Kinase consensus site	note
Rpb1 (192KDa)	$(YS^{2}PTS^{5}PS)_{27}$	CKI, GSK-3, MAPK	Heptapeptide repeat found in the CTD
Spt5 (116KDa)	$(S^{1}AWGGQ)_{5}$	-	Hexapeptide repeat found in the CTD
Rad6 (20KDa)	$NPAS^{120}PAN$	-	-

Tat-SF1 (Fong and Zhou 2001), The Ski-interacting protein (SKIP) (Bres, Gomes et al. 2005), and the cap binding complex (CBC) (Lenasi, Peterlin et al. 2011).

The mammalian homologue of Cus2 is Tat-SF1 (Yan, Perriman et al. 1998), a molecule identified as a general transcription factor, and splicing factor (Fong and Zhou 2001). Tat-SF1 has been shown to pull down spliceosomal U snRNP, and is part of the U2 snRNP. Immunoprecipitated Tat-SF1-U snRNP complex can stimulate transcription elongation at intron-less genes by binding and activating the P-TEFb complex (Fong and Zhou 2001). Tat-SF1 sequence is very similar to *CUS2* in yeast, except that it has an extended acidic C-term. This C-term can be phosphorylated by P-TEFb, and is necessary for binding of Tat-SF1 to the CycT1 subunit (Fong and Zhou 2001). The Ski-interacting protein (SKIP), a second step splicing factor, was also found to associate with P-TEFb and could function as a transcriptional coactivator by stimulating the activity of P-TEFb at certain genes (Bres, Gomes et al. 2005; Bres, Yoshida et al. 2009). Most recently, the P-TEFb complex was shown to associate with another splicing factor in mammalian cells, the cap binding complex (CBC) (Lenasi, Peterlin et al. 2011). In this study, the CBC was shown to help recruit the P-TEFb complex to the RNA polymerase, which led to the serine 2 phosphorylation of the CTD. This phosphorylation event promotes the recruitment of SR proteins and other

splicing factors to the CTD of RNA Pol II leading to increased splicing activity (Pandit, Wang et al. 2008; Lenasi, Peterlin et al. 2011). Interestingly, recent work from our laboratory in the budding yeast has demonstrated that the CBC can recruit the Bur complex to the RNA Pol-II through physical interactions (work in preparation).

In our previous work (Chapter 2) we have shown that the Bur complex plays an important transcription independent role in pre-mRNA splicing regulation. We demonstrated that the Bur complex interacts with the U1 snRNP as well as with the prespliceosome suggesting that the Bur complex regulate early spliceosome assembly. Furthermore we show that the phosphorylation activity of the Bur1 kinase is important for splicing regulation, and that activation of the Bur1 kinase is essential for formation of catalytic spliceosomes *in vitro*. In this study we analyze potential substrates of the Bur complex. We first look at the homologues of Tat-SF1 (Cus2) and the CBC proteins (Cbp20 and Cbp80) to investigate their phosphorylation status in yeast. Next we show that the yeast spliceosomal complexes harbor multiple phosphorylated splicing factors, some of which are affected by mutation of the Bur kinase activity. Finally we show that subtle changes within the conformation of the Bur complex may help regulate pre-mRNA splicing.

Section 3.2: Results

Cus2 is phosphorylated by the Bur complex *in vitro*.

In order to identify substrates of the Bur activity, we decided to directly analyze the phosphorylation state of the yeast counterparts of known P-TEFb substrates and determine if their phosphorylation, if observed, was dependent upon Bur kinase activity *in vitro*. *CUS2* is a non-essential gene in which mutations show genetic interactions with components of the U2 snRNP (Perriman and Ares 2000), however its deletion does not cause splicing defects (Yan, Perriman et al. 1998). Cus2 is important for splicing fidelity and promotes U2 snRNA rearrangements throughout splicing (Perriman and Ares 2007). Sequence analysis reveals that *CUS2* contains two RNA recognition motifs (RRM), and is terminated with a short (25 amino acids) acidic C-terminal domain. A putative casein kinase II (CKII) site is found within the C-terminal domain of Cus2 (Yan, Perriman et al. 1998). Given the diversity of Bur1 phosphorylation sites this CKII site (NEDS^{278}EVE) could serve as a substrate for Bur1 activity. Indeed, in mammalian cells P-TEFb can phosphorylate Tat-SF1 in the extended acidic domain which contains multiple CKII sites (Yan, Perriman et al. 1998; Fong and Zhou 2001), therefore we investigated if the Bur complex was able to phosphorylate Cus2 *in vitro*.

Bacterially expressed recombinant Cus2 protein (rCus2) was mixed with immunoprecipitated Bur complex from yeast splicing extracts (Figure 3.1). In the absence of Bur complex rCus2 is not phosphorylated (lane 1), however after addition of immunoprecipitated kinase complex, rCus2 becomes phosphorylated (lane 2). Increasing the amount of rCus2 present in the reaction results in increased protein phosphorylation (lanes 2-4). The bacterially expressed rCus2 is found in two main polypeptide species: the full length protein (f.l.) and a truncation protein (trunc.) transcribed from a cryptic internal start site found between the two RRMs (Ares communications). After expressing only the truncated C-terminal half of Cus2 (rCus2-C) we observed higher levels of phosphorylation of this peptide when compared to the full length protein relative to their respective protein levels. Conversely, the N-terminal half of Cus2 when expressed (rCus2-N) does not get phosphorylated by the Bur complex (lane 8). These results confirm that the Cus2 is phosphorylated in its C-Terminal half as predicted, and indicate that the N-terminal half of the molecule, while not phosphorylated, could modulate phosphorylation of the C-terminal half. Since rCus2 is a target of the Bur complex kinase activity *in vitro*, we next investigated the status of Cus2 phosphorylation within the context of a splicing extract to determine if this phosphorylation was solely dependent upon Bur kinase activity.

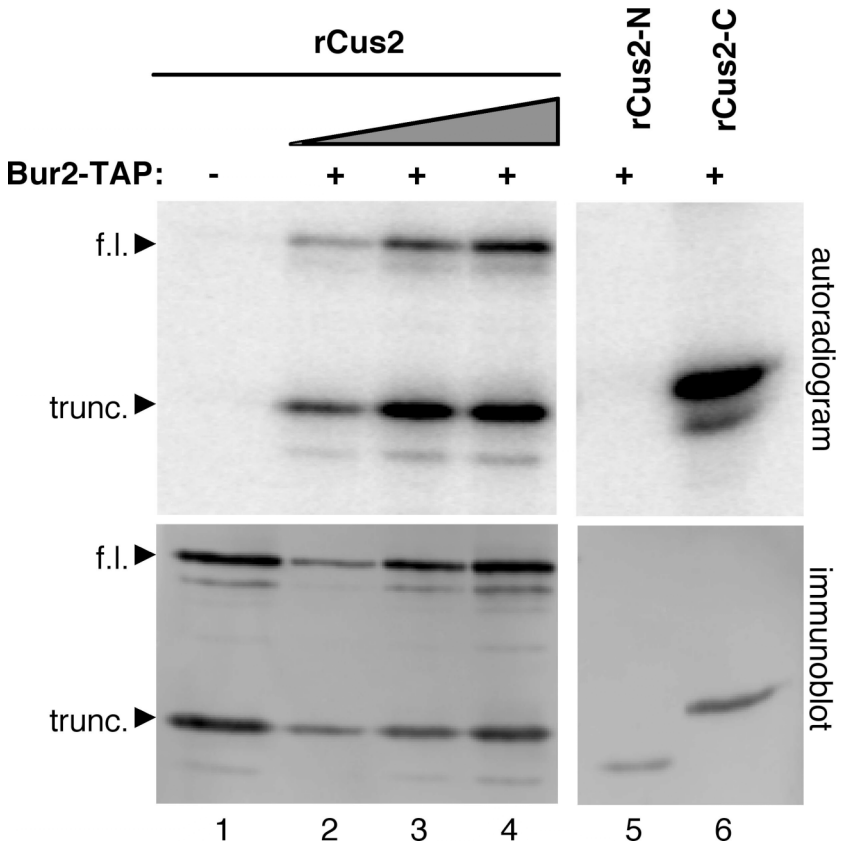


Figure 3.1. Recombinant Cus2 protein acts as a substrate for Bur phosphorylation. 150μCi [γ^{32}P] ATP was added to rCus2 (8μg used in lane 1 and 4-16μg used in lanes 3-4), in the absence or in the presence of immunoprecipitated Bur2-TAP. Cus2 truncations rCus2-N (in lane 5) and rCus2-C (lane 6) were also tested in the presence of immunoprecipitated Bur2-TAP. Autoradiogram showing protein phosphorylation (top panel), and immunoblot showing protein levels (bottom panel) are shown. Full length (f.l.) rCus2 can be distinguished from truncated peptide (trunc.).

Cus2 is phosphorylated in splicing extracts in a Bur2 independent manner.

To test the phosphorylation status of Cus2 in splicing extracts, we added rCus2 to WT, *cus2Δ*, *bur2Δ*, and *bur2Δcus2Δ* extracts. Following a kinase assay we immunoprecipitated rCus2 and analyzed its phosphorylation status by PAGE analysis (Figure 3.2). Both full length and truncated rCus2 were detectably phosphorylated in all extracts even in the absence of the cyclin Bur2, indicating that Cus2 is phosphorylated in splicing extracts, but that this phosphorylation is not dependent solely on the activity of the Bur complex. Interestingly we observe a phosphorylated peptide with a mobility similar to Cus2 (~32kDa) when looking at B and A spliceosomal complexes in native gels (Figure 3.4 B) however this phosphorylation event is not affected by the lack of Bur kinase activity again suggesting that this phosphorylation event may not be completely Bur dependent. While the Bur complex can phosphorylate Cus2 both *in vitro* and in extracts, this phosphorylation event does no seem to be unique to the Bur1 kinase. Interestingly some Bur1 substrates (Rpb1 and Spt5) have been shown to be phosphorylated by other CDKs *in vitro*, thus highlighting the promiscuity of kinases in different environments (Keogh, Podolny et al. 2003). Nonetheless, Cus2 phosphorylation in its C-terminal domain (possibly at the CKII site) is

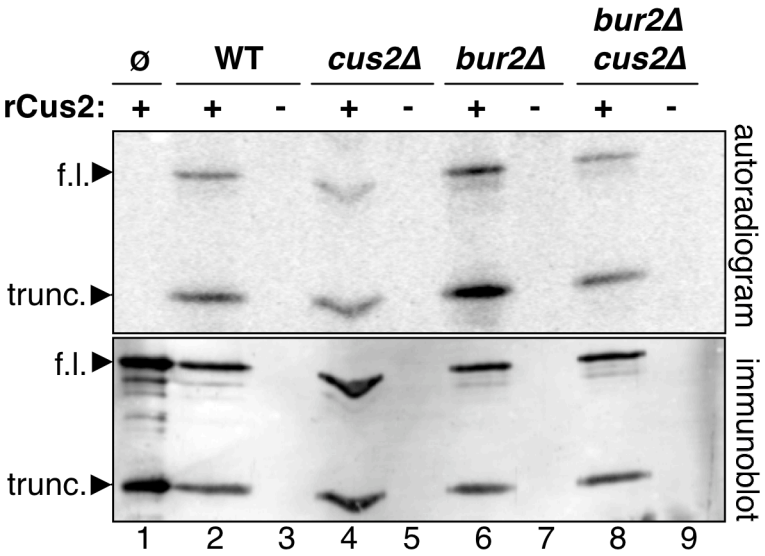


Figure 3.2. Recombinant Cus2 protein is phosphorylated in splicing extracts. Autoradiogram of the kinase assay (top panel) shows phosphorylation levels of rCus2 (4μg) in the absence of splicing extract (lane1) or with WT, *cus2Δ*, *bur2Δ*, *bur2Δ cus2Δ* extracts (lanes 2-3, 4-5, 6-7, and 8-9 respectively). Recombinant Cus2 is phosphorylated in all extracts in the presence of 150μCi [γ^{32}P] ATP. Bottom panel shows recombinant protein levels by immunoblot. Full length (f.l.) rCus2 can be distinguished from truncated peptide (trunc.).

interesting because of its proximity to residues important for U2 snRNA interactions (Yan, Perriman et al. 1998).

Cbp80 is not phosphorylated *in vitro*.

The CBC, composed of Cbp20 and Cbp80, binds to the newly synthesized 5' m^7G cap of the nascent RNA molecule. The CBC is important for splicing both *in vivo* and *in vitro*: it has been shown to be important for the recruitment of the U1 snRNP to the 5'SS, and for the recruitment of the U2 snRNA to the BP (Fortes, Bilbao-Cortes et al. 1999; Fortes, Kufel et al. 1999). Furthermore the CBC has been implicated in the formation of the catalytic spliceosome (Hage, Tung et al. 2009). Additionally the CBC has been demonstrated to be necessary for co-transcriptional spliceosome assembly (Gornemann, Kotovic et al. 2005; Bragulat, Meyer et al. 2010). Strikingly the CBC has been shown both in mammals (Lenasi, Peterlin et al. 2011), and in budding yeast (unpublished data from our laboratory) to physically interact with the Bur complex (P-TEFb in mammals). Considering the physical interaction as well as the remarkably similar roles played by these complexes in pre-mRNA splicing based upon our previous data, we decided to investigate the phosphorylation status of the CBC when exposed to the Bur1 kinase. Interestingly the CBC subunit Cbp80 has been shown to be phosphorylated in

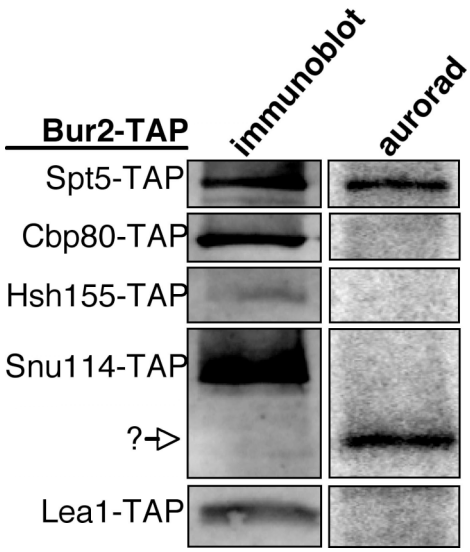


Figure 3.3. Testing phosphorylation of candidate splicing factors reveals a potential substrate of Bur1. *In vitro* kinase assay with immunoprecipitated Bur complex (Bur2-TAP) and candidate splicing factors (TAP tagged) in the presence of 150μCi [γ^{32}P] ATP. Immunoblot (left panels) indicates protein levels and autoradiogram (right panels) indicates phosphorylation levels. Spt5 is used as a positive control for a known Bur1 substrate and Lea1 is used as a potential negative control as there is no known indication of Lea1 phosphorylation. An unidentified 100 KDa phosphoprotein is co-immunoprecipitated with Snu114-TAP (indicated by "?").

mammalian cells by the S6 kinase, a serine/threonine kinase like Bur1/P-TEFb (Wilson, Wu et al. 2000).

To assay for a possible Bur-dependent phosphorylation of Cbp80, we immunoprecipiteted both Bur2-TAP and Cbp80-TAP and perfomormed an *in vitro* kinase assay (Figure 3.3). Despite evidence of physical interactions between the Bur complex and the CBC, we did not detect any phosphorylation of Cbp80-TAP. As a positive control we immunoprecipitated Spt5-TAP and were able to detect Bur dependent phosphoryltion upon the known Bur1 substrate (Figure 3.3). While this preliminary experiment did not support a role for the Bur complex in phosphorylating Cbp80 *in vitro*, it does not preclude the possibilty of CBC phosphorylation *in vivo*.

The Bur complex is important for phosphorylation of spliceosomal proteins.

Following the directed kinase assay experiments testing Cus2 and Cbp80 phosphorylation, we decided to survey both prespliceosomes and spliceosomes for phosphorylation events. To achieve this we performed *in vitro* spliceosome assembly assay in the presence of *in vitro* synthesized acting pre-mRNA. To label and visualize phosphoproteins within the spliceosome we added [γ^{32}P] ATP to the splicing reactions. The spliceosomes

were resolved using a native gel system. Upon separation of the different complexes, we isolated both the prespliceosomal and spliceosomal gel fractions, and electro-eluted the proteins contained in these complexes. Finally we concentrated the eluted proteins using TCA precipitation and ran SDS-PAGE to resolve the proteins based on mobility (Figure 3.4). In order to test the role of the Bur kinase in phosphorylating splicing factors we compared WT extracts to *bur1-T240A* and *bur2Δ* extracts. Furthermore we reconstituted the Bur mutant extracts using micrococcal nuclease (MN) treated extracts similarly to experiments shown in Chapter 2. The protein levels shown in the silver stain gel reflects the spliceosome assembly kinetics observed in the respective extracts: after 10 minutes of spliceosome assembly the WT extract forms predominantly spliceosomal A complex, the *bur1-T240A* accumulates B and B' complex while the reconstituted *bur1-T240A* with MN-WT forms some catalytic A complexes. Without reconstitution, the *bur2Δ* extract does not form pre-mRNA containing spliceosomal complexes, and with reconstitution only forms a fraction of the complexes seen in the WT and *bur1-T240A* extracts (Figure 3.4 A).

Distinct phosphorylation events occur within both spliceosomal complexes (Figure 3.4 B). While some phosphoproteins are observed in all extracts, the availability of a functional Bur1 kinase dramatically alters the

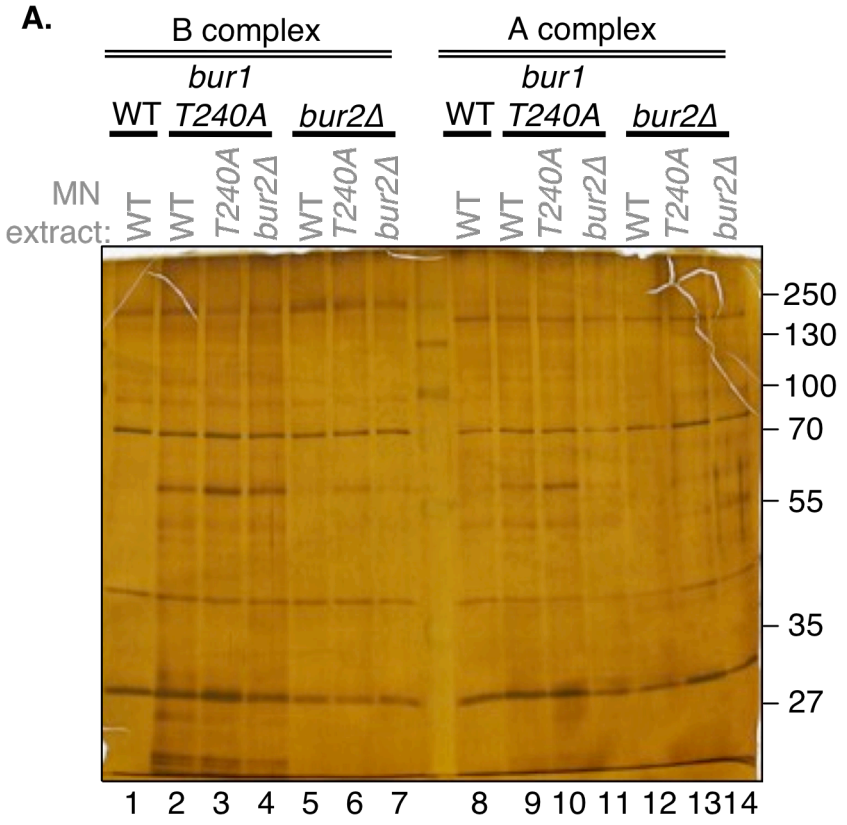


Figure 3.4. The Bur complex is important for phosphorylation of splicing factors. A. *In vitro* splicing (10 min reaction time) was performed using non-radioactive actin pre-mRNA in the presence of 150μCi [γ^{32}P] ATP and loaded on a native gel. Equivalent amounts of splicing extract (black) were reconstituted with MN-treated extracts (grey). Proteins from WT control (lane 1 and 8), reconstituted *bur1-T240A* (lanes 2-4, and 9-11), and reconstituted *bur2Δ* (lanes 5-7, and 12-14) were extracted by electro-elution from complex gel regions corresponding to B (lanes 107) and A (lanes 8-14) complexes and ran on a 10% SDS PAGE. The gel was silver stain to show protein levels. **B.** Autoradiograph of the gel in part A showing phosphorylation events with spliceosomes. In the absence of efficient Bur kinase activity, protein phosphorylation throughout the spliceosomes is decreased. Apparent Bur specific phosphorylation events are listed in tables 3.2 and 3.3.

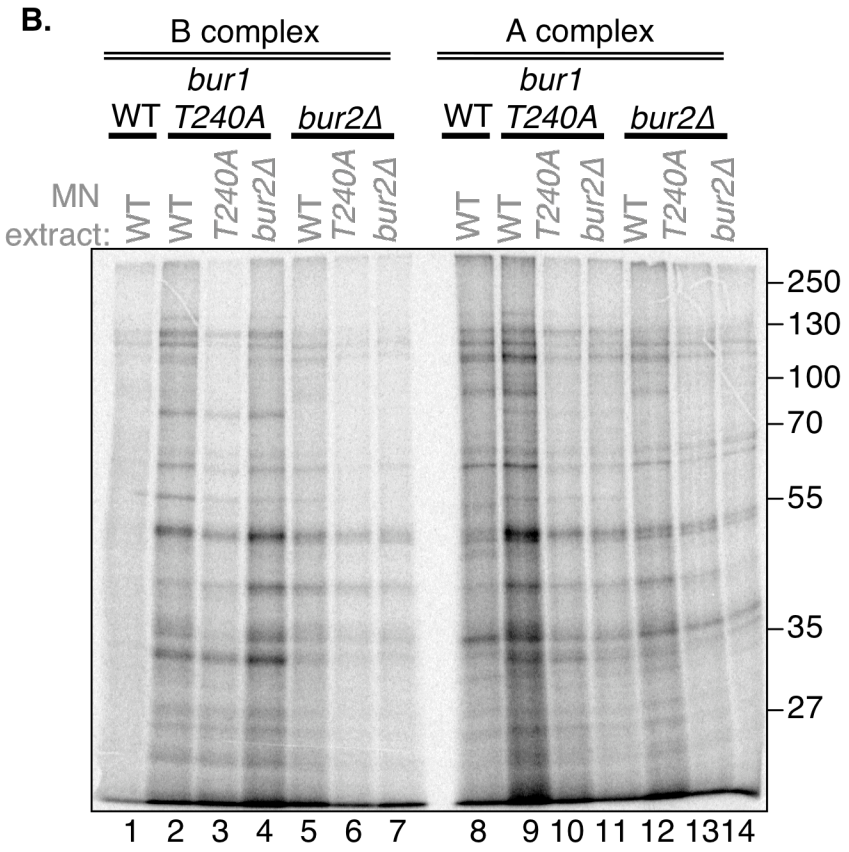


Figure 3.4. The Bur complex is important for phosphorylation of splicing factors, continued.

phosphorylation events within both B and A complexes. Indeed, phosphorylation of a protein or proteins in the molecular weight range of 120 to 200 kDa seems to be altered in both the *bur1-T240A* and *bur2Δ* mutant extracts (lanes 2 vs. 3 and 5 vs. 7 in B complexes; lanes 9 vs. 10 and 12 vs. 14 in A complexes). In the A complex specifically, a 100 kDa protein is phosphorylated only in extracts being reconstituted with a WT complement of proteins (lanes 8, 9, and 12). Interestingly a protein with a mobility of about 80 kDa could be the Bur1 kinase (74 kDa) that is auto-phosphorylated. Since the proteins that were separated by SDS PAGE were isolated from splicing complexes, we infer that we are analyzing mostly splicing factors, however it is possible that proteins not associated with the spliceosomes migrate in that particular region of the native gel.

Using information provided by this assay we attempted to identify the potential substrates of Bur within the spliceosome. We used mobility as an indicator for the molecular mass of candidate Bur substrates and identified splicing factors of similar weight that have been shown to be phosphorylated using the *S. cerevisiae* genome database (Table 3.2). Remarkable we found that the likely candidates are mostly essential RNA helicases of the DExD/H family. Prp2 and Prp28 stand out as potential targets of the Bur1 kinase because they are involved in the catalytic activation of the spliceosome, a

Table 3.2: List of known phosphorylation events in B and A complexes

Complex	MW (Kda)	Candidate	known phosphorylation sites	Kinase consensus site	function in splicing
B/A	120	Prp16 (122KDa)	ELDS[109]PVQ	-	second catalytic step
A	120	Snu114 (114KDa)	NTQS[85]PQT SPQT[88]PLV	CKI MAPK	spliceosome activation
A	100	Prp2 (100KDa)	VAAT[284]SVA	-	spliceosome activation
B/A	90	Prp43 (88KDa)	RRFS[8]SEH RFSS[9] EHP VERS[18]IPE	cAPK - CKII	recycling
B/A	65	Prp28 (67KDa)	KVNS[69]RDD	GSK-3	U5 snRNP
B/A	60	Mud2 (61KDa)	RQKS[49]DGE	CKII	U2 snRNP
B/A	30	Cus2 (32KDa)	KAKS[163]EGE NEDS[278]EVE	CKII CKII	U2 snRNP

blocked assembly step in the Bur1 mutant, Bur1-T240A. Of course the phosphorylation events observed could be due to splicing factors not known to be phosphorylated in yeast, such as Cbp80 and Prp45 (Table 3.3), the homologs of which have been shown to interact with the Bur homolog in metazoans. A preliminary survey of known phosphorylated sites in splicing factors show at least a couple Bur1-like motifs such as S^{109} of Prp16, S^{85} of Snu114 and S^{18} of Prp43 (Table 3.1 vs. Table 3.2). Furthermore Bur1 was shown to phosphorylate serine 5 of the heptapeptide repeat of the CTD of Rbp1 *in vitro* which is part of a GSK-3 kinase motif, a motif also found in Prp28. While prediction of Bur dependent phosphorylation using kinase consensus site is at best indicative of potential substrates, this survey can be a powerful tool for choosing candidates for future directed analysis.

To validate our analysis we selected two potential targets from Tables 3.2 and 3.3. First we tested the Bur-dependent phosphorylation of Snu114, a factor involved in tri-snRNP addition, which contains a putative CKI site similar to the phosphorylation site contained within the heptapeptide repeat of Rpb1 (tables 3.2 and 3.1 respectively). We also tested phosphorylation of Hsh155, which has been shown to be dynamically regulated by phosphorylation in the mammalian spliceosome. While neither splicing factors show Bur-dependent phosphorylation, the Snu114-TAP co-purifies with a phosphorylated 100 KDa

Table 3.3: List of potential phosphorylation events in B and A complexes

Complex	MW (KDa)	Candidate	function in splicing
B	110	Hsh155	prespliceosome formation
A	104	Prp6	component of the tri-snRNP
A	100	Cbp80	prespliceosome formation and catalytic activation
B/A	43	Prp45	second catalytic step

protein (Figure 3.3). Remarkably, we observed a striking Bur-dependent phosphorylation event for a 100 KDa protein in the A complex in our spliceosomal phosphorylation survey (Figure 3.4). In large scale interaction studies Snu114 has been shown to interact with three splicing factors in the 100KDa range: Cbp80, Prp2 and Prp6 (Brenner and Guthrie 2005; Gavin, Aloy et al. 2006; Krogan, Cagney et al. 2006; Collins, Kemmeren et al. 2007; Lardelli, Thompson et al. 2010). Further experiments will be necessary to positively identify this 100 KDa potential Bur1 substrate.

These results indicate that multiple phosphorylation events occur within spliceosomes, and that splicing factors are differentially phosphorylated throughout the pre-mRNA splicing reaction. Furthermore the Bur1 kinase activity seems to be required for some (but not all) of the spliceosome's phosphorylation events. To further understand the role of the Bur1 kinase activity in pre-mRNA splicing regulation, we next tested a modular analog sensitive Bur1 kinase.

Dissecting the role of the Bur complex in splicing using an analog-sensitive Bur1 mutant and ATP analogs.

In order to elucidate the mechanism by which the Bur complex regulates splicing and to identify possible spliceosomal substrates of the Bur1

kinase, we have obtained an analogue sensitive (as) Bur1 mutant (Liu, Warfield et al. 2009). The *bur1-L149G* (*bur1-as*) has a mutation at the gatekeeper amino acid of the ATP binding pocket that allows the mutant kinase to accept analogs such as 3MB-PP1 (Figure 3.5) to partially inhibit the kinase activity (Gregan, Zhang et al. 2007; Liu, Warfield et al. 2009). These analogs share a similar backbone as an adenine molecule but include extra aromatic rings making them suitable for analog-sensitive kinases with enlarged ATP-binding pockets, while other kinases will not be able to bind to these bulkier analogs. Based on previous studies, the Bur1-as kinase activity can be decreased in the presence of 3MB-PP1 inhibitor, impairing the regulatory role of the Bur complex in transcription elongation (Liu, Warfield et al. 2009). Remarkably the presence of the analog inhibitor, used in a range of concentrations in previous studies, did not cause cell death indicating that some basal Bur1 activity still occurs since complete loss of kinase activity is lethal (Liu, Warfield et al. 2009).

The *bur1-as* mutant was tested both *in vivo* (Figure 3.6) in the presence of the 3MB-PP1 (3MB) analog. Using a U3 primer extension assay, we were unable to detect an effect on splicing *in vivo*. For this assay we grew cells in the presence of 6μM 3MB-PP1, a concentration sufficient to detect decreased *in vivo* phosphorylation of Spt5 (Liu, Warfield et al. 2009). Interestingly

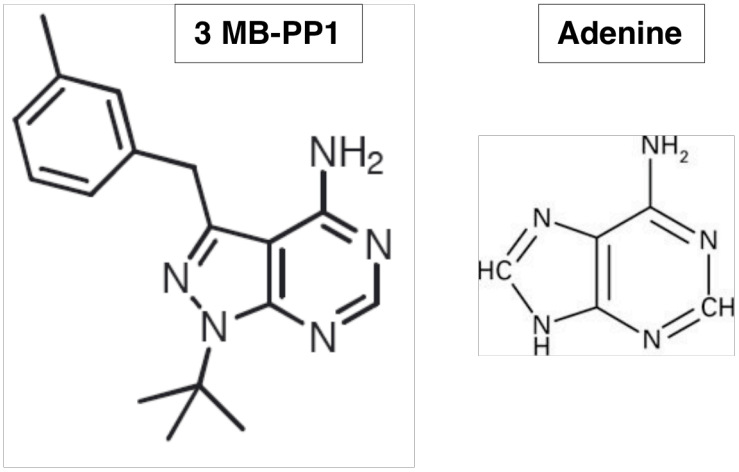


Figure 3.5. Structure of Bur1-as inhibitor 3-methylbenzyl-pyrazolopyrimidine. Structure of 3-methylbenzyl-pyrazolopyrimidine (3MB-PP1) compared to the structure of adenine.

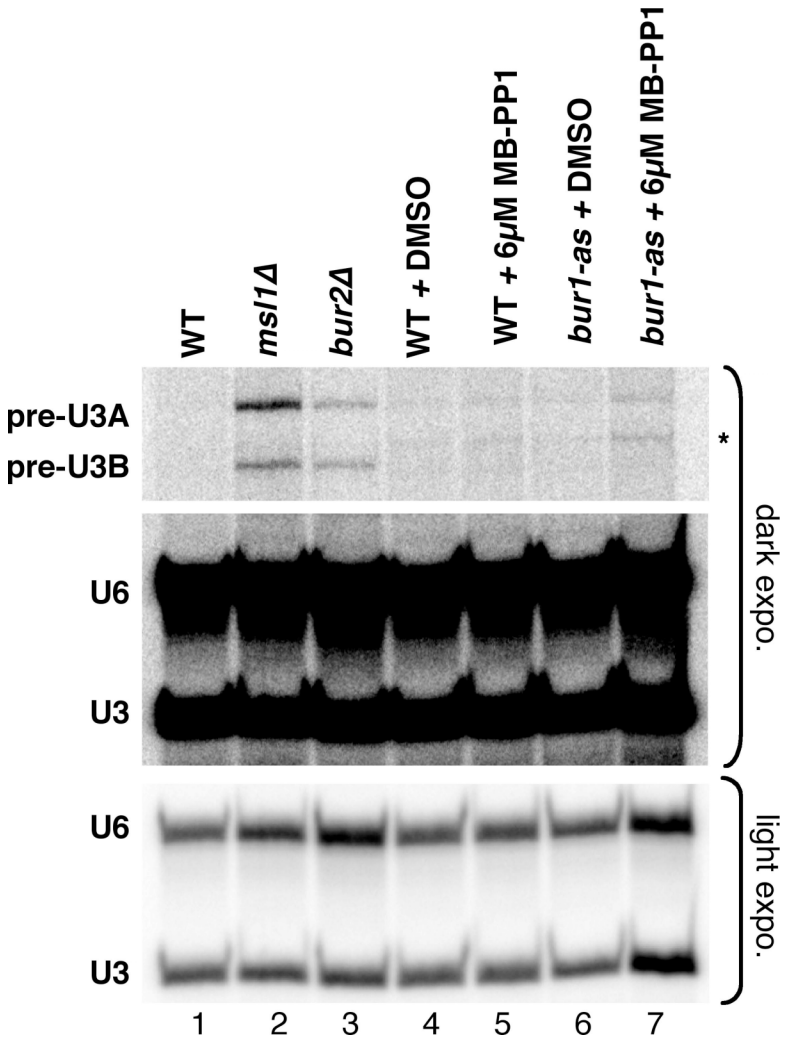


Figure 3.6. Directed inhibition of Bur1 kinase activity does not result in a *in vivo* splicing defect. Primer extension assay of U3A, U3B, and U6 RNA using total RNA extracted from WT, *msl1Δ*, *bur2Δ*, and *bur1-as*. Prior to RNA extraction, WT and *bur1-as* yeast were grown in the presence of 6μM 3MB-PP1 dissolved in DMSO, or DMSO alone as a control. Light exposure is shown to indicate loading levels using the U6 control. Asterisk (*) denotes an unknown product observed in samples treated with DMSO.

presence of DMSO (used as a solvent for the analogs) was sufficient to partially inhibit pre-mRNA splicing and caused formation of non-specific products possibly hindering our ability to resolve a subtle splicing defect in the analog sensitive cells. Using a *bur1-T240A* mutant we have previously shown that some pre-mRNA accumulates *in vivo* in the *BUR1* mutant strain, yet the splicing defect observed is subtle and only detectable with a highly sensitive assay (such as low cycle PCR analysis of *ACT1* in Chapter 2). Therefore we hypothesized that the U3 primer extension assay sensitivity limit and the observed non-specific effect of DMSO prevented detection of unspliced products, so we next tested the Bur1-as inhibition using an *in vitro* splicing assay.

Using *ACT1* radiolabeled pre-mRNA as a substrate, we compared *in vitro* splicing in the presence of increasing amounts of inhibitor in a WT extract and a *bur1-as* extract. We were surprised that no effect on *in vitro* splicing could be observed even at high levels of 3MB-PP1 (Figure 3.7 A). Considering that ATP was in twenty fold excess compared to the highest level of 3MB-PP1 (2mM and 0.1mM respectively), we reasoned that ATP was out-competing the Bur1 inhibitor for occupancy of the Bur1-as ATP-binding pocket. We therefore decided to repeat this experiment at low ATP concentrations.

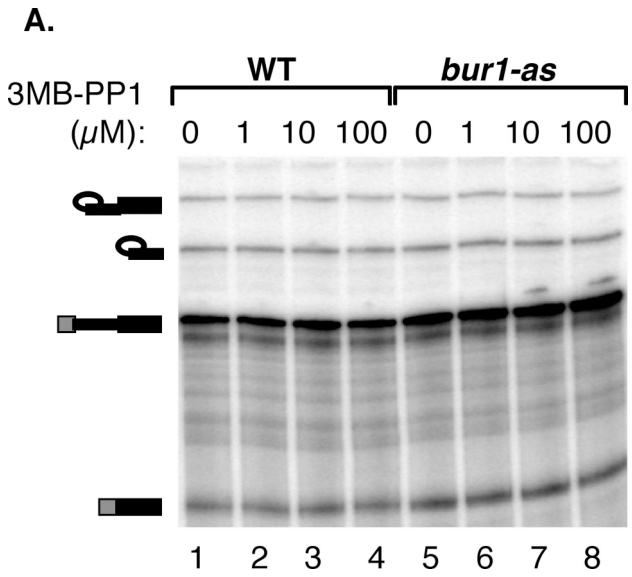


Figure 3.7. 3MB-PP1 affects the activity of Bur1-as at low ATP concentrations. **A.** *In vitro* splicing assay using radiolabeled *ACT1* pre-mRNA as a substrate shows splicing for WT (lanes 1-4) and *bur1-as* (lanes 5-8) extract in the presence of 2mM exogenous ATP and increasing amounts of 3MB-PP1. **B.** *In vitro* assay shows splicing for WT (lanes 1-8) and *bur1-as* (lanes 9-16) extract in the absence or presence of 100*μ*M exogenous ATP and increasing amounts of 3MB-PP1. **C.** *In vitro* assay shows splicing for WT (lanes 1-4, 9-12) and *bur1-as* (lanes 5-8, 13-16) extract in the absence or presence of 100*μ*M exogenous ATP and Bur1-as inhibitors (100*μ*M of 3MB-PP1, CZ31, and CZ42).

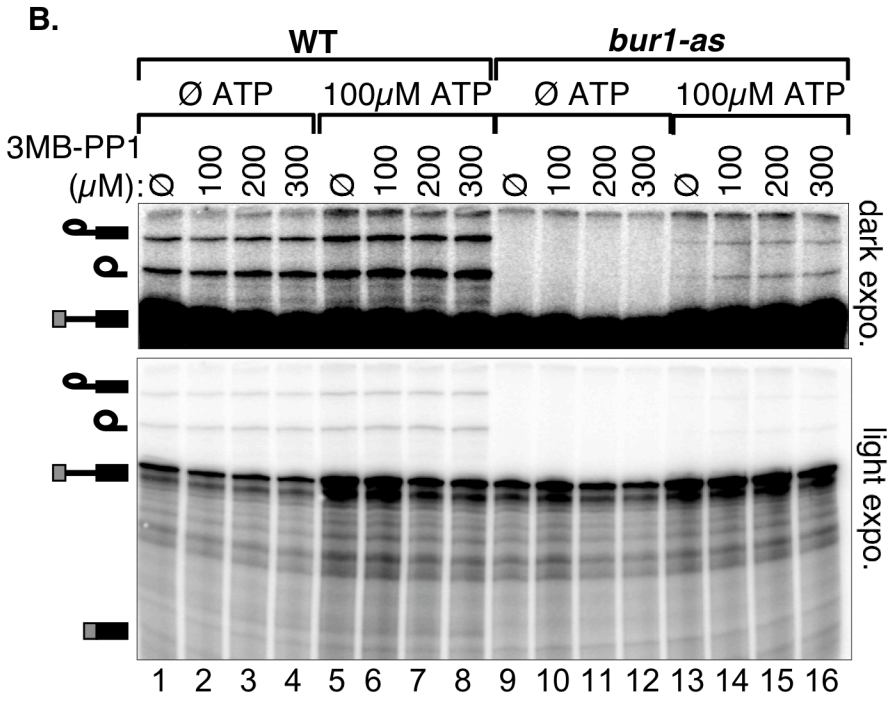


Figure 3.7. 3MB-PP1 affect the activity of Bur1-as at low ATP concentrations, continued.

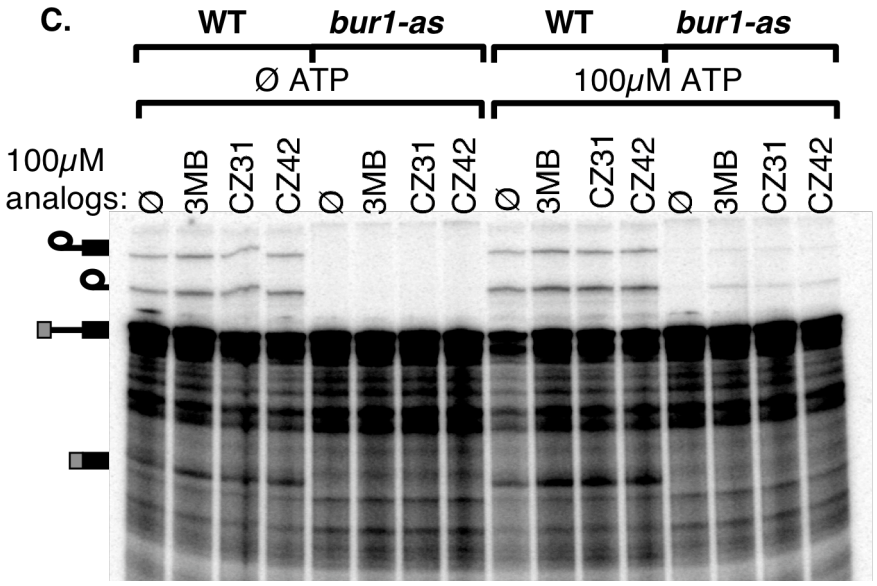


Figure 3.7. 3MB-PP1 affect the activity of Bur1-as at low ATP concentrations, continued.

We next assayed splicing at equivalent ATP and 3MB concentrations (Figure 3.7 B). Pre-mRNA splicing *in vitro* requires at least 500μM ATP for progression through spliceosome assembly (Lin, Newman et al. 1985), so we were surprised to see basal splicing activity in the WT extract which indicated presence of endogenous ATP in this extract (lanes 1-4). The addition of 100μM ATP to WT stimulated splicing (lanes 5-8), and the presence of increased amount of 3MB did not affect WT splicing at either ATP concentrations as expected (Lanes 1 vs. 2-4, and 5 vs. 6-8). In the absence of exogenous ATP the *bur1-as* extract did not show any splicing indicating a lower amount of endogenous ATP as compared to the WT extract (lanes 9-12). As expected addition of 100μM ATP to the mutant extract stimulated splicing, yet surprisingly addition of 3MB analog further stimulated splicing (lane 13 vs. 14-16). This analog specific stimulation did not increase proportionally to the increased amount of analog indicating that the stimulatory effect was due to only a fraction of the 3MB present in the reactions. To test if this stimulatory effect was cause specifically by the binding of Bur1-as to the analog, we assayed two other Bur1-as inhibitors: CZ31 and CZ42 (structure unknown, provided by Steven Hahn). Upon testing all analogs with both WT and *bur1-as* extract at low ATP concentrations we observed similar stimulatory effect for all three analogs when added to the analog-sensitive extract, while there was no noticeable effect (either positive or negative) upon the WT

extract (Figure 3.7 C). These results indicate that the analog sensitive Bur1 kinase, when bound to a non-hydrolysable ATP analog, can promote pre-mRNA splicing *in vitro*. In an attempt to test the effect of analogs upon WT at lower ATP concentration (similar to *bur1-as* ATP levels), we depleted the endogenous ATP in the WT extract using hexokinase and glucose (Figure 3.8). ATP depletion was achieved with moderate amount of hexokinase, however we were unable to reconstitute splicing activity with excess (2mM) exogenous ATP.

Recent work with analog-sensitive kinases has shown that binding to the analog does not always inhibit their activities. Indeed analog binding can also induce activation of some kinases through conformational changes within the kinase (Han, Lerner et al. 2009). At least one mechanism by which this has been shown to occur is through analog induced conformational changes that result in hyperphosphorylation of the activation site, threonine 240 in the case of Bur1 (Okuzumi, Fiedler et al. 2009). To test this possibility we looked at the phosphorylation status of Bur1-flag and Bur1-as-flag in extracts (Figure 3.9). As previously described, phosphorylated Bur1 on T240 migrates slower in SDS-PAGE analysis (Yao and Prelich 2002). To our surprise both Bur1-flag and Bur1-as-flag were found to be mostly activated in the extract, however while the some unphosphorylated Bur1-flag was detected, all the Bur1-as-flag

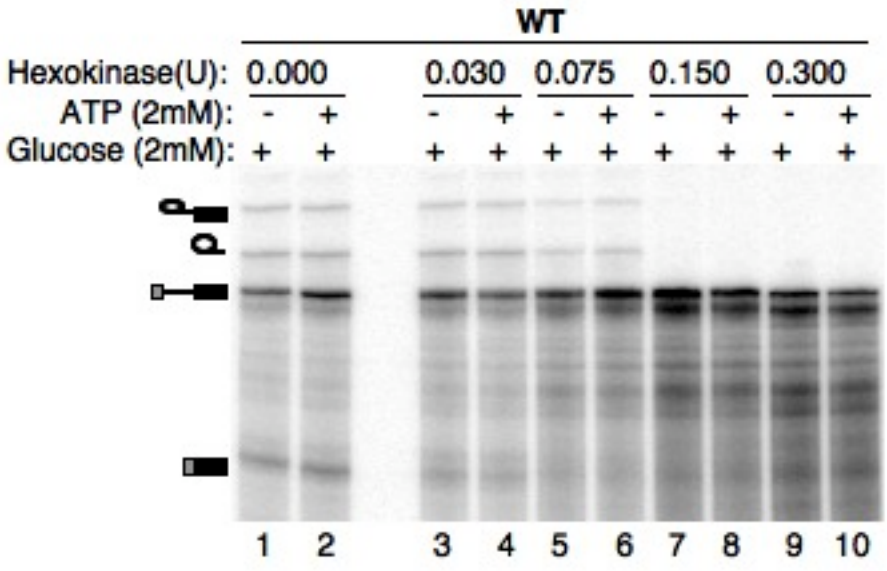


Figure 3.8. WT extract can be depleted of endogenous ATP, but splicing cannot be reconstituted with exogenous ATP. *In vitro* splicing is abrogated in a WT extract upon depletion of endogenous ATP using hexokinase (0.15U and above) and glucose (2mM). Addition of 2mM ATP following depletion reaction did not reconstitute splicing (lanes 8 and 10).

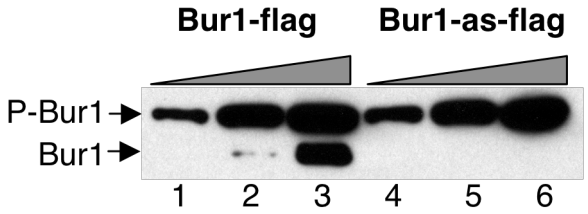


Figure 3.9. A majority of Bur1 kinase is phosphorylated in splicing extracts. Splicing extracts containing Bur1-flag or Bur1-as-flag were analyzed on 7.5% SDS PAGE and immunoprobed using anti-flag antibodies. Increasing amount of splicing extract were loaded (1-3*μ*l) for *BUR1-FLAG* (lanes 1-3) and *bur1-as-FLAG* (lanes 4-6). Lower mobility band is labeled “Bur1”, while the higher mobility band is hypothesized to be phosphorylated Bur1 and is labeled “P-Bur1”.

detected was phosphorylated. This preliminary results suggest that in splicing extracts both Bur1 kinases exist predominantly in a phosphorylated form, and surprisingly the AS kinase did not appear to be unphosphorylated in our extracts. We hypothesize that splicing extracts, which are a form of nuclear extract, contain an enriched population of phosphorylated and activated Bur1 kinase, presumably because activated Bur1 is involved in pre-mRNA splicing regulation.

In order to investigate the role of this activated Bur1-kinase we tested complex formation at low ATP concentrations in the presence of 3MB analog (Figure 3.10). Consistent with our data, the Bur1-as extract stimulates splicing when exposed to 3MB and ATP (lanes 8-9 vs. 11-12). Unfortunately this stimulatory effect was not clearly noticeable when we ran a native gel to assay for spliceosome formation over time (Figure 3.10 lower panel). Further experiments will be necessary to elucidate the effects of the analogs upon spliceosome formation

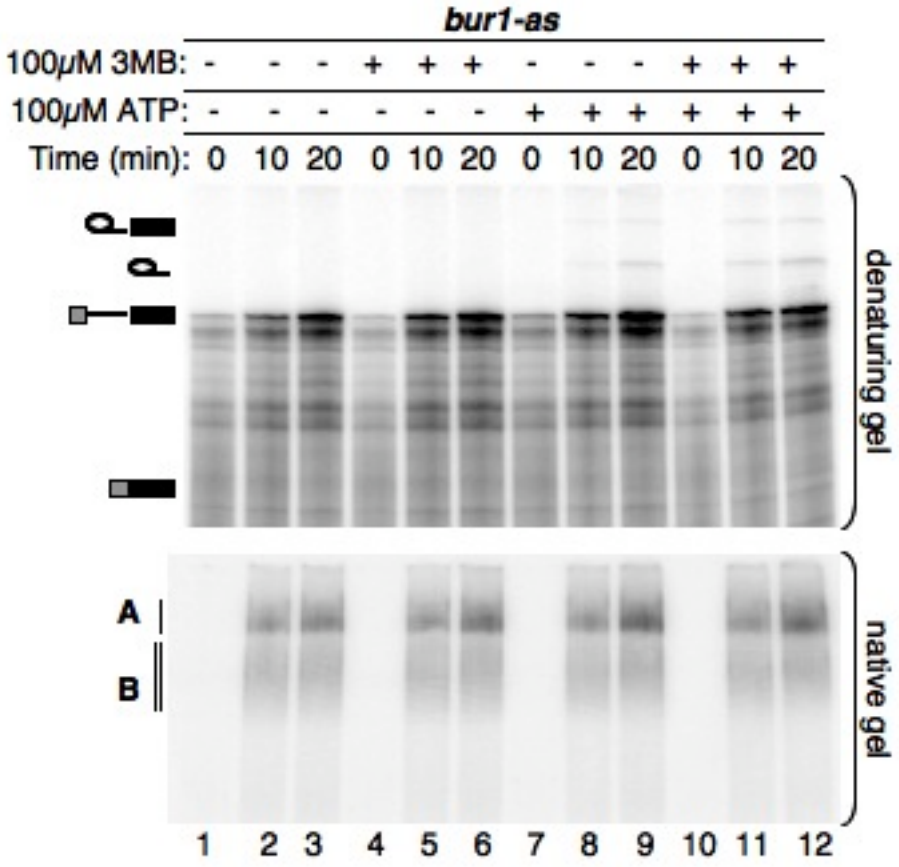


Figure 3.10. Effects of 3MB-PP1 on complex are subtle. Time course *In vitro* splicing assay showing splicing (top panel) and spliceosome assembly (bottom panel) for a *bur1-as* extract in the absence or presence of 100μM ATP and μM 3MB-PP1.

Section 3.3: Discussion

In this study we investigate the role and function of Bur1 dependent phosphorylation activity. First we look at potential substrates, the U2 snRNP factor Cus2 and the U1 snRNP complex CBC. We first analyzed the Tat-SF1 homolog, Cus2, and we found that the Bur complex can phosphorylate the C-terminal half of Cus2 efficiently *in vitro*. While we show that Cus2 was also phosphorylated in extract, this phosphorylation could occur in a Bur2 independent manner. This data shows that in splicing extracts, an unidentified kinase is also able to phosphorylate Cus2. Cus2 contains at least two potential phosphorylation sites (Table 3.2), so it is possible that Bur and the unknown kinase phosphorylate Cus2 at different sites, possibly differentially regulating Cus2's activity. Furthermore one of the known *in vivo* substrate of the Bur1 kinase, Rpb1, is phosphorylated by multiple cyclin dependent kinases upon the same residues, yet these phosphorylation events have different regulatory functions throughout transcription. We therefore cannot exclude the possibility that Cus2 serves as an *in vivo* substrate of Bur, yet Cus2's role in splicing regulation alone does not explain the phenotypes we have observed previously with Bur1/2 mutants. Therefore, we next tested the phosphorylation of another candidate Bur substrate, Cbp80. Despite evidence of direct interaction in mammalian (Lenasi, Peterlin et al. 2011) and yeast (unpublished

data from our laboratory) cells between the Bur complex and CBC, we did not observe *in vitro* Bur1 kinase activity upon Cbp80. Future experiments will aim to assay CBC phosphorylation status in splicing extracts, a molecular environment more closely related to *in vivo* conditions.

We next decided to survey the phosphorylation events within spliceosomes, and to test whether some phosphorylations were dependent upon functional Bur complex. Remarkably we found that phosphorylation of splicing factors is prevalent and in both prespliceosomes as well as in pre-catalytic and catalytic spliceosomes. Furthermore some phosphoproteins were only observed at specific stages of spliceosome assembly suggesting that phosphorylation may play an important role in directing rearrangements of the spliceosome. Analysis of Bur-dependent protein phosphorylation suggest it is important for particular phosphorylation events in both B and A complexes. Remarkably most of these Bur-dependent phosphorylations coincided with essential spliceosomal RNA helicases that have been shown in large scale studies to be phosphorylated. While many candidates stand out, a 100 KDa protein (possibly Prp2 or Prp6) and 65 KDa protein (possibly Prp28) are interesting potential substrates of Bur because of their involvement in the catalytic activation of the spliceosome. This study will be a powerful tool for planning further analysis of Bur substrates (described bellow).

Finally to better understand the role of Bur's kinase activity in regulating pre-mRNA splicing we used an analog sensitive Bur1 kinase mutant. Addition of inhibitor failed to block splicing suggesting that pre-mRNA splicing regulation only requires low kinase activity from the Bur complex. Interestingly, in the presence of the analog, we uncovered an unexpected stimulatory role for Bur1-as in promoting pre-catalytic spliceosome formation, and in stimulating splicing under low ATP conditions. We speculated that these results could be due to high levels of conformational activation of Bur1-as when bound to the inhibitor similar to results observed in a number of mammalian studies. For example, studies with the analog sensitive Akt kinase showed that in the presence of the analog, the kinase is hyperphosphorylated on its T-loop domain, which results in hyperactive kinase activity when the inhibitor was removed form the reaction (Okuzumi, Fiedler et al. 2009). The Bur1 kinase is activated by phosphorylation of threonine 240 in the kinase T-loop domain by Cak1 (Yao and Prelich 2002). Our preliminary results indicate that the majority of Bur1 in extract is phosphorylated. While we cannot explain why such a high fraction of Bur1 kinase is phosphorylated in our extracts we reasoned that the splicing competent extracts contain a majority of the spliceosomal associated Bur complex, most of which is activated to promote various steps in spliceosome assembly.

These data depict an interesting model, consistent with our previous data, whereby the Bur complex activation (possibly through conformational changes involving T-loop phosphorylation) promotes spliceosome assembly. Furthermore the Bur1 kinase seems to play an important role in the phosphorylation of the prespliceosomes as well as the pre-catalytic and catalytic spliceosomes. To test this model, future experiment will involve an in-depth analysis of potential substrate phosphorylation status *in vivo*. For substrates, such as Cus2, that can be phosphorylated by multiple kinases, identification of the secondary kinases might be important to fully understand the regulation of the substrate. This identification could be achieved through purification of the substrate proteins from both whole cell extracts and splicing extracts, followed by mass spectroscopy analysis. Furthermore the candidates listed in tables 3.2 and 3.3 shall be tested, using *in vitro* and in extracts kinase assay (similar to experiments shown in Figure 3.3 and Figure 3.2 respectively), for potential Bur dependent phosphorylation events. In particular this study will assay Prp45, whose mammalian homolog, SKIP, is known to associate with the homolog of the Bur complex, P-TEFb. Once candidates have been confirmed as substrates for the Bur1 kinase, they will be subjected to mutational analysis using both *in vivo* and *in vitro* splicing assays to understand the regulatory role of each Bur-dependent phosphorylation event.

Further investigation will also be necessary for uncovering the phosphorylation-independent role for the Bur complex in spliceosome assembly. First, *in vitro* kinase assays with Bur1-as and its activator kinase Cak1, with or without 3MB, will be necessary to understand if binding of Bur1-as to the analog leads to the hyperphosphorylation of the T-loop. In addition to these experiments, future studies will focus on identifying interactions between the spliceosome and conformationaly active Bur1 (possibly Bur1-as bound to 3MB) as well as conformationaly inactive Bur1 (Bur1-T240A). If successful, these studies will paint an accurate model for the role of the Bur cyclin dependent kinase complex in regulating multiple steps during spliceosome assembly.

Chapter 4: Discussion

Section 4.1: Summary of data

The work described in this dissertation highlights the role for a cyclin dependent kinase complex in regulating spliceosome assembly. We have shown that deletion of the regulatory subunit of the Bur complex, the cyclin *BUR2*, leads to a pre-mRNA accumulation *in vivo*. While the Bur complex is involved in transcriptional regulation, the splicing defect observed in Bur mutants, *bur1-T240A* and *bur2Δ*, persist in an *in vitro* assay where splicing and transcription are uncoupled. Furthermore, splicing complexes that form in the presence of Bur1 mutants or Bur2 deleted extracts show dramatic blocks in early spliceosome assembly, indicating a role for the CDK complex in regulating the addition and/or rearrangements of the snRNPs. Interaction studies in native gels showed that the Bur complex migrates with the prespliceosome, and co-immunoprecipitation demonstrates a strong interaction between the Bur1 kinase and the U1 snRNA.

Since the Bur complex possesses kinase activity, we undertook a search of potential substrates of this activity within the spliceosomes. First we investigated the phosphorylation status of early and late spliceosomes with the

presence of normal and mutant Bur complex. These studies reveal that there are numerous phosphorylated proteins that comigrate with B and A complexes, presumably as components of these complexes, some of which could be Bur mediated. Directed analysis of the U2 snRNP factor Cus2 showed that while the protein can be phosphorylated by the Bur complex, this phosphorylation was not unique to the CDK complex. Interestingly, a 100 KDa phosphoprotein present in the A complex appears to be a substrate of the Bur1 kinase activity. To further elucidate the mechanism by which the Bur1 kinase effects splicing, an analog sensitive Bur1 kinase was utilized. Surprisingly these studies revealed that analog binding to Bur1-as may alter the protein's conformation such that it is increasingly susceptible to phosphorylation and more importantly may adopt a conformation that stimulates splicing. While we were unable to confirm that complete inhibition of Bur1 activity (sufficient to eliminate splicing) was achievable, it is apparent from this work that Bur1 adopts a conformation that is important for splicing, corroborating the results we observed with Bur1-T240A.

These data reveal a previously uncharacterized role for a CDK in spliceosome assembly possibly through effects on snRNP rearrangements and phosphorylation of splicing factors. This is the first reported phosphorylation event in the yeast spliceosome. Furthermore, and possibly

most importantly, this body of work hints at a possible coupling mechanism between the transcription and splicing machinery.

Section 4.2: Phosphorylation of the spliceosomes by the Bur complex

Our results show that activation of Bur1 kinase activity both through binding of the cyclin Bur2 and through T-loop phosphorylation is necessary for proper spliceosome assembly. Furthermore we have shown that the Bur complex can phosphorylate known splicing factors. Remarkably, many interesting spliceosome phosphorylation events appear to be Bur1-dependent. Potential candidate substrates of Bur include essential splicing factors such as Prp28, which has been shown to be phosphorylated in metazoans (Mathew, Hartmuth et al. 2008). These data indicate that kinase activity of the Bur complex plays a major role in activation of the spliceosome.

While there are several examples of post translational modifications in mammalian spliceosomes, in the budding yeast only a few examples have been reported (reviewed in (McKay and Johnson 2010)). Remarkably there have been no specific kinases identified that affect splicing in budding yeast. In the mammalian spliceosome, SR protein kinases are known to phosphorylate spliceosomal proteins to facilitate dynamic rearrangements of

the spliceosome (Mathew, Hartmuth et al. 2008; Cho, Hoang et al. 2011). Our studies have now uncovered a similar regulatory mechanism in *Saccharomyces cerevisiae*.

Section 4.3: The role for the Bur complex in splicing provides an opportunity for stress-dependent splicing regulation

In this dissertation I show that the Bur1/2 CDK complex affects pre-mRNA splicing independently from its role in transcription. Notably these conclusions are based upon strains deleted of *BUR2* or in which a mutation of *BUR1* prevents its activity. It is therefore tempting to speculate that proper activity of the Bur complex is necessary for progression through pre-mRNA splicing, and that by modulating Bur's activity in response to stress, budding yeast may be able to affect spliceosome assembly. Indeed, when exposed to environmental stresses, budding yeast have been shown to rapidly shut-off pre-mRNA splicing (Pleiss, Whitworth et al. 2007).

The Bur1/2 complex is not a cell cycle CDK and the levels of Bur2 are relatively unchanged through the cell cycle (Yao, Neiman et al. 2000). However, the levels of the Bur2 cyclin do change when the cells are exposed to stress (Gasch, Spellman et al. 2000). A downregulation of Bur2 levels

would lead to a decrease in prespliceosome formation, similarly to the splicing defects observed in *bur2Δ* cells. Blocking spliceosome formation is all the more important in cells experiencing a decrease in Bur's activity, since unproductive elongation would ultimately lead to abortive transcription. *In vivo*, pre-mRNA splicing occurs in the context of transcription. Thus Bur-dependent splicing may serve as a mechanism by which splicing is coordinated with transcription in response to stresses present in the cell's environment.

Since the Bur complex can affect splicing independently of its role in transcription, we would expect that expression of *BUR1/2* genes would mirror the expression of early acting splicing factors, especially when the cells are exposed to stresses. It is therefore not surprising that in the same data set for expression patterns in response to stress, expression of the *BUR2* cyclin was closely clustered with U1 snRNP factors *PRP39*, *MUD2*, and *CBP80* (Gasch, Spellman et al. 2000).

Section 4.4: Coupling transcription, chromatin modification, and splicing

The Bur complex has been shown to affect both the transcription machinery and the chromatin modifying factors. Phosphorylation of the CTD of RNA pol II at serine 2 of the heptapeptide repeats by Bur1 is important for

promoter clearance (Qiu, Hu et al. 2009). The Bur complex also phosphorylates Rad6 to promote Histone 2B (H2B) mono-ubiquitination (Wood, Schneider et al. 2005), and the CTD of Spt5, which recruits the PAF elongation complex (Liu, Warfield et al. 2009; Zhou, Kuo et al. 2009). This results in the recruitment of histone methylases which, along with other elongation factors, promote an open state of the chromatin to facilitate transcription (reviewed in (Krebs 2007)). Interestingly these interactions appear to be conserved as the fission yeast homolog of Bur has been implicated in both chromatin modification (Guiguen, Soutourina et al. 2007) and in the recruitment of the RNA capping enzymes (Pei, Schwer et al. 2003), while the mammalian homolog (P-TEFb), which is known for its interaction with chromatin remodeling factors (Yang, Yik et al. 2005), was recently shown to interact directly with the cap binding complex (Lenasi, Peterlin et al. 2011). Taken together these studies provide evidence for a spectacular array of interactions between transcription and chromatin modifying factors, capping enzymes, and splicing factors perhaps to enhance and dynamically couple these processes. Indeed the Bur complex appears to play a key role in mediating and assuring productive transcription elongation while promoting co-transcriptional spliceosome recruitment and assembly upon the nascent pre-mRNA.

Another interesting co-transcriptional spliceosome assembly network emerges from recent studies with the SAGA complex. The acetyl-transferase subunit of the SAGA complex, Gcn5, was shown to be important for the co-transcriptional recruitment of the U2 snRNP (Gunderson and Johnson 2009). Interestingly the Bur complex activity upon the Rad6 ubiquitin-conjugating enzyme is helpful for the efficient recruitment of SAGA through ubiquitination of histone 2B (Daniel, Torok et al. 2004). This provides an interesting model whereby the Bur complex promotes the recruitment of the U1 snRNP to the nascent pre-mRNA as well as the facilitating the recruitment of SAGA, which in turn can help recruit the U2 snRNP co-transcriptionally. More work will be necessary for uncovering the mechanisms and direct interactions which mediate co-transcriptional spliceosome assembly.

Section 4.5: Future directions

In order to better understand the regulation of spliceosome assembly by the Bur complex it will be necessary to uncover all the substrates of the Bur1 kinase within the spliceosome. One possible approach is to use analog sensitive Bur1 kinase. Using liquid chromatography coupled with mass spectroscopy (LC-MS) we can investigate the phosphorylation pattern of fractionated spliceosome from splicing extracts. The Bur1-as kinase is able to

accept ATP-analogs which differ substantially in atomic mass from normal ATP (Liu, Warfield et al. 2009). We can then compare spliceosome phosphorylation when the splicing extracts have been exposed to ATP alone, or a mix of ATP and ATP-analog. Using this approach we may be able to identify splicing factors that are phosphorylated by the Bur1-as kinase through a small differential in the mass of these factors when exposed to the ATP-analog.

Another advantage of such method is the ability of LC-MS analysis to pinpoint the likely phosphorylated amino acids within each Bur substrate. Using this information future work should focus on engineering phosphomimetic and unphosphorylatable mutants of Bur substrates. These new mutant strains would then be analyzed genetically for viability and growth phenotypes, and biochemically for their effect on pre-mRNA splicing regulation and spliceosome assembly. Using a combination of these mutants we should be able to mimic the splicing phenotypes observed with the *BUR1/2* mutants. Accumulated data from this mutational analysis will reveal a regulatory model to explain the role of Bur mediated phosphorylation in mediating spliceosome assembly. Furthermore these splicing phosphomutants will be important for characterizing the role of phosphorylation in facilitating co-transcriptional spliceosome assembly.

In this dissertation I discuss a model in which the Bur complex, through its kinase activity as well as through physical interactions, facilitates the recruitment of the U1 snRNP and promotes the catalytic activation of the spliceosome (Chapter 2). Using specific phosphomutants of Bur substrates it would be very interesting to investigate the co-transcriptional spliceosome recruitment by chromatin immunoprecipitation (ChIP). For instance if a particular phosphorylation event leads to the recruitment of the U1 snRNP to the 5' SS, then a phosphomimetic mutant should show increase recruitment of the U1 snRNP even in absence of Bur activity, while an unphosphorylatable mutant would either display reduced or abrogated recruitment of the U1 snRNP. This analysis would provide a better understanding for the timing of regulatory phosphorylation events that are involved in co-transcriptional spliceosome assembly.

Section 4.6: Implication for human diseases

The experiments described above would substantially advance our understanding of dynamic gene expression regulation. Furthermore, these studies may enable us to create directed therapies to treat diseases caused by missregulated expression.

Defects in pre-mRNA splicing are known to cause a variety of diseases in humans (reviewed in (Cooper, Wan et al. 2009)). Some have even speculated that half of the disease-causing mutations may affect some step of splicing regulation (Lopez-Bigas, Audit et al. 2005). However most of these diseases are caused by mutations affecting the sequence of DNA thus altering splice site or enhancer sequences. There have been few examples of diseases caused by missregulated spliceosome assembly. One instance of faulty spliceosome activity has been described in retinitis pigmentosa patients where a mutated tri-snRNP leads to reduced splicing efficiency of specific pre-mRNAs (Mordes, Luo et al. 2006; Mordes, Yuan et al. 2007). While it has been hypothesized that cancers may harbor defects in the splicing machinery, the molecular mechanisms behind such defects have been difficult to identify (Cooper, Wan et al. 2009). Such observations highlight the importance of discovering and mapping the regulatory networks regulating splicing in the context of gene expression.

The work presented in this dissertation along with the proposed future experiments could potentially reveal a new function for the well studied human P-TEFb complex. While P-TEFb activity is essential for proper gene expression and development in multiple tissues, missregulation of this activity leads to severe disease (reviewed in (Kohoutek 2009)). Indeed recent studies

have demonstrated that P-TEFb activity was enhanced in many human cancers, and surprisingly activation of P-TEFb is sufficient to promote tumorigenesis in certain forms of breast cancer (He, Jahchan et al. 2008). Cardiac hypertrophy, a disease characterized by overgrown cardiac cells, is caused in large part by P-TEFb missregulation which leads to increased transcription levels (Sano, Abdellatif et al. 2002; Huang, Wagner et al. 2004). Finally the P-TEFb complex has been heavily studied because of its prominent role in the transcription of HIV-1 (reviewed in (Wang, Liu et al. 2009)). The search for therapies resulting from such studies has led to the development of a myriad of CDK9 inhibitors from Flavopiridol (Chao, Fujinaga et al. 2000; Chao and Price 2001), to the recently characterized anti-angiogenesis CAN508 inhibitor (Krystof, Rarova et al. 2011). With the more prevalent use of CDK9 inhibitors, a complete understanding of the role of P-TEFb in cells has become paramount. Since the Bur complex and the P-TEFb complex display conserved activities, it is likely that the mammalian complex may also play a role in regulating splicing. Thus a complete characterization of the interactions between P-TEFb and the spliceosome, made easier by studies such as those described here, will be essential to create future treatments for cancer, HIV replication and cardiac hypertrophy.

Chapter 5: Materials and methods

Yeast strains and DNA constructs

A list of budding yeast strains used in this study is shown in table 5.1. All *S.cerevisiae* strains are isogenic to BY4743 (*his3Δ leu2Δ ura3Δ*) from Open Biosystems. A list of plasmid DNA constructs is shown in table 5.2. Standard LiAc transformation method was used for plasmid transformation, followed by selective media plating for plasmid selection. Plasmid shuffling was done on 5-fluoroorotic acid.

Standard protocols were used for yeast growth, matting, sporulation tetrad dissection and transformation (Burke, Dawson et al. 2000).

Primer extension analysis

Total RNA was isolated using hot-phenol extraction method. Primer extension analysis was performed as previously described for the U3 genes, with a U6 RNA loading control as opposed to a U1 control (Stevens and Abelson 2002). The same technique was employed for snRNA analysis following IP using U1, U2, U4, U5 and U6 snRNAs specific primers. A list of primers used in this study is shown in table 5.3.

ACT1 cDNA and PCR analysis

Generation of *ACT1* total cDNA was done using Maxima First Strand cDNA kit (Fermentas). Full transcript *ACT1* cDNA was synthesized using a gene specific primer and SuperScript II kit (Invitrogen). cDNA sets were analyzed by PCR, 19 cycles, with Phire Hot Start II DNA polymerase (Finnzymes) according to manufacturer protocol. PCR products were run on a 8% polyacrylamide 1xTBE gel, and post stained for 25min in 1xTBE buffer with SYBR-green-I dye (Lonza). Gels were scanned and visualized on Typhoon 9400 scanner and quantitated with Image Quant 5.2 software. A list of primers used in this study is shown in table 5.3.

Splicing extracts preparation and *in vitro* splicing and spliceosome assembly reactions

Splicing extracts were prepared using the liquid nitrogen method (Stevens and Abelson 2002). *In vitro* splicing reactions and spliceosome assembly assay were conducted as previously described (Stevens and Abelson 2002). For micrococcal nuclease (MN) digestion 16μl of splicing extract were incubated with 300U of micrococcal nuclease in the presence of 1mM $CaCl_2$. The MN reaction was stopped with the addition of EGTA (2.5mM final concentration). For reconstitution of splicing and spliceosome assembly the non-treated extract and MN-treated extracts were combined at a 1:3 ratio

(Figure 2.4) and at 1:1 ratio (Figure 2.5). Unless otherwise indicated *in vitro* splicing reactions were incubated for 30 min at room temperature.

RNA immunoprecipitation

Splicing reactions were immunoprecipitated using pre-washed and blocked anti-HA conjugated beads (Thermo Scientific). For each IP, 10μl of bead slurry was blocked with 100μg BSA and 1μg sheared salmon sperm DNA in binding buffer (20mM KCl, 20mM HEPES, 0.2mM EDTA, 2.5% glycerol, 0.5mM DTT). IP were placed on nutator for one hour at 4˚C. Beads were vigorously washed twice with wash buffer (500mM or 700mM KCl, and 20mM HEPES, 0.2mM EDTA, 2.5% glycerol, 0.5mM DTT). RNA was eluted from beads after 1hr incubation with proteinase K (NEB) at 37˚C. RNA was then purified using acid phenol and precipitated in ethanol.

Immunoprecipitation

Splicing reactions were immunoprecipitated using anti-HA conjugated beads (Thermo Scientific or anti-HA antibody from Cell Signaling Tech) and/or IgG beads (for TAP purification, Amersham). For each IP, 100μl beads were pre-washed in binding buffer (20mM KCl, 20mM HEPES, 0.2mM EDTA, 2.5% glycerol, 0.5mM DTT). The beads were incubated with 15μl of splicing extract in a total volume of 500μl with binding buffer. For reactions containing anti-HA

antibody 10 μl was used. Finally the beads were washed 3 times with binding buffer. Proteins were eluted using SDS loading dye and incubation at 95˚C.

Western analysis

Electrophoretic transfer of native gels to PVDF membrane was done using the BIO-RAD Transblot SD semi-dry cell according to manufacturer's instructions. PVDF membrane was blocked using milk-TBS buffer and probed using IgG-HRP conjugated antibody in TBS buffer. Membranes were washed three times with TBS+0.01%Tween.

For SDS-PAGE gels, transfer was done using Bio-Rad wet transfer apparatus. PVDF membrane was blocked using milk-TBS buffer and probed using IgG-HRP conjugated antibody in TBS buffer. Membranes were washed three times with TBS+0.01%Tween.

Kinase assay

For kinase assay in splicing extracts, 10μl of extract was incubated and 1μl of gamma [^{32}P] ATP for 30 min at 30˚C. For rCus2 assay, 2μg recombinant protein was used per reaction. Tagged proteins were then IP with specific beads, and eluted in SDS loading dye then incubated shortly at 95˚C before loading the gel.

For in vitro kinase assay, IP proteins of interests were incubated in equal proportion (usually 15μl bead slurry) with 1μl of gamma [^{32}P] ATP for 30 min at 30˚C. The beads were then washed three times using binding buffer. Proteins were eluted in SDS loading dye then incubated shortly at 95˚C before loading the gel.

Recombinant protein

Recombinant Cus2-6HIS was expressed from a pET24b vector. BL21-DE3 cells (Stratagene) expressing the vector were grown in LB+Kan until an OD_{600} of 0.5 and then induced with 1mM IPTG. Cell pellets were frozen and then sonicated according to manufacturer specification. Recombinant protein was purified on Ni-NTA columns according to manufacturer specification (Qiagen).

Table 5.1. Strains used in this study

Name	Phenotype	Source
TJY1700	*WT*	Open Biosystems
TJY1987	*bur2Δ::KanMX4 [URA3 BUR2]*	Open Biosystems
TJY2840	*bur1Δ::KanMX4 [URA3 BUR1][LEU2 bur1-T240A-HA]*	Open Biosystems
TJY0396	*msl1Δ::KanMX4*	Open Biosystems
TJY0462	*lea1Δ::KanMX4*	Open Biosystems
TJY1652	*BUR1-TAP:HIS3*	Open Biosystems
TJY1653	*BUR2-TAP:HIS3*	Open Biosystems
TJY2176	*WT [LEU2 BUR1-HA]*	Open Biosystems
TJY2179	*WT [LEU2 bur1-T240A-HA]*	Open Biosystems
TJY0124	*cus2Δ::KanMX4*	Open Biosystems
TJY2024	*bur2Δ::KanMX4 cus2Δ::KanMX4 [URA3 BUR2]*	This study
TJY3564	*bur1-L149G-Flag::KanMX4*	(Liu et al. 2009)
TJY4317	*spt5Δ::KanMX4 [HIS3 spt5ΔC-15]*	(Liu et al. 2009)

Table 5.2. Plasmids used in this study

Name	Backbone	Reference
BUR1-HA	pRS315	(Keogh et al., 2003)
bur1-T240A-HA	pRS315	(Keogh et al., 2003)
BUR1	pRS316	(Keogh et al., 2003)
BUR2	pRS316	This study
spt5ΔC-15	pRS313	(Liu et al., 2009)
rCUS2	pET24b	Ares lab.

Table 5.3. Primers used in this study

Name	Sequence (5' to 3')
ACT1-RT	CAG TGC TTA AAC ACG TCT TTT CC
ACT1-In-F	TCG ATT GCT TCA TTC TTT TTG TTG C
ACT1-Ex-F	TTG GTT ATT GAT AAC GGT TCT GG
ACT1-Ex-R	CTT GGT GTC TTG GTC TAC CG
U1 snRNA	GAA TGG AAA CGT CAG CAA ACA C
U2 snRNA	GGT GCC AAA AAA TGT GTA TTG
U4 snRNA	ACC ATG AGG AGA CGG TCT GG
U5 snRNA	TTC GTT ATA AGT TCT ATA GGC
U6 snRNA	AGG GGA ACT GCT GAT C
U3 snoRNA	CCA AGT TGG ATT CAG TGG CTC

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