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Genetic and biochemical analysis of the role of the DEAD-box protein sub2 in pre-mRNA splicing in Saccharomyces cerevisiae

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Genetic And Biochemical Analysis Of The Role Of The DEAD-Box Protein Sub2 In Pre-mRNA Splicing In *Saccharomyces cerevisiae*

by

Amy L. Kistler

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DEDICATION

FOR DAVID

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I would not have been able to carry out the work in this thesis without the generous help and support of numerous thoughtful people at UCSF and in my personal life. It is with a great deal of gratitude and happiness that I take this time to acknowledge them and their contributions towards the successful completion of my dissertation.

UCSF has been a wonderful place and community to carry out my graduate studies. I first became interested in coming to UCSF after one of my favorite undergraduate professors wistfully referred to UCSF as "Nirvana-land" having recently returned from a visit to the Department of Biochemistry. After being here for several years, I can personally attest to this evaluation. UCSF is a place where hard, high quality science is performed in an amazingly friendly, open, and collegial atmosphere. There is a standard of community and generosity among the students, postdocs, faculty, and administrative staff that greatly facilitates learning and scientific progress. I have learned so much more than I had ever imagined I would during graduate school. I am grateful for the existence of this graduate program and the dedication of those involved in it who somehow managed to create an incredibly challenging and stimulating, and friendly environment that accommodates so many different people and styles of advanced thinking. It has truly been a privilege to be a part of this institution.

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there were many days where clarity would not be the word I would use to describe my research progress over the years!). During these meetings, Christine was always able to take a step back and objectively help define the key issues that needed to be addressed, or pinpoint the experimental test required to validate or disprove a particular model we might be tossing around. This skill combined with her tenacious curiosity and endurance for intellectual pursuits was really a marvel to witness and be a part of as a graduate student. Equally impressive was to also experience and see these same skills applied to help students and others who knocked on her door, through very difficult personal times. As one of those people, I am greatly impressed with her patience, dedication, and faith in me during not only the good times, but some less than stellar moments. It is truly difficult to find words to express my gratitude for the life experience and knowledge of so many different aspects of being a scientist that she has shared with me.

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Genetic And Biochemical Analysis Of The Role Of The DEAD-Box Protein Sub2 In Pre-mRNA Splicing In *Saccharomyces cerevisiae*

Amy L. Kistler

ABSTRACT

Accurate removal of introns during pre-mRNA splicing is essential. The splice sites within a transcript are recognized and removed in a two-step trans-esterification reaction by the spliceosome. The spliceosome is a dynamic ribonucleoprotein complex that assembles de novo on each splicing substrate in an ordered series of ATP-dependent interactions between the spliceosome and the pre-mRNA substrate. Through this process, multiple recognition events occur at the intron splice sites, both before and during catalysis. Spliceosomal ATPases have been implicated to play an important role in the fidelity of these recognition events, but their mechanism remains unclear.

To better understand how spliceosomal ATPases affect fidelity during pre-mRNA splicing, I undertook genetic and biochemical analysis of the role of the Sub2 ATPase on early recognition events at the branchpoint sequence (BPS). Sub2 has high homology to the mammalian ATPase UAP56, which associates with the U2AF65 splicing factor and is required for the ATP-dependent U2-BPS basepairing interaction. Genetic and biochemical analysis of *sub2* alleles suggests that Sub2 functions to stabilize the ATP-independent BPS binding of BBP and Mud2, the yeast U2AF65 homologue; however, in a subsequent ATP-dependent step, Sub2 likely destabilizes these factors at the BPS, thereby promoting the U2-BPS interaction. Further genetic analysis of *SUB2* interactions supports this model. Biochemical analyses of a *Δsub2Δmud2* extract suggest that the

balance of BBP and U2 snRNA binding to the BPS is altered in the absence of Sub2 and Mud2. Taken together these studies suggest that Sub2 may promote accurate BPS recognition by influencing a balance of affinities between BBP, Mud2, and the U2 snRNP at the BPS.

The genetic link between *SUB2* and the snRNP biogenesis factor *BRR1* implicates a role for Sub2 in snRNP biogenesis. *SUB2* and *BRR1* exhibit a complex series of genetic interactions, influenced by the levels of each factor. Overexpression of *SUB2* partially suppresses the decrease in snRNA levels observed in a *brr1-1* strain. Moreover, snRNP levels are similarly reduced in extracts derived from *sub2-1* and *Δbrr1* strains. Taken together, these data suggest that Sub2 may play additional roles in the cell related to snRNP biogenesis or stability.

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The goal of this thesis work was to gain a deeper understanding of how the cell accurately transmits genetic information during the process of pre-mRNA splicing. In pre-mRNA splicing, intervening, non-coding sequences (introns) residing between coding sequences (exons) of a nascently transcribed pre-mRNA molecule, are removed by the cell to create an mRNA molecule which typically contains the appropriate sequences necessary to generate a functional protein. This process likely occurs co-transcriptionally in vivo. It proceeds by a two-step transesterification reaction, and is carried out by a large ribonucleoprotein complex, the spliceosome.

This step of gene expression poses a particularly daunting challenge to the fidelity of gene expression in the cell for two reasons. First, the signals that distinguish the boundaries of introns destined to be spliced from a pre-mRNA transcript are short, ranging from only three to seven nucleotides in length. Although conserved, these sequences do exhibit some degeneracy in yeast. This is even more of a problem in the context of higher eukaryotes where splice site consensus signals are shorter, and significantly more degenerate. Second, the spliceosome is not a preformed enzymatic machine, but a dynamic ribonucleoprotein complex that appears to assemble de novo in an ordered pathway on each splicing substrate (Staley and Guthrie 1998). Through this seemingly baroque strategy, the spliceosome is able to recognize the short consensus splicing signals flanking and within the introns, and juxtapose them in the appropriate configuration for subsequent cleavage and removal from the transcript. To complete the splicing process, the spliceosome must also retain and catalyze the ligation of the cleaved exons.

In contrast to other cellular processes related to the maintenance or expression of the genetic material within the cell, such as DNA replication, telomere maintenance, RNA transcription, or protein translation, the juxtaposition of exons, cleavage of the intron, and ligation of the exons in pre-mRNA splicing are not template-guided reactions. At the outset of these studies, I was extremely interested in understanding how the fidelity of gene expression is maintained during such a complex process in the absence of a template. I therefore embarked upon an analysis of the mechanism by which the spliceosome recognizes one of these short consensus splicing signals, the branchpoint sequence (BPS), in the early stages of spliceosome assembly, before splicing catalysis occurs.

When I joined the laboratory, a number of exciting new advances in the field of pre-mRNA splicing had occurred which shed light on some of the possible mechanisms by which the fidelity of gene expression might be achieved during this process. Work in the lab by Sean Burgess implicated a role for a spliceosomal DEAD-box ATPase in the fidelity of splice site recognition at the branchpoint sequence (BPS) after the first step of splicing (Burgess et al. 1990; Burgess and Guthrie 1993b). Genetic and biochemical analysis of *PRP16* provided evidence for the possibility of a kinetic proofreading mechanism operating to maintain fidelity during pre-mRNA splicing (Burgess and Guthrie 1993b). In kinetic proofreading, multiple and independent kinetic competitions between productive and discard pathways occur coupled to NTP hydrolysis steps (Burgess and Guthrie 1993a). This model fit nicely with the genetic and biochemical impact of *prp16* alleles which were isolated on the basis of a defect in fidelity of BPS recognition or utilizaton (Burgess and Guthrie 1993b). These alleles displayed a lower

rate of ATP hydrolysis during pre-mRNA splicing, and the stability of the lariat intermediate product that Prp16 binds to in the context of the spliceosome was increased.

Evidence for multiple recognition events driving the fidelity of splice site recognition during pre-mRNA splicing had also been observed early in splicing, before the first catalytic step. Independent studies examining the basepairing interactions of the snRNA component of the splicesome with the 5' splice site (5'ss) suggested that the fidelity of 5'ss usage is influenced by a series of multiple, mutually exclusive RNA-RNA interactions at the 5'ss (Kandels and Séraphin 1993; Lesser and Guthrie 1993; Sontheimer and Steitz 1993). The exchange of these interactions at the 5'ss is coupled to ATP hydrolysis. These observations raised the possibility that a spliceosomal DEAD-box ATPase might also be involved in either monitoring, or catalyzing the exchange of RNA-RNA interactions at this splice site as well.

Concomitant with these studies, genetic and biochemical work in yeast implicated an additional spliceosomal ATPase, *PRP5*, in facilitating the recognition of the BPS by the U2 snRNA at an early stage in spliceosome assembly, before the first step of splicing (Ruby et al. 1993). A combination of genetic and biochemical analysis identified additional yeast factors required for the ATP-dependent basepairing interaction between the U2 snRNA with the BPS. Mutant alleles of the pre-mRNA processing factors, *PRP5*, *PRP9*, *PRP11*, and *PRP21* were all demonstrated to be defective in formation of the U2-BPS complex, the prespliceosome (PS), the first ATP-dependent splicing complex that forms on a pre-mRNA substrate (Ruby et al. 1993). Independent work in both yeast and mammals also identified a complex of proteins required for formation of the functional 17S U2 snRNP, which is competent to add to the pre-mRNA substrate (Bennett and Reed

1993; Brosi et al. 1993; Legrain and Chapon 1993). *PRP9*, *PRP11*, and *PRP21* correspond to this U2 subcomplex (Legrain and Chapon 1993). In vitro analysis of the yeast components of this complex and the Prp5 protein implicate a function for Prp5 in increasing the accessibility of the U2 snRNP BPS-interacting domain through interactions with the *PRP9-PRP11-PRP21* component of the U2 snRNP (O'Day et al. 1996; Wiest et al. 1996). However, a mammalian homolog of the Prp5 DEAD box had not been identified, despite the fact that a clear requirement for ATP in formation of the mammalian PS had been demonstrated.

A great deal of progress had also been made in both mammals and yeast in identification of a set of factors that recognize the BPS in an ATP-independent step. In mammalian extracts, a U2 auxiliary factor of 65 kilodaltons (U2AF65) was identified to interact with the polypyrimidine tract adjacent to the branchpoint region (Ruskin et al. 1988; Zamore et al. 1992). This factor was demonstrated to bind pre-mRNA in an ATP-independent manner, yet was required for the subsequent association of U2 with the BPS (Ruskin et al. 1988). U2AF65 was also identified in affinity purified early (E) ATP-independent complexes which formed on biotinylated pre-mRNA substrates, but was not detectable in later complexes, after U2 association (Bennett et al. 1992).

In yeast a similar ATP-independent complex requiring an intact BPS was also observed. In vitro analysis demonstrated that the formation of this complex on a pre-mRNA substrate could commit that substrate to the splicing pathway (Séraphin et al. 1988; Séraphin and Rosbash 1991a). This ATP-independent complex is detectable by native gel electrophoresis and was named the commitment complex (CC) (Legrain et al. 1988; Séraphin and Rosbash 1989). Two forms of CC have been defined based on their

kinetics of appearance and migration on a native gel. Commitment complex 1 (CC1) migrates faster on a native gel, appears first kinetically, contains the U1 snRNP and other proteins, and does not require an intact BPS to form (Séraphin and Rosbash 1991a). It is required for the formation of the subsequent commitment complex, CC2, and mutation or deletion of the 5'ss abolishes its formation. CC2 formation requires the presence of an intact BPS (Séraphin and Rosbash 1991a). One of the components of CC2, Mud2, bears some similarity in both function and sequence to U2AF65 (Abovich et al. 1994). It acts at the same stage of splicing, binds directly to the pre-mRNA substrate, and contains a homologous RNA binding domain (Abovich et al. 1994). However, in contrast to mammals, in yeast, Mud2 is not strictly required for U2 snRNP association with the BPS. Additionally, MUD2 is not essential for viability. Moreover, the homology between Mud2 and U2AF65 is confined to an RNA binding domain, and does not extend to the additional linker or RS domains present in U2AF65. Thus, whether Mud2 was indeed the functional homolog of U2AF65 was an unresolved question (Abovich et al. 1994). Differences between the sequence conservation of the BPS and the relative contribution of the BPS and the polypyrimidine tract in yeast and mammals also raised the possibility that both the ATP-independent and the subsequent ATP-dependent steps leading to U2 snRNP association with the BPS might differ in these two systems.

Evidence supporting a strong similarity in the players involved in this step of splicing in both systems became available through further genetic analysis of factors interacting with Mud2. A synthetic lethal screen for factors required for viability in the absence of Mud2 identified another component of the commitment complex, *MSL5*, more commonly referred to as branchpoint binding protein (BBP) (Abovich and Rosbash

1997). Independently in mammals, a homolog of this factor was identified biochemically (Kramer 1992; Berglund et al. 1997). BBP was demonstrated to bind the BPS in a sequence-specific manner (Berglund et al. 1997), in collaboration with U2AF65 (Berglund et al. 1998a). Similar interactions were proposed for BBP and Mud2 in yeast (Berglund et al. 1997). These results raised the possibility that the ATPase activity of Prp5 was likely required to catalyze the exchange of BBP and Mud2 bound at the BPS for the U2 snRNP (Berglund et al. 1998a). Indeed, soon after the identification of BBP and analysis of its interactions at the BPS, a DEAD box factor that interacts with U2AF65 was identified in mammals through 2 hybrid screens for U2AF65 associating proteins (Fleckner et al. 1997). UAP56 is a 56 kilodalton protein that associates with U2AF65 both by 2 hybrid and by co-immunoprecipitation. UAP56 associates with pre-mRNA in vitro in a U2AF65 dependent manner, and depletion of U2AF65 leads to a defect in pre-mRNA splicing in vitro. In such extracts, association of U2 snRNA with a sensitized pre-mRNA substrate is inhibited. Given this apparent parallel in function with Prp5, it was proposed that UAP56 might function as the Prp5 homolog in mammals.

However, independent experiments in our lab pointed to the possibility that UAP56 actually corresponded to a distinct DEAD box ATPase in yeast. Although UAP56 is homologous to Prp5, another ATPase in yeast, Sub2, was more homologous to UAP56 than Prp5. Sub2 was identified on the basis of its ability to suppress the growth defect conferred by a cold sensitive mutant version of *BRR1*, a factor required for snRNP biogenesis in yeast (Noble 1995). The identification of this UAP56 homolog raised the possibility that Sub2 might function directly in pre-mRNA splicing in yeast. If Sub2 functioned similarly to UAP56, at the stage of PS formation, this would suggest that two

spliceosomal ATPases might be required for U2 addition during pre-mRNA splicing in yeast. Understanding how this additional spliceosomal ATPase might function during PS formation provided an attractive inroad to a deeper understanding of the mechanism by which the BPS recognition at an early stage of splicing occurs. Such studies were particularly appealing since the potential substrates for the ATPases involved in PS formation are likely more simple, and ultimately more biochemically tractable for detailed analysis than the substrates of spliceosomal DEAD box factors functioning in later stages of the pre-mRNA splicing pathway. Thus, I undertook a genetic and biochemical analysis of the role of the *SUB2* gene in pre-mRNA splicing in yeast.

Since *SUB2* was identified on the basis of its ability to suppress the growth defect in a snRNP biogenesis factor, rather than a direct function in splicing, I first set out to determine if Sub2 did indeed play a direct role in pre-mRNA splicing in yeast. In chapter 1, I describe the genetic and biochemical characterization of the interactions and splicing defects detected in conditional alleles of *SUB2*. Through PCR mutagenesis of the *SUB2* ORF, two classes of conditional alleles of *SUB2* were generated. One class of alleles obtained in these studies exhibited a cold sensitive (cs-) growth defect, while a second class exhibited both temperature sensitive and cold sensitive (cs-/ts-) growth defects. I was able to infer a role for Sub2 in pre-mRNA splicing through genetic and biochemical analysis of the two strongest alleles from each of these classes. The cs-/ts- *sub2-1* allele exhibited a defect in splicing activity upon preincubation at the non-permissive temperature. Surprisingly however, this mutant confers a defect after PS formation, rather than before PS formation, as we'd expected based on the immunodepletion studies of UAP56 in mammalian extracts. In contrast, the cs- *sub2-5* allele exhibited no

detectable defect in splicing in vitro. Moreover, genetic analysis suggested that both of these alleles are defective for different aspects of interactions with Mud2: deletion of MUD2 suppressed the ts- phenotype of *sub2-1*, while it exacerbated the cs- phenotype of *sub2-5*. Surprisingly, deletion of MUD2 actually bypassed the requirement for Sub2 function in vivo. Taken together, these data suggested that Sub2 functions both in collaboration with Mud2 and in opposition to Mud2 function in an early stage of splicing to influence the formation of a productive PS complex. In the absence of MUD2, the function of Sub2 appeared to be dispensable. Thus we hypothesized that Sub2 was required for the removal of Mud2 (and perhaps BBP) at the BPS of a pre-mRNA substrate to facilitate the formation of a productive PS complex.

In chapter 2, I describe a series of follow-up genetic and biochemical experiments designed to test this model for Sub2 function during pre-mRNA splicing in yeast. Newly generated conditional alleles of BBP allowed me to assess the prediction that Sub2 might function in collaboration and opposition with BBP in a similar manner to that observed with Mud2. As observed with the genetic interactions with the null allele of MUD2, a distinct series of genetic interactions were detected between the conditional alleles of BBP and the *sub2-1* and *sub2-5* alleles. Additionally, the *msl5-5* allele of BBP phenocopied some of the genetic interactions of the Δmud2 allele: it suppressed the *sub2-1* ts- growth defect as well as bypassed the requirement for Sub2 function in vivo.

Additional distinct genetic interactions detected between the *sub2-5* allele and the other conditional alleles of BBP that we tested provided further support for our hypothesis of distinct functional defects in the *sub2-1* and *sub2-5* alleles. Moreover, the genetic interactions detected between *sub2-5* and a subset of BBP alleles defective for

pre-mRNA retention in the nucleus raises the possibility that the *sub2-5* allele may be defective in a function tied to nuclear retention of pre-mRNA or targeting of nascent pre-mRNA transcripts to the splicing pathway.

Analysis of genetic interactions between the *sub2* alleles and factors known to influence the association of the U2 snRNP with the BPS tested the prediction that Sub2 function might be linked to PS formation. The *sub2* alleles appeared to exacerbate the growth defects conferred by *prp*9, *prp*11, and *prp*21. Moreover, preliminary genetic analysis of *CUS2-SUB2* genetic interactions suggest that disruption of *CUS2*, which normally allows for ATP-independent PS formation, is capable of bypassing the requirement for Sub2 in vivo at low temperature. Thus, although further biochemical and genetic analysis will be required to confirm these preliminary results, it does appear that Sub2 may influence the functions of factors required for PS formation in yeast.

Finally, biochemical analysis of the splicing defects conferred by extracts derived from the viable *Δsub2 Δmud2* strain are were also examined. These experiments allowed a test of our predictions for splicing in the absence of Sub2. Overall, the results from these experiments were consistent with our expectations. Through these experiments we have been able to confirm and refine our model for Sub2 function during pre-mRNA splicing.

In chapter 3, I describe further experiments exploring the original link to Sub2 and snRNP biogenesis factor *BRR1*. Again, I observed a complex series of genetic interactions between mutant alleles of these two factors, consistent with the possibility of a functional interaction between Sub2 and Brr1. These interactions were strongly influenced by the levels of each of the factors in the cell. Analysis of the impact of high

copy *SUB2* on the different phenotypes conferred by the *brr1-1* mutant suggests that overexpression of *SUB2* may suppress the defects conferred by *brr1-1* through a direct effect on snRNA stability or biogenesis, rather than an indirect effect via an enhancement of splicing efficiency. Analysis of snRNP levels in *sub2-1* and *sub2-5* extracts also revealed that although *sub2-5* snRNP levels appear similar to a wildtype strain, the *sub2-1* snRNP profile seems to phenocopy that of the Δ*brr1* strain. In all cases, regardless of preincubation regime, the *sub2-1* extract appears to have lower levels of spliceosomal snRNPs than a wildtype extract. Whether this altered snRNP profile is a cause or an effect of the *sub2-1* mutant is still unclear.

Taken together, the experiments I have completed during my thesis provide strong evidence for a function for Sub2 in the early stages of BPS recognition and PS formation in yeast. Many questions remain to be resolved with regard to the mechanism(s) by which Sub2 performs this function in yeast, and how such functions may be linked to the fidelity of BPS recognition before splicing catalysis. Thus, our understanding of Sub2 function remains far from complete. In the epilogue, I summarize these studies and the implications of our work in the context of parallel advances in our understanding of the recognition events at the splice sites. I also describe recent advances in our understanding of the functions of spliceosomal ATPases during pre-mRNA splicing, and highlight how our work with Sub2 contributes to these studies.

In addition to the data related to a possible function in snRNP biogenesis presented in chapter 3, Sub2 has also been identified to interact with factors involved in processes ranging from transcription mediated hyper-recombination to RNA transport. I summarize this evidence for a role for Sub2 in other RNA processing events, and how

CHAPTER 1

Deletion of MUD2, the yeast homolog of U2AF65, can bypass the requirement for Sub2, an essential spliceosomal ATPase

ABSTRACT

Mammalian U2AF65 and UAP56 are required for pre-spliceosome (PS) formation. We tested the predictions that the yeast UAP56 homologue, *SUB2*, is required for the same step, and functions collaboratively with *MUD2*, the yeast homologue of U2AF65. Unexpectedly, *sub2-1* extracts accumulate PS-like complexes. Moreover, deletion of *MUD2* exacerbates the cs phenotype of *sub2* alleles, yet suppresses both the ts *sub2-1* and the lethal *Δsub2* phenotypes. We propose that Sub2 functionally interacts with Mud2 both before and after PS formation. In the absence of Mud2, Sub2 function becomes dispensable.

INTRODUCTION

The assembly, function, and disassembly of the spliceosome is a multi-step, ATP-dependent pathway that requires the participation of at least seven members of the "DEAD-box" protein family (Staley and Guthrie 1998). These proteins exhibit RNA-stimulated ATP binding/hydrolysis activity and, in some cases, ATP-dependent RNA unwinding (Wagner et al. 1998; Wang et al. 1998). In several cases, these ATPases appear to catalyze conformational rearrangements which result in mutually exclusive RNA "switches" (Staley and Guthrie 1998), providing a plausible strategy for the precise coordination of this multi-step pathway. However, the molecular mechanisms by which these factors function remain unclear.

The earliest ATP-dependent step in spliceosome assembly in both yeast and mammals is the basepairing of the U2 snRNA with the branchpoint region of the intron to form the prespliceosome (PS) (Konarska and Sharp 1986; Cheng and Abelson 1987;

Konarska and Sharp 1987; Liao et al. 1992; Das et al. 2000). In mammals, this event requires prior binding of the branchpoint region by Branchpoint Binding Protein, mBBP/SF1, and of the pyrimidine tract by U2AF65 in an ATP-independent, early (E) complex (Ruskin et al. 1988; Kramer and Utans 1991; Michaud and Reed 1991). Although the U2 snRNP has been detected in the mammalian E complex, mutations in the branchpoint sequence (BPS) which affect base-pairing do not affect this association, suggesting that the U2 present in E complex is not base-paired with the BPS (Das et al. 2000). It is likely that the sequence-specific binding of mBBP is mutually exclusive with the subsequent basepairing of the U2 snRNA with the BPS (Berglund et al. 1997; Berglund et al. 1998a). An additional factor, the U2AF65-associated protein of 56kD, UAP56, is a DEAD-box protein that is also required in mammals for U2 snRNP addition in vitro (Fleckner et al. 1997). The mechanism by which UAP56 promotes PS formation in mammals is unknown. It has been proposed to facilitate the association of the U2 snRNP with the pre-mRNA substrate through either affecting the availability of the branchpoint basepairing region of the U2 snRNA or by modulating the accessibility of the branchpoint region of the pre-mRNA substrate (Fleckner et al. 1997).

Yeast contain homologues of both BBP, yBBP/ScSF1, (Abovich and Rosbash 1997) and U2AF65, Mud2 (Abovich et al. 1994). These factors bind the BPS in the absence of ATP to form the commitment complex (CC2) prior to PS formation (Abovich et al. 1994; Abovich and Rosbash 1997). The DEAD-box protein Prp5 is required for stable, ATP-dependent association of the U2 snRNP with the BPS to form the PS (Ruby et al. 1993). Available data suggest that Prp5 promotes a conformational rearrangement of the U2 snRNP (O'Day et al. 1996; Wiest et al. 1996). It was originally proposed that

UAP56 was the mammalian counterpart of Prp5 (Fleckner et al. 1997). Subsequently, as we describe here, *SUB2* has been identified as the UAP56 homologue. Moreover, a human homologue of Prp5 has recently been identified (C. Newnham and C. Query, personal communication). Thus, two ATPases may be required to promote efficient PS formation during splicing in both yeast and mammals.

Here, we report biochemical and genetic analyses that suggest that Sub2 may play multiple roles in the splicing pathway, both before and after PS formation. Remarkably, the essential function(s) of Sub2 can be partially bypassed by deletion of the non-essential *MUD2* gene, suggesting that in the absence of this likely protein target, the essential function of Sub2 becomes dispensable.

RESULTS AND DISCUSSION

SUB2 Encodes An Essential Homologue Of Human UAP56

We initially identified the *su*ppressor of *brr1-1*, (*SUB2*), in a selection for high copy suppressors of the cold-sensitive growth defect of this snRNP biogenesis mutant (Noble 1995; Noble and Guthrie 1996b). *SUB2* encodes a DEAD-box protein with 63% identity and 78% similarity to human UAP56 (Figure 1). This homology extends beyond the conserved helicase domain of the two proteins (Figure 1). Moreover, this level of homology is comparable to that previously reported for yeast and human spliceosomal DEXH-box proteins with demonstrated similarity in function (Ono et al. 1994; Gee et al. 1997; Zhou and Reed 1998).

To test the prediction that *SUB2* encodes a functional homologue of UAP56, we first examined the impact of a loss of Sub2 function. Previous systematic deletion studies

in yeast have shown that deletion of the *SUB2* ORF has variable effects on viability depending on strain background (Lopez et al. 1998). In our hands, deletion of *SUB2* is lethal in two different strain backgrounds (Materials and Methods). To examine the impact on splicing, we generated a strain in which *SUB2* expression was under control of the glucose-repressible *GAL1* promoter. A shift to glucose media caused growth to slow after 12 hours, then halt after 28 hours (data not shown). In vivo genetic depletion of *SUB2* causes U3 pre-mRNA levels to increase up to 6-fold, as monitored by primer extension of RNA derived from this strain (data not shown). Taken together, these data indicate Sub2 plays an early, essential role in pre-mRNA splicing.

Isolation of two classes of conditional sub2 mutants

We next used mutagenic PCR (Leung et al. 1989) to generate conditional *sub2* alleles. We obtained six alleles, four of which (*sub2-1* through *sub2-4*) are both cold-sensitive and temperature-sensitive (cs/ts), while two (*sub2-5* and *sub2-6*) are cold-sensitive (cs). We focused on the alleles from each class with the strongest growth defect, *sub2-1* and *sub2-5*. The minimal number of mutations required to confer the growth defects for each allele has not been determined; nonetheless, we note an interesting correlation in the location of the mutations and the different growth phenotypes of *sub2-1* and *sub2-5*.

The cs *sub2-5* allele contains a single mutation (Q308R) near helicase motif IV (Figure 1). Available crystal structures of superfamily 2 (SF2) helicases and related superfamily 1 (SF1) helicases place this motif in a structural domain implicated in RNA binding/helicase activity (Pause et al. 1993; Korolev et al. 1997; Yao et al. 1997; Bird et

al. 1998; Korolev et al. 1998; Velankar et al. 1999). In contrast, the *sub2-1* allele contains multiple mutations (D22G, E83G, L142M, and I146T) in the vicinity of the SF2 helicase motifs I and Ia (Figure 1). These motifs have been shown to be required for ATP binding/hydrolysis and thus the coordination of conformational changes in the ATP binding domain with the function of the other motifs of SF2 helicases (Pause and Sonenberg 1992; Hotz and Schwer 1998; Edwalds-Gilbert et al. 2000). Recent crystal structures of SF1 and SF2 helicases place motifs I and Ia in a structural domain distinct from that containing motif IV (Bird et al. 1998; Korolev et al. 1998; Johnson and McKay 1999). Thus, the differences in growth phenotypes of *sub2-1* and *sub2-5* likely reflect inactivation of different aspects of wildtype Sub2 function.

Evidence for Sub2 function after U2 addition

We analyzed the in vitro splicing activity of temperature-inactivated extracts derived from *sub2-1* and *sub2-5* cells grown at permissive temperature. We have been unable to detect any defects in splicing activity or spliceosome assembly in *sub2-5* extracts (data not shown). Possibly, the *sub2-5* allele is defective for an in vivo splicing function which is not rate-limiting in vitro.

In contrast, after 37°C preincubation, *sub2-1* extracts accumulate uncleaved pre-mRNA, with a concomitant decrease in lariat intermediate, excised lariat, and mRNA (Figure 2a, lanes 1-4). This early splicing defect is likely due to a block in activity, rather than reduced kinetics, since incubation up to 120 minutes did not increase the amount of splicing intermediates and products (data not shown). This phenotype is specifically due to heat inactivation of *sub2-1*, since *sub2-1* extracts preincubated on ice (lanes 5-8) and

SUB2 extracts preincubated at 37°C (lanes 9-12) or on ice (lanes 13-16) show similar levels of splicing activity. Thus, consistent with our expectation for a functional homologue of UAP56, Sub2 function is required early in splicing.

Native gel analysis (Cheng and Abelson 1987) was performed to test the prediction that heat-inactivated *sub2-1* extracts would be blocked prior to formation of the PS. Surprisingly, a complex that co-migrates with the PS in *SUB2* extracts preincubated at 37°C (Figure 2b, lane 2) accumulates in the *sub2-1* heat-inactivated extracts (Figure 2b, lane 1). Depletion of ATP prior to addition of labeled pre-actin substrate abolishes formation of the *sub2-1* PS (data not shown). Northern analysis of the native gel shown in Figure 2B confirmed the presence of U2 snRNA in the *sub2-1* complex (data not shown). Taken together, these data indicate that Sub2 may function after, rather than before, PS formation in yeast.

To determine if the *sub2-1* complex is a bona fide splicing intermediate, we tested if the splicing activity defect of heat-inactivated *sub2-1* extracts could be chased. We first incubated radiolabeled pre-mRNA in heat-inactivated *sub2-1* extracts and then performed a second (chase) incubation with different extracts in the presence of cold competitor pre-mRNA (Figure 2C, Materials and Methods). As expected, heat-inactivated *sub2-1* extracts were inactive in the chase (lane 1). In contrast, U2-inactivated extracts (Figure 2C, lane 3), mock U2-inactivated extracts (Figure 2C, lane 4), and mock-preincubated *sub2-1* extracts (Figure 2C, lane 5) were all competent to chase the labeled pre-actin substrate into splicing intermediates and products. These data suggest that the PS complex which accumulates in the *sub2-1* extract is a competent splicing intermediate, rather than an aberrant, "dead-end" complex.

Our efforts to complement the *sub2-1* splicing defect with Sub2 purified from *E. coli* or yeast have been unsuccessful to date, despite the fact that both of these versions of Sub2 fully complement a *Δsub2* allele when expressed in yeast (data not shown). Furthermore, efficient immunoprecipitation of epitope tagged Sub2 from splicing extract requires high salt (data not shown), suggesting that Sub2 may be part of a complex. It may be significant in this regard that a U2-inactivated extract (Figure 2C, lane 2) is only partially competent to chase the precursor that accumulates in the *sub2-1* heat-inactivated extract (Figure 2C, lane 3). This same U2-inactivated extract can be fully chased by a mock U2-inactivated extract (Figure 2C, lane 6). While we cannot rule out a non-specific impact of combining two defective extracts, these data raise the possibility that heat inactivation of the *sub2-1* extract may also affect Sub2-interacting factors in the U2 snRNP. Consistent with this possibility, Northern analysis of U2 snRNA levels suggests a slight decrease (less than 2 fold, data not shown) in levels of U2 in the *sub2-1* extracts regardless of preincubation treatment. Whether this is a cause or effect of the presence of the *sub2-1* allele is unclear, but further indicates an interaction between *sub2-1* and U2 snRNP function.

Evidence For Functional Interactions Between Sub2 And Mud2

Since UAP56 interacts with U2AF65, we tested for genetic interactions between the *sub2* alleles and *MUD2* (Table 1). Deletion of the non-essential *MUD2* gene exacerbates the cold-sensitive (cs) growth defects of both *sub2-1* and *sub2-5*. In contrast, the temperature-sensitive (ts) growth defect of *sub2-1* is partially suppressed by the *Δmud2* allele. Exacerbation of the cs growth defect is consistent with a defect in a

collaborative function between Sub2 and Mud2, while suppression of the ts growth defect of *sub2-1* suggests an antagonistic interaction between Sub2 and Mud2.

Since the *sub2-1* mutant contains mutations in ATPase motifs, the absence of Mud2 would be predicted to suppress a defect in an ATP-dependent function of Sub2. An extreme extension of this hypothesis is that the sole function of Sub2 is to remove Mud2 to promote the formation of a productive PS. To test this hypothesis, we asked if deletion of *MUD2* could suppress a *SUB2* deletion. Strikingly, deletion of *MUD2* restores viability to the otherwise lethal deletion of *SUB2* at 25°C (Table 1). In the absence of both *MUD2* and *SUB2* plasmids, the *Δsub2Δmud2* strain is capable of growth (Figure 3, lower segment). The presence of *MUD2* abrogates the growth of the *Δsub2Δmud2* strain (Figure 3 middle segment), while a *SUB2* plasmid confers wildtype growth to the *Δsub2Δmud2* strain (Figure 3, top segment). These genetic interactions indicate that Sub2 may function in an ATP-dependent step to promote removal of Mud2. Thus, in the absence of Mud2, Sub2 function becomes dispensable.

A Sub2-Mud2 Dependent Splicing Pathway

Taken together, our data are consistent with a two-step model for Sub2 function (Figure 4, left panel). The exacerbation of the cs growth phenotype of *sub2-5* by deletion of *MUD2* suggests that Sub2 may first function in collaboration with Mud2. Sub2 may interact in an ATP-independent fashion with the pre-mRNA substrate via Mud2, or via Mud2 and other components of the CC2 complex. Indeed, such a function is predicted for Sub2 based on previous studies of the human homologue, UAP56 (Fleckner et al. 1997). Although we have been unable to detect a biochemical defect in CC formation or

stability in *sub2-5* extracts (data not shown), this hypothesis for Sub2 function is supported by results in the accompanying communications.

In *sub2-100* extracts, the formation of CC2 complexes is blocked (M. Zhang and M.R. Green, submitted), while in *sub2-201* extracts, CC2 accumulates with a concomitant decrease in PS (D. Libri et al, submitted). We think it is likely that the variability in the severity of the biochemical defects detected before PS formation reflects differences in the mutations present in *sub2-5* (Q308R), *sub2-100* (D175G), and *sub2-201* (D175G, L405F). The fact that all three groups have obtained independent genetic or biochemical evidence implicating defects in Sub2 function before PS formation strongly supports the hypothesis that wildtype Sub2 may first function early in splicing, before U2 addition.

In contrast, *Δmud2* suppression of the ts phenotype of the *sub2-1* allele indicates a second function of Sub2, antagonistic to Mud2. This interpretation is strongly supported by the *Δmud2* bypass suppression of *Δsub2* lethality (Figure 3). Since Mud2 has been detected in CC2 complexes, but not PS complexes (Abovich et al. 1994; Rutz and Seraphin 1999), the ATP-dependent function of Sub2 may be to promote the removal of Mud2 (and perhaps BBP) from the branchpoint region to facilitate the stable/productive interaction of the U2 snRNP with the pre-mRNA substrate. An important future goal is to test whether Mud2 is present in the the PS complex that accumulates in the *sub2-1* extracts (Figure 4, left panel, bracketed intermediate).

A Sub2-Mud2 Independent Splicing Pathway

The fact that *MUD2* is inessential has been thought to reflect the diminished role of the pyrimidine tract in yeast vs. mammalian introns (Abovich et al. 1994; Abovich and Rosbash 1997). Indeed, although Mud2 enhances the efficiency with which yeast BBP binds to introns, this reaction can occur in the complete absence of Mud2 (Berglund et al. 1997; Berglund et al. 1998a). In the Sub2-Mud2 independent pathway proposed in Figure 4 (right panel), BBP binds in the absence of Mud2, albeit more weakly, and can be displaced from the BPS without the requirement for Sub2.

It remains to be seen whether the removal of BBP is also affected in *sub2* mutants. We have been unable to detect genetic interactions between our *sub2* alleles and the original BBP mutant, *msl5* (Abovich and Rosbash 1997). Newly reported mutant alleles may be informative in this regard (Rutz and Seraphin 2000). However, the finding that several conditional *msl5* mutants show no impairment of splicing in vitro (Rutz and Seraphin 2000) suggests that BBP may also be dispensable in the Sub2-Mud2 independent pathway.

Implications

While our finding that the essential requirement for the *SUB2* ATPase can be bypassed by deletion of the non-essential splicing factor *MUD2* was unanticipated, we believe that these results have wider significance with regards to the role of ATP in splicing. Prp28 is an essential DEAD-box protein required to promote the exchange of U1 for U6 at the 5' splice site; reduced function *prp28* alleles become lethal in the presence of a hyperstabilized base-pairing interaction between U1 and the 5'ss (Staley

and Guthrie 1999). As reported by Chang and colleagues (T.-H. Chang et al., submitted), mutations in the RNA-binding domain of the U1C snRNP protein render Prp28 dispensable, and suppress the lethal phenotype conferred by combining *prp28-1* with a hyperstabilized U1-5'ss interaction. Thus, the ATP-dependent role of Prp28 appears to be essential only for counteracting the stabilizing effect of U1C on the U1-5'ss interaction. Similarly, the ATP-dependent function of Sub2 appears to be essential only for counteracting the interaction of Mud2 (and thus, perhaps BBP) at the BPS. Taken together, these results strengthen the notion that the ATP-dependent steps of splicing are not an essential mechanistic feature, but rather have evolved for the refinement of the splicing pathway, allowing multiple opportunities for regulation and fidelity maintenance.

It will be extremely interesting to further investigate these and other scenarios in which energy-requiring transitions in splicing can be bypassed. For example, deletion of the non-essential splicing factor *CUS2* allows formation of the *PRP5*-dependent PS in the absence of ATP in vitro (Perriman and Ares 2000). Such studies provide a promising inroad to a deeper mechanistic understanding of how DEAD-box proteins utilize ATP to catalyze conformational rearrangements during the course of splicing, and how such events contribute to the accuracy and activity of the essential function of the spliceosome.

MATERIALS AND METHODS

Yeast Methods

All methods for manipulation of yeast, including media preparation, growth conditions, transformation, plasmid recovery, and 5-fluoro-orotic acid (5-FOA) selection were

performed according to standard methods (Guthrie and Fink 1991). Additional techniques utilized in this study are noted where applied. The strains utilized in this work are derived from W303, unless otherwise indicated.

SUB2 plasmid shuffle strain

To obtain a haploid *SUB2* shuffle strain, the *SUB2* ORF was replaced by *the HIS3* coding sequences through one-step gene replacement into the wildtype diploid strain yCG465. Disruption of the chromosomal *SUB2* in the resulting *HIS+* strain, yCG466, was confirmed by whole cell PCR. yCG466 was transformed with pCG788 (*SUB2* in pRS316 (Sikorski and Hieter 1989)), and sporulated. Dissection of tetrads yielded 4 viable spores in which the *HIS+* phenotype segregated 2:2. All viable *HIS+* haploids were also *URA+*, and inviable on 5-FOA media. yCG470 (*Δsub2::HIS3/p*CG788) was used as the *SUB2* shuffle strain. Disruption of the chromosomal *SUB2* locus with the kan-MX6 marker (Longtine et al. 1998) was also lethal in a haploid in the SS330 strain background (Ruby et al. 1993).

Strains for analysis of genetic interactions between sub2 and Δmud2

Strain yCG472 (*Δsub2Δmud2*) was generated from yCG470 by replacement of the chromosomal *MUD2* with the kan-MX6 marker through one step gene replacement (Longtine et al. 1998). Disruption of *MUD2* in yCG472 was confirmed by whole cell PCR. yCG472 was transformed with wildtype *SUB2* (pCG817), *sub2-1* (pCG838), and *sub2-5* (pCG840) in the *LEU2* CEN vector pRS315 (Sikorski and Hieter 1989). The growth of these and an isogenic *MUD2* (yCG470) strain was assessed on YEPD after loss

of pCG788 on 5-FOA (Table 1). Additionally, yCG472 and its isogenic parent, yCG470, were transformed with pRS315, *MUD2*-pRS315 (Abovich et al. 1994), or *SUB2*-pRS315 (pCG817). Growth of these strains in the absence of pCG788 was assessed on 5-FOA-LEU plates. Growth of viable strains was tested on YEPD (Table 1).

Plasmids

The original *SUB2* plasmid isolate, pSN120, was identified in a selection for high copy suppressors of the cold-sensitive growth defect of the *brr1-1* mutant (Noble 1995) using a GAL-cDNA library (Liu et al. 1992). A plasmid bearing the genomic version of *SUB2* (ORF YDL084W) was cloned in two steps. The 1939bp SalI-XbaI *SUB2* fragment from the S. cerevisiae lambda cosmids 70700 and 70751 (American Type Culture Collection) was cloned into a SalI-XbaI cleaved pBS (KS+) vector (Stratagene) to generate pCG782, and the remaining 181bp of upstream 5'UTR sequences was PCR amplified from the same lambda cosmid DNA, and subcloned into pBS (KS+) to make pCG783. pCG788 (YDL084W in pRS316) was generated by 3-way ligation of KpnI-XbaI digested pRS316, KpnI-SalI digested pCG783, and SalI-XbaI digested pCG782. A*SUB2 LEU2*-CEN plasmid (pCG817) was generated by subcloning the ApaI-NotI *SUB2* fragment from pCG788 into pRS315.

Generation of conditional alleles of SUB2

Mutagenic PCR conditions (Leung et al. 1989) were used to amplify the *SUB2* ORF (final concentrations of Mn^{2+} used in PCR reactions ranged from 0mM $MnCl_2$ - 0.01mM $MnCl_2$), using pCG817 as a template, and primers oCG252

(5'CCTTGTTTTATATATTCATTGTTCA3') and oCG253 (5'GAAAATTTCAAGATTTTTTCCAAAT3'). The resulting PCR products were co-transformed into yCG470 with the purified 7171bp PstI-NdeI vector fragment of pCG817 (Muhlrad et al. 1992). Transformants were selected on SD-LEU media at 25°C, and then replica-plated to 5-FOA media at 25°C, 16°C, and 37°C. The efficiency of mutagenesis ranged from 1.7% to 24.6%, depending on [Mn^{2+}]. A total of 25,000 transformants were screened. Six *sub2* alleles (*sub2-1* though *sub2-6*; pCG838 through pCG843) were growth-impaired at 16°C or both 37°C and 16°, and were sequenced by the Biomolecular Resource Center DNA sequencing facility at UCSF. The software program EditView was used in conjunction with the NCBI BLAST alignment server (http://www.ncbi.nlm.nih.gov/BLAST) to identify mutations in these recovered *sub2* alleles.

In vitro splicing assays and native gel analysis

Splicing extracts derived from yCG484 (*sub2-1* strain), yCG485 (*sub2-5* strain) and yCG483 (*SUB2* strain) were prepared (Umen and Guthrie 1996). Spliceosome assembly analysis was performed by native gel analysis (Cheng and Abelson 1987). Preincubation conditions required to inactivate *sub2-1* extracts but not *SUB2* extracts varied depending on protein concentration of each extract prep and the presence or absence of splicing reaction buffer. Effective conditions remained consistent with each extract preparation, and ranged from 15 minute preincubation at 37°C or 40°C in the presence of splicing buffer components (2mM ATP, 2.5mM MgCl2, 0.6M Kphoshate, pH7, 3% PEG 8000), to 45 minute preincubation at 37°C in buffer D (20mM Hepes

pH7.9, 2mM EDTA, 1.5mM $MgCl_2$, 50mM KCl, 20% glycerol, 0.5mM DTT). Mock preincubations were performed on ice.

In vitro chase experiments

Heat inactivated *sub2-1* extracts or U2-inactivated extracts were first incubated under standard splicing conditions (40% extract, 2mM ATP, 2.5mM MgCl2, 0.06M Kphosphate buffer (pH7), 3% PEG 8000) in a volume of 5ul with 0.4nM of uniformly labeled α-[^{32}P] pre-ACT1 substrate for 10 minutes at 23°C. A second, chase incubation was then carried out for another 10 minutes at 23°C. This chase incubation was initiated in the presence of 100 fold molar excess cold pre-ACT1, added in addition to 5ul of splicing mix containing heat-preincubated or mock-preincubated *sub2-1* extracts, U2-depleted, or U2 mock-depleted splicing extract.

In vitro U2 snRNA depletions

U2 snRNA depletions were performed as previously described, using a final concentration of 1uM U2 oligo (5'-CAGATACTACACTTG-3') (McPheeters et al. 1989), and incubating for 15 minutes at 25°C as recently described (Perriman and Ares 2000). In mock depleted extracts, water was substituted for the U2 oligo during the preincubation at 25°C.

Figure 1. Sub2 is highly homologous to UAP56.

Alignment of UAP56 and Sub2. Sequences were aligned with CLUSTALW and MacBoxShade. Dark grey boxes highlight identical amino acids, light grey boxes highlight similar residues. Thick black bars above the aligned sequences highlight the seven conserved motifs (I, Ia, II-VI) of helicase superfamily 2. Mutations present in *sub2-1* (plain letters) and *sub2-5* (circled letters) are indicated below the altered residue.

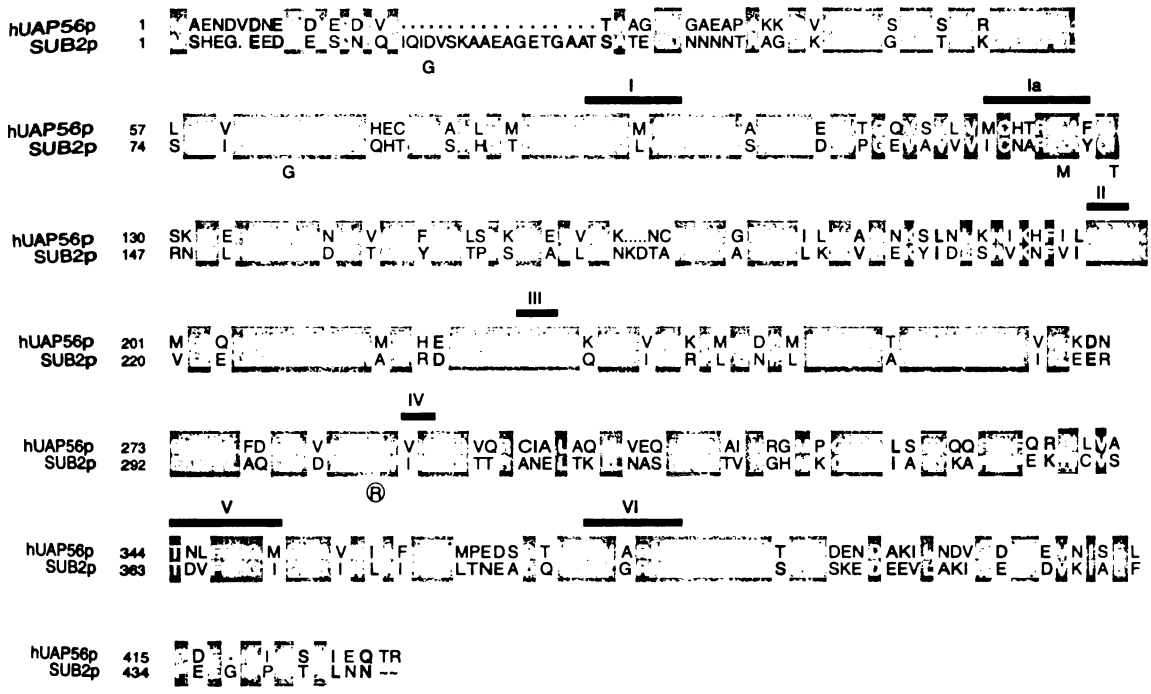

hUAP56p
SUB2p
G
I
Ia
II
III
IV
V
VI

Figure 2. Biochemical phenotype of heat inactivated *sub2-1* mutant. A. Time course of in vitro splicing activity of *sub2-1* extracts. *sub2-1* (lanes 1-8) or *SUB2* (lanes 9-16) extracts were preincubated at 37°C (lanes 1-4 and lanes 9-12) or on ice (lanes 5-8 and lanes 13-16) for 15 minutes, then assayed for splicing activity with radiolabeled pre-actin RNA at 23°C for 0, 8, 16, and 30 minutes. B. Spliceosome assembly phenotype of *sub2-1* extract. Spliceosome assembly of 37°C preincubated samples from the 16 minute timepoint shown in figure 2A (*sub2-1*, lane 3 and *SUB2*, lane 11) was assessed by native gel analysis (Cheng and Abelson, 1986). Spliceosome complexes corresponding to H (heterogenous), PS (prespliceosome), and S (spliceosome) are indicated at left; lane 1, *sub2-1*; lane 2, *SUB2*. C . Chase of splicing defect in *sub2-1* extract. Heat preincubated *sub2-1* (lanes 1, 3-5) or U2-inactivated extracts (lanes 2 and 6) were incubated with radiolabeled pre-actin RNA in a two step incubation schematized in the box above the gel. Extracts used in each stage of the incubation experiment are indicated above each lane. *sub2-1Δ*, 37°C preincubated extract; U2-, U2 oligo-inactivated extract; *sub2-1+*, mock (ice) preincubated extract; U2+, mock oligo-inactivated extract.

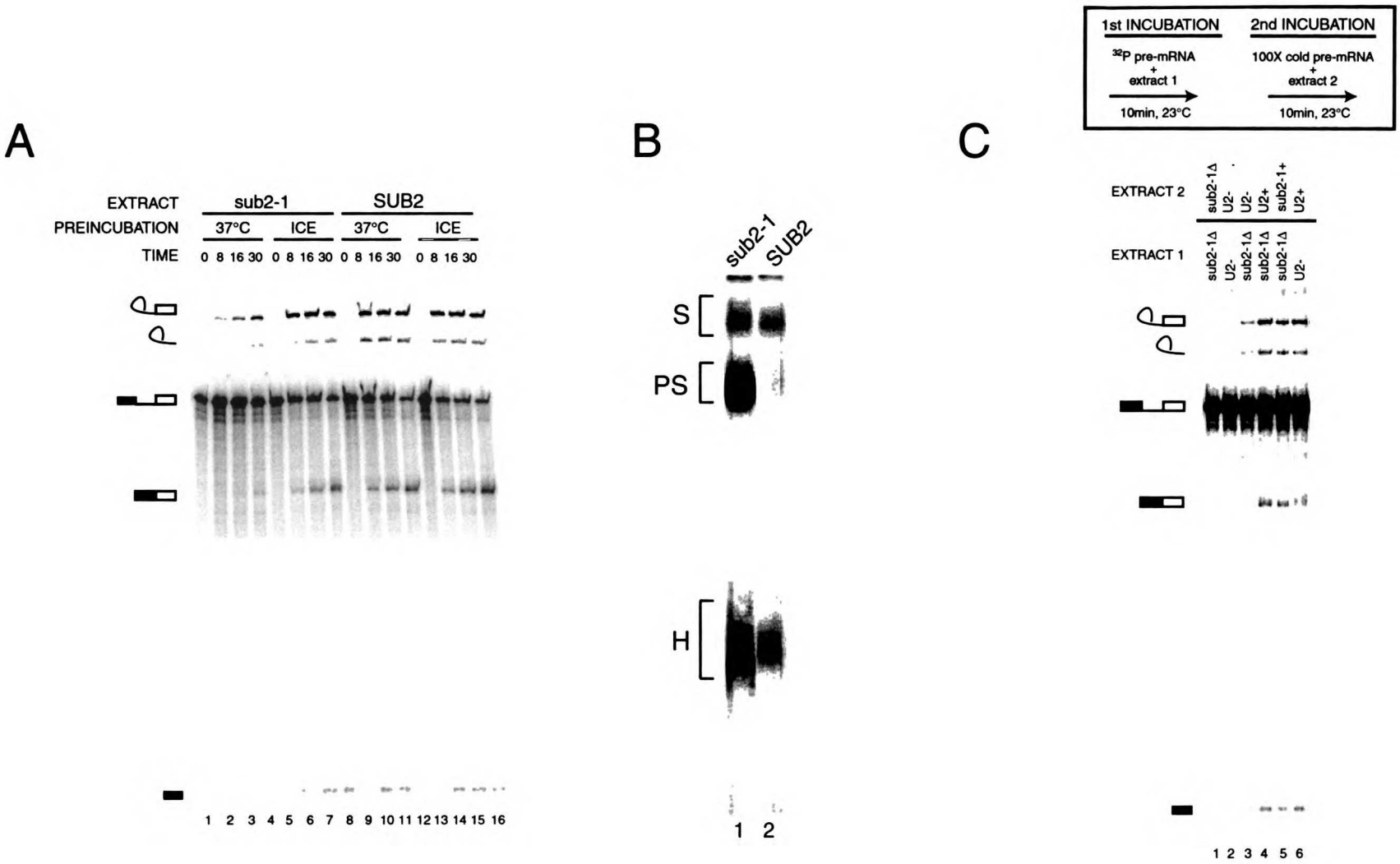

A
EXTRACT
sub2-1
SUB2
PREINCUBATION
37°C
ICE
37°C
ICE
TIME
0 8 16 30 0 8 16 30 0 8 16 30 0 8 16 30
pre
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
sub2-1
SUB2
S
PS
H
1 2
C
1st INCUBATION
2nd INCUBATION
32P pre-mRNA
+
extract 1
10min, 23°C
100X cold pre-mRNA
+
extract 2
10min, 23°C
EXTRACT 2
sub2-1Δ
U2-
U2-
U2+
sub2-1+
U2+
EXTRACT 1
sub2-1Δ
U2-
sub2-1Δ
sub2-1Δ
sub2-1Δ
U2-
pre
1 2 3 4 5 6

Figure 3. Impact of *Δmud2* on the lethality of *Δsub2*. Growth on 5-FOA-LEU media of *Δsub2MUD2/pSUB-URA3 CEN* strain (left side of plate) compared with growth of *Δsub2Δmud2/pSUB2-URA3 CEN* strain (right side of plate) transformed with either *pSUB2-LEU2 CEN* (upper segment), *pMUD2-LEU2 CEN* (middle segment), or *pLEU2 CEN* (lower segment) plasmids.

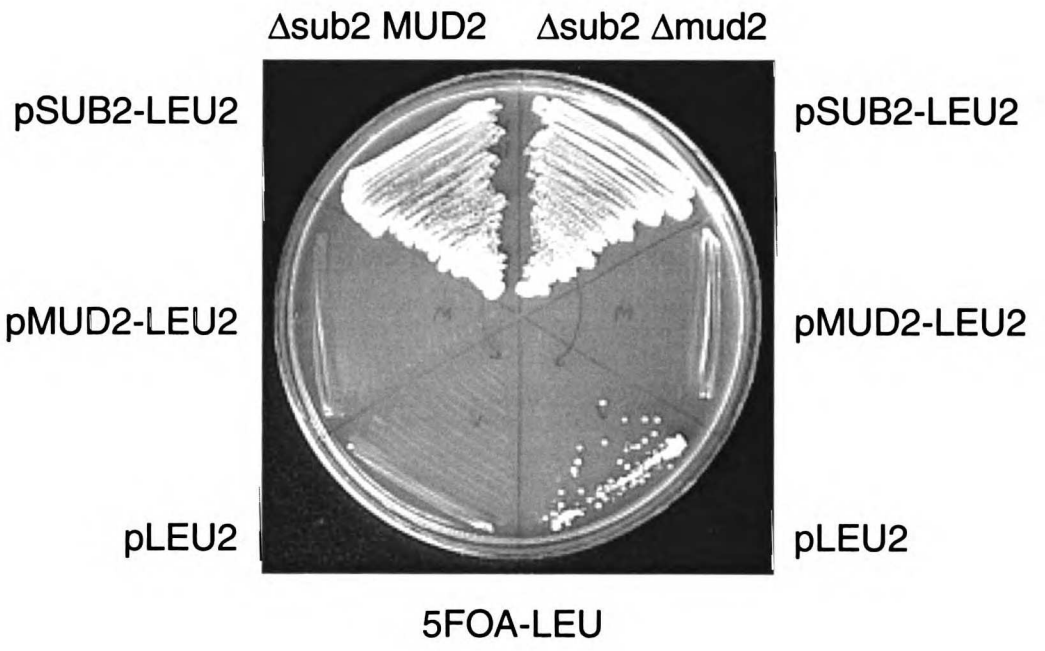

Δsub2 MUD2
Δsub2 Δmud2
pSUB2-LEU2
pSUB2-LEU2
pMUD2-LEU2
pMUD2-LEU2
pLEU2
pLEU2
5FOA-LEU

Figure 4. Models for a Sub2-Mud2 dependent splicing pathway and a Sub2-Mud2 independent splicing pathway. Spliceosomal complexes formed during the early ATP-independent and ATP-dependent stages of splicing are diagrammed. CC1, commitment complex 1; CC2, commitment complex 2; PS, prespliceosome. Circled B, BBP; Circled M, Mud2. The U1 snRNP, U2 snRNP, and Sub2, Prp5, and Cus2 are denoted by labeled shapes. Hypothesized defects in Sub2 functions by different *sub2* alleles are indicated at right in the Sub2-Mud2 Dependent Pathway model. Small arrows from Prp5 and Sub2 indicate activity on potential substrates in the presence of ATP. A possible scenario for the functions of both Prp5 and Sub2 during the ATP-dependent conversion of CC2 to PS complex is shown surrounded by brackets in the Sub2-Mud2 Dependent Pathway model. Potentially less stable binding of BBP with the BPS in the absence of Mud2 is denoted by a dotted outline for the BBP symbol in the Sub2-Mud2 Independent Pathway.

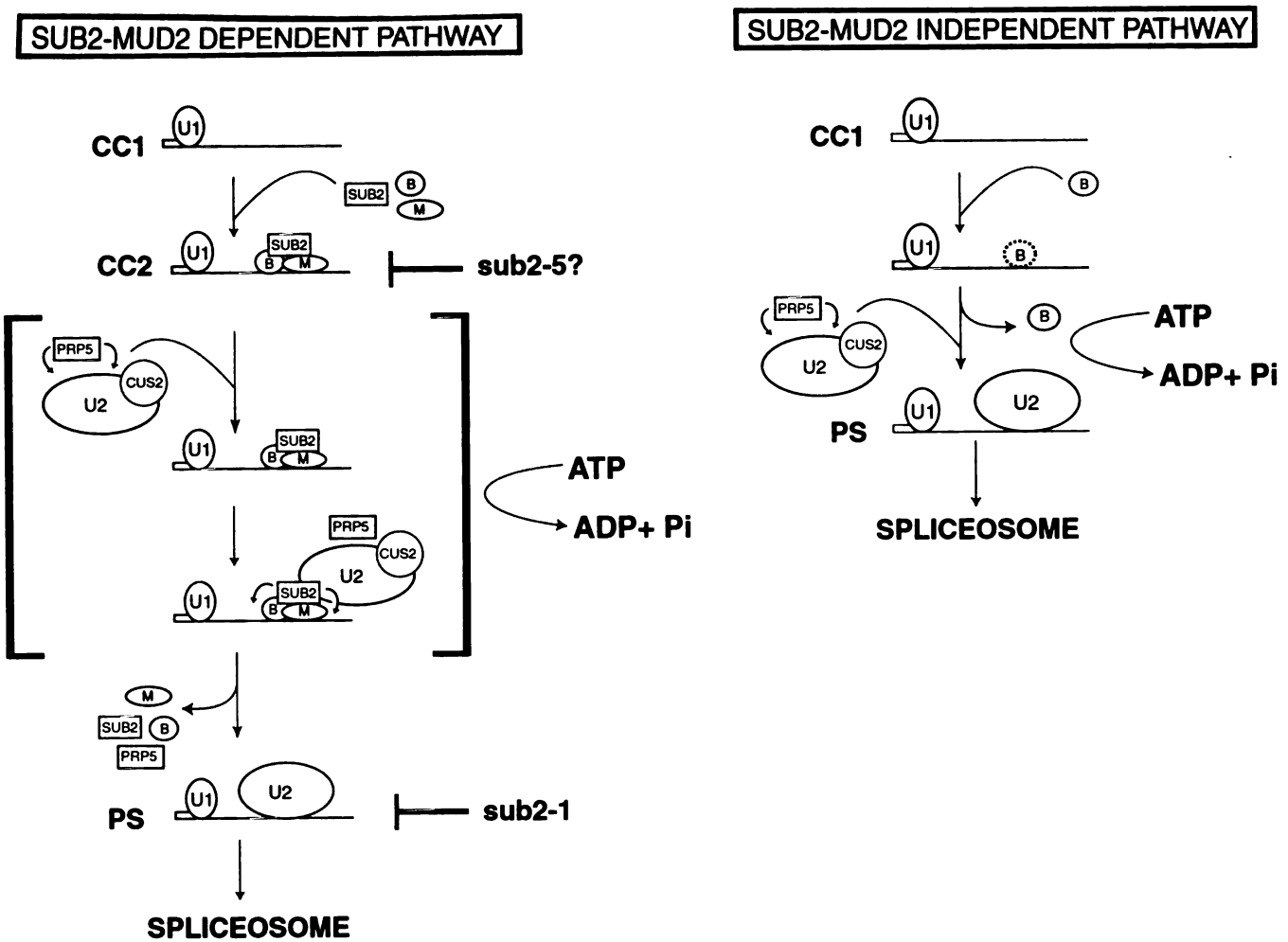

SUB2-MUD2 DEPENDENT PATHWAY
CC1
U1
SUB2
B
M
CC2
sub2-5?
PRP5
CUS2
U2
ATP
ADP+ Pi
PS
sub2-1
SPLICEOSOME
SUB2-MUD2 INDEPENDENT PATHWAY

Table 1. Genetic interactions between *Δmud2* and *sub2* mutants.

Relevant Genotype	Growth phenotype at different temperatures		
SUB2, MUD2	16°C[a]	25°C[b]	37°C[c]
SUB2, MUD2	+++	+++	+++
SUB2, Δmud2	+++	+++	+++
sub2-1, MUD2	+	++	+/-
sub2-1, Δmud2	+/-	++	+
sub2-5, MUD2	+/-	++	++
sub2-5, Δmud2	-	++	++
Δsub2, MUD2	-	-	-
Δsub2, Δmud2	-	++	-

Growth on YEPD plates by streaking isogenic strains bearing the designated *sub2* allele covering a *Δsub2* at different temperatures in the presence or absence of a chromosomal *MUD2* (wildtype growth, +++; mild defect, ++; poor growth, +; severe defects, +/-; no growth -).

[a]Growth at 16°C after 14 days.

[b]Growth at 25°C after 5 days.

[c]Growth at 37°C after 5 days.

CHAPTER 2

Testing the model for Sub2 functions in pre-mRNA splicing

ABSTRACT

Genetic and biochemical analysis of conditional alleles of *SUB2* suggests that Sub2 functionally interacts with Mud2 (and perhaps BBP) to promote the formation of a functional prespliceosome (PS) complex on the branchpoint sequence (BPS) of a pre-mRNA substrate. We tested this model for Sub2 function through further directed genetics and biochemical analysis of our recently generated *sub2* mutant strains. Allele-specific genetic interactions between recently generated conditional alleles of BBP and *sub2-1, sub2-5*, and the null allele of *SUB2* lend further support to this model for Sub2 function. Detection of allele-specific interactions between the *msl5-3* and *msl5-9* alleles of BBP, defective for both CC1 and pre-mRNA retention in the nucleus, support previous data suggesting an early, ATP-independent function for Sub2. Detection of genetic interactions similar to that previously reported for a null allele of MUD2, between *sub2-1* and the *sub2* null allele and the *msl5-5* allele of BBP lend further support to the hypothesized subsequent, ATP-dependent function of Sub2. Consistent with a functional link to PS formation, *sub2* mutants also exacerbate the growth defects of mutant alleles of the splicing factors *PRP5*, *PRP9*, *PRP11*, and *PRP21*, which all exhibit defects in PS formation in yeast. Moreover, preliminary analysis suggests that disruption of *CUS2*, which allows for ATP-independent PS formation, also bypasses the requirement for Sub2 function in vivo. Intriguingly, this *Δsub2Δcus2* strain is only viable at low temperature. The splicing activity and spliceosome assembly phenotypes of extracts derived from a *Δsub2Δmud2* strain suggest suggest that growth defects detected in this strain may reflect a balance of affinity between BBP and the U2 snRNP for the BPS. These results support and refine our previous model for Sub2 function during pre-mRNA splicing and raise a

number of parallels between the recognition events occuring at the BPS to those at the 5' splice site before the first step of splicing.

INTRODUCTION

Genetic and biochemical analysis of *SUB2* described in Chapter 2 and elsewhere (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001) led us to hypothesize a role for Sub2 both before and after PS formation during splicing in yeast. Extracts genetically depleted of *SUB2* or derived from the *sub2-100* mutant are defective in splicing and blocked before formation of the ATP-independent commitment complex 2 (CC2) on the BPS of a pre-mRNA substrate. Additionally, deletion of MUD2 exacerbates the cold sensitive growth defect of the *sub2-5* (Kistler and Guthrie 2001). Taken together, these data suggest an early, ATP-independent function for Sub2, in collaboration with Mud2 (and perhaps BBP) to promote the formation or stabilization of CC2 at the branchpoint sequence (BPS). In contrast, independent biochemical analyses of the *sub2-1* and *sub2-201* mutant extracts demonstrate a defect in spliceosome assembly after PS formation (Kistler and Guthrie 2001; Libri et al. 2001). Deletion of MUD2 suppresses the temperature sensitive growth defect conferred by *sub2-1*. These data suggest that Sub2 may also function in a subsequent, ATP-dependent step antagonistic to Mud2 (and thus perhaps BBP) at the BPS to promote the formation of a productive prespliceosome (PS) complex. The observation that deletion of MUD2 bypasses the requirement for Sub2 function in vivo, supports this hypothesis, and led us to hypothesize a the existence of a Sub2-Mud2 independent splicing pathway (Kistler and Guthrie 2001). We speculate that splicing can occur in this pathway in the absence of Sub2 because BBP binding to the BPS is reduced in the absence of Mud2 (Berglund et al. 1998a). Under such conditions, Sub2 may no longer be required to displace BBP in order

for productive PS formation to occur. Rather, the U2 snRNP may compete directly with BBP for binding to the BPS of the pre-mRNA substrate.

These models for splicing in the presence and absence of Sub2 raised several experimentally tractable predictions. The model for splicing in the presence of Sub2, where Sub2 function in collaboration with and later antagonistically to Mud2 and BBP during pre-mRNA splicing, predicts that *sub2* mutants would interact genetically with mutant alleles of BBP. Moreover, if Sub2 is required to promote the removal of both BBP and Mud2 during splicing, we might also expect that like the null allele of MUD2, a null allele or mutant allele of BBP may be able to bypass the requirement for Sub2 function. This model also predicts that *sub2* mutants would interact genetically with mutant alleles of factors required for PS formation in yeast. It is likely that the growth phenotypes of such double mutants would be exacerbated, since two steps required for PS formation would be defective in this context. Thus, to assess our model for Sub2 function during splicing, we examined the genetic interactions between *SUB2* and a series of conditional alleles of BBP and several factors required for PS formation, *PRP5*, *PRP9*, *PRP11*, *PRP21*, and *CUS2*.

In our model for splicing in the absence of both Sub2 and Mud2, it is likely that splicing activity and spliceosome assembly in extracts derived from *Δsub2Δmud2* strains are sensitized to the relative binding affinities of BBP and the U2 snRNP for the BPS. The defects in growth detected in this strain at both high (37°C) and low (16°C) temperatures may reflect defects in splicing activity and spliceosome assembly due to the alterations in BBP binding and thus BPS accessibility for U2 snRNP association. To assess this model for a Sub2-Mud2 independent splicing pathway, we also examined the

in vitro splicing activity and spliceosome assembly phenotype of extracts derived from the *Δsub2 Δmud2* extract at both permissive and non-permissive temperature.

RESULTS

Prediction 1:

sub2 mutants will interact genetically with mutant alleles of BBP

Our observation of a distinct series of genetic interactions between *SUB2* and MUD2 led us to hypothesize that Mud2, and perhaps BBP, might be functional targets of Sub2. In our initial directed genetic analysis, we were unable to detect any genetic interaction between the *sub2* alleles and the only available allele of BBP, *msl5-1* (Abovich and Rosbash 1997). However, the *msl5-1* allele of BBP has no phenotype on its own (Abovich and Rosbash 1997). In light of this negative result, it remained unclear if the lack of detectable genetic interaction between *msl5-1* and the *sub2* alleles reflected a lack of a functional interaction between Sub2p and BBP or merely the weakness of the *msl5-1* allele. New conditional alleles of BBP have since been generated (Rutz and Seraphin 2000). Since these alleles have phenotypes on their own, it was likely that they might be more sensitized to perturbations in Sub2 function. We therefore compared the growth phenotypes of strains containing both the *sub2* mutants and these newly generated BBP conditional alleles.

We first examined the impact of *sub2* mutants on the growth defect of strains bearing the ts- *msl5-2* allele. Strains bearing both the *msl5-2* allele and the *sub2-5* allele were viable, and the growth defects of this double mutant were not significantly altered when compared to strains bearing either of the single mutants (Table 1; data not shown).

In contrast, the ts- *msl5-2* allele and the *sub2-1* allele were inviable at the permissive temperature (Table 1; data not shown). We were unable to recover a strain containing both these alleles at any of the temperatures tested (data not shown). Thus, it is likely that the combination of the *sub2-1* and *msl5-2* alleles is synthetically lethal.

In contrast to the *msl5-2* allele, the ts- growth defect of the *msl5-3* and *msl5-9* alleles was weakly suppressed by *sub2-1* (Table 1, Figure 2, middle panel). In contrast, the *sub2-5* allele significantly suppressed the ts- growth defect of these alleles (Table 1, Figure 2, bottom panel). Intriguingly, the combination of *sub2-5* with *msl5-3* had significant defects at lower temperatures. At temperatures less than or equal to 25°C, the growth of this double mutant was significantly slower than either of the single mutants combined (Table 1, Figure 1). The exacerbation of the cs- *sub2-5* defect is notable despite the fact that the cs- growth defect of *sub2-5* seems to be generally exacerbated by the presence of a conditional allele of BBP (Table 1). This exacerbation of the cs- growth by *msl5-3* appears to be specific to the *sub2-5* allele; the cs- growth defect of the *sub2-1* mutant is not similarly affected by the *msl5-3* allele (Table 1, Figure 1). Moreover, the *msl5-3* allele does not exhibit cold sensitive defects on its own. Finally, the *msl5-3* mutant is not the strongest ts- *msl5* allele tested. Thus it seems unlikely that the exacerbation of the cs- growth defect of *sub2-5* at the permissive temperature by *msl5-3* merely reflects a general additive effect of the combined growth defects of these two mutants. Rather, the detected genetic interactions between *sub2-5* and *msl5-3* seem to reflect an allele-specific interaction, consistent with a hypothesized functional interaction between these two factors.

A distinct set of genetic interactions was also detected between the *sub2* alleles and the cold sensitive allele, *msl5-5*. The *msl5-5* allele strongly suppresses the *sub2-1* ts- growth defect at 37°C, but does not affect the cs- growth (Table 1, Figure 2, middle panel). In the cold, the growth of this strain was similar to that observed in the *msl5-5* single mutant, which has a stronger cs- growth defect than the *sub2-1* single mutant (Table 1). Similarly, the *msl5-5* mutant only slightly exacerbates the *sub2-5* cs- growth defect at the semi-permissive temperature of 20°C, and does not exacerbate the growth of *sub2-5* at temperatures above 20°C (Table 1, Figure 1 and Figure 2). Thus, the *msl5-5* allele of BBP appears to be an allele-specific suppressor of the *sub2-1* allele. Taken together, these observations, as well as those described above, demonstrate the existence of distinct allele-specific genetic interactions between both *sub2-1* and *sub2-5* and these newly isolated alleles of BBP. These data provide genetic evidence for a functional interaction Sub2 and BBP during pre-mRNA splicing in yeast.

Prediction 2:

Mutation or deletion of BBP may also bypass the requirement for Sub2 function in vivo

The allele specific suppression phenotype of the *msl5-5* allele of BBP is reminiscent of the genetic interactions detected between *SUB2* and MUD2 (Kistler and Guthrie 2001). Our previous detection of bypass suppression of the lethality of a *SUB2* deletion conferred by a null allele of MUD2 raised the possibility that MUD2, or both MUD2 and BBP might be functional targets for *SUB2* during splicing. In this model, Sub2 promotes the removal of both BBP and Mud2 from the BPS, and by so doing, promotes the basepairing interaction of the U2 snRNP with the BPS to form a stable,

productive PS (Kistler and Guthrie 2001). Since disruption of BBP is lethal, it is unlikely that a null allele of BBP would similarly bypass the requirement for *SUB2* in vivo. However, recent genetic analysis have demonstrated that certain conditional alleles of the essential splicing factor U1C are capable of bypassing the requirement for the essential spliceosomal ATPase, Prp28 (Chen et al. 2001). To address whether deletion or mutation of BBP bypasses the requirement for Sub2 function in vivo, we tested the null allele of BBP and the available conditional alleles of BBP for growth in the absence of *SUB2*.

Strains bearing the wildtype *MSL5* and *SUB2* genes on a *URA3* marked plasmids, were transformed with an empty *LEU2* plasmid vector, or a *LEU2* marked plasmid bearing wildtype *MSL5* or the conditional *msl5* alleles. These strains were tested for growth in the absence of *SUB2* by streaking on 5-FOA-LEU media at different temperatures (Table 2 and Figure 3) to counterselect for the presence of both of the wildtype copies of the *SUB2* and *MSL5* on the *URA3* marked plasmids. As expected, the strain bearing the wildtype *MSL5 LEU2* plasmid was unable to grow in the absence of *SUB2* at any temperature tested (Table 2 and Figure 3). Likewise, the strain in which both *MSL5* and *SUB2* are deleted is also inviable under all temperatures tested (Table 2 and Figure 3). However, there was clear growth in the absence of *SUB2* in the strains bearing the *msl5-5* allele of BBP. In contrast, the other BBP alleles were unable to support growth in the absence of *SUB2* at any temperature tested (Table 2, and Figure 3). The *Δsub2 msl5-5* strain is able to grow at 23°C, 25°C, and 30°C. Introduction of WT-*MSL5* abrogrates the growth of this *Δsub2 msl5-5* strain (Figure 4), confirming that the viability of the *Δsub2 msl5-5* strain is due to specifically to the defective *msl5-5* allele. Thus it appears that a conditional allele of BBP can bypass the requirement for Sub2

function in vivo in a manner similar to that previously observed with a null allele of MUD2. The observation that loss of function alleles of both of these CC2 complex components can bypass the requirement for Sub2 bolsters our hypothesis that a function of Sub2 may be to remove or destabilize these factors at the BPS during splicing.

Prediction 3:

sub2 mutants may exacerbate PRP5, PRP9, PRP11, PRP21, and CUS2 mutant phenotypes

Genetic and biochemical characterization of a number of *sub2* alleles suggest that wildtype Sub2 may perform multiple functions during splicing, both before and after PS formation (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). Based on our genetic analysis, we have hypothesized that the ATP-dependent function of Sub2 may be to promote the removal of Mud2 and BBP bound at the branchpoint region to facilitate the productive interaction of the U2 snRNP with the pre-mRNA substrate. If this hypothesis is correct, it is likely that mutant alleles of *SUB2* would exacerbate the growth defects conferred by mutant alleles of genes known to be required for U2 snRNP association with the BPS. To test this prediction, we examined the impact of mutant alleles of *sub2* on the growth defect conferred by conditional alleles of a subset of factors required for PS formation.

The splicing factors Prp 5, Prp9, Prp11, and Prp21 (collectively termed "PS Prps" in this chapter) are all required for PS formation in yeast (Ruby et al. 1993). Prp 5 is a DEAD-box ATPase which has been implicated in modulating the accessibility of the branchpoint interacting region of the U2 (Dalbadie-McFarland and Abelson 1990; O'Day

et al. 1996). It interacts genetically with the U2 snRNA, Cus2, and Prp9, Prp11, and Prp21 (ref Ruby&Abelson; Perriman and Ares, 2000). Prp9, Prp11, and Prp21 are components of the U2 snRNP, homologous to components of the mammalian SF3a complex which is required for formation of the 17S form of the functional U2 snRNP (Krämer 1996). An additional factor, Cus2 interacts genetically with U2, Prp11, and Prp 5 (Yan et al. 1998; Perriman and Ares 2000). Deletion of *CUS2* has no growth phenotype, but allows for PS formation in the absence of ATP (Yan et al. 1998; Perriman and Ares 2000).

Both biochemical and genetic data have demonstrated that these factors interact with each other in a complex which in turn, interacts with U2 as part of the U2 snRNP (Ruby et al. 1993; Wells and Ares 1994; Krämer 1996; Wiest et al. 1996; Yan and Ares 1996; Yan et al. 1998). In yeast, each of the PS Prp factors are synthetically lethal with one another, as well as with U2 snRNA mutants defective for association with the BPS (Wells and Ares 1994; Yan and Ares 1996; Yan et al. 1998). Thus the growth phenotype of these PS Prps are definitely sensitized to the presence of factors which influence PS formation in yeast.

A link has also been detected between some of these PS Prps and factors required for the ATP-independent CC2 complex formation. Prp11 has been linked through 2-hybrid genetic interactions to Mud2 (Abovich et al. 1994). *CUS2* and *PRP11* also display 2-hybrid interactions with each other (Yan et al. 1998). Additionally, the S. pomble homolog of *CUS2* has also been reported to interact with the S. pombe homolog of U2AF65 (McKinney et al. 1997). Thus, ample genetic and biochemical data

suggested that these PS Prps might be good candidates to test in directed genetic analysis for functional interactions with Sub2.

Because the phenotypes of these PS Prps are so tight on their own (see Table 3), there is only a very small temperature window for investigation of the impact of the *sub2* mutants on the growth phenotype of the PS Prps. Nonetheless, we were able to detect an impact of the *sub2* alleles on the growth of the PS Prps at 20°C, 23°C, and 25°C. At all three temperatures, the *sub2-1* and the *sub2-5* mutations exacerbated the growth defects of these PS Prps (Figure 5-10). At 20°C and 23°C, the growth of *prp5-3*, *prp9-1*, *prp11-1*, and *prp21-1* were all exacerbated when combined with either mutant allele of *sub2* (Figures 5 and 7). There was also a slight exacerbation of the growth of the *prp5-1* strain at these temperatures (Figures 6 and 8). At 25°C, the exacerbation of growth defects of the *prp5* alleles and *prp9-1* allele by the *sub2* mutants was no longer detectable (Figures 9 and 10). However, the exacerbation of the growth defects of *prp11-1* and *prp21-1* persisted at 25°C (Figures 9 and 10). Not surprisingly, preliminary analysis did not reveal any bypass suppression of the *sub2* null allele by any of these conditional PS Prp mutants (data not shown).

Detailed analysis of the impact of the *sub2* alleles on the growth phenotype of a *CUS2* disruption strain has not been completed, but preliminary analysis of the impact of the *CUS2* disruption on the lethality of a *Δsub2* strain has been performed. Surprisingly, disruption of *CUS2* can bypass the requirement for Sub2, but only below room temperature (Figure 11). This is in contrast with the bypass suppression observed with both the *msl5-5* allele (Table 2) and the Δmud2 allele (Kistler and Guthrie 2001) which can grow between 20°C and 33°C. The viability of this *Δsub2 Δcus2* strain was

unexpected. However, the growth of this strain may reflect the increased sensitivity of the splicing pathway to variation in the relative binding affinities of BBP and the U2 snRNP for the BPS of a pre-mRNA substrate in the absence of Sub2 (see Prediction 4 below and Discussion). Further analysis will be required to confirm this result and clarify our understanding of the *Δsub2 Δcus2* viability. Regardless, taken together the genetic interactions detected between the *sub2* mutants in combination with these PS *prps* are consistent with our expectations for a functional role for Sub2 in the formation of a productive PS complex in yeast.

Prediction 4:

The growth phenotype of Δsub2Δmud2 strain may reflect the influence of temperature on the binding interactions of BBP with the BPS

Identification of a strain in which the requirement for Sub2 in vivo is bypassed by deletion of MUD2, led us to hypothesize the existence of a Sub2-Mud2 independent splicing pathway that is capable of supporting splicing and viability in yeast (Kistler and Guthrie 2001). However, the growth of the *Δsub2 Δmud2* strain is slow in comparison to a wildtype strain at the permissive temperature (Kistler and Guthrie 2001): the doubling time of the *Δsub2 Δmud2* strain is approximately 5 hours at 30°C in YEPD, compared to 1.5 hours for a wildtype *SUB2 MUD2* strain grown in the same media (data not shown). Additionally, the *Δsub2 Δmud2* strain is both ts- and cs- (Kistler and Guthrie 2001). Thus although the Sub2-Mud2 independent splicing pathway is capable of supporting viability, it is clearly unable to do so under all temperature conditions.

If the hypothesis that Sub2 normally functions both in collaboration with, and later antagonistically to Mud2 and BBP function is correct, we can make several testable predictions about the splicing phenotypes we might observe in the *Δsub2 Δmud2* extracts at different temperatures. In the Sub2-Mud2 independent pathway, the binding of BBP is likely to strongly influence the splicing activity and spliceosome assembly phenotype in the *Δsub2 Δmud2* extracts. At permissive temperature in these extracts, BBP binding is likely destabilized due to the absence of Mud2 (Berglund et al. 1998a) and perhaps also Sub2 (Kistler and Guthrie 2001; Zhang and Green 2001). Thus, in *Δsub2 Δmud2* extracts, the U2 snRNP may be able to compete directly with BBP for interaction with the BPS. However, potential interactions between Sub2 and Mud2 with the U2 snRNP that may facilitate PS formation are also lost in this context. Thus at permissive temperature, the observed growth defects may be due to an overall decrease in spliceosome assembly due to the absence of both Sub2 and Mud2, which in turn causes a decrease in splicing efficiency.

At higher temperatures, the BBP-BPS interaction might be further destabilized, due to an increase in the off-rate of BBP. As a consequence, the availability of the BPS of a pre-mRNA substrate would increase. In the absence of Sub2 and Mud2, decreased BBP binding to the BPS may actually facilitate the association of the U2 snRNP with the BPS of the pre-mRNA substrate. However, uncoupling the addition of the U2 snRNP from the normal Sub2-Mud2 dependent splicing pathway may lead to the formation of less active PS or non-productive PS complexes. In this case, we would expect to see an accumulation of PS complexes relative to that detected in extracts prepared from a wildtype strain. It is also likely that defects in splicing accuracy and efficiency due to

these prematurely added/less functional PS might be detected at high temperature in the *Δsub2 Δmud2* extracts.

Low temperature may also exacerbate the splicing efficiency defect in the Sub2-Mud2 independent pathway by influencing the binding of BBP with the BPS of a pre-mRNA substrate. In this case, it is possible that the low temperature may have a stabilizing effect on the interaction of BBP with the BPS, despite the absence of both Sub2 and Mud2. However, the absence of Sub2 in conditions where BBP binding to the BPS is relatively stable may prove deleterious for subsequent U2 addition, leading to decreased splicing activity and defects in spliceosome assembly before PS formation.

Analysis of the defects in splicing activity and spliceosome assembly at the permissive and non-permissive temperatures in the absence of Sub2 would directly test these predictions. Insight from such experiments may help to further confirm or refine the model for Sub2 function in pre-mRNA splicing. Thus, we compared the effect of temperature on the in vitro splicing activity and assembly of extracts derived from *Δsub2 Δmud2*, *SUB2Δmud2*, and wildtype strains grown in parallel at the permissive temperature.

1. Analysis of splicing activity at different temperatures

In preliminary analysis of a time course of the splicing activity of these extracts at room temperature (approximately 23°C), the first and second step of splicing appears slightly slowed in the *Δsub2 Δmud2* extract relative to the *SUB2 Δmud2* extract and the wildtype extract (Figure 12). In the *Δsub2 Δmud2* extract, the most easily detectable product of the first step of splicing, free exon 1, becomes detectable at 10 minutes,

whereas in the *SUB2 Δmud2* extract and wildtype extract this product is detected at the 5 minutes timepoint. Moreover, the appearance of excised lariat or mRNA, products of the second step of splicing, are not detected until the 15 minutes timepoint in the *Δsub2 Δmud2* extract. In contrast, at least one of these products is present as early as 5 minutes in both the *SUB2 Δmud2* extract and the wildtype extract. Taken together, these data suggest that the slow growth phenotype of the *Δsub2 Δmud2* strain at the permissive temperature may be due to a decrease in the overall efficiency of pre-mRNA splicing.

Further preliminary analysis of the splicing activity of the *Δsub2 Δmud2* extract was performed at both high and low temperatures. At 33°C, the splicing activity of *the Δsub2 Δmud2* strain appeared more similar to the other strains than at 23°C (Figure 13). In the timecourse at this temperature, the products of the first step of splicing, lariat intermediate and exon 1, were detectable at the 5 minute timepoint in all three extracts. The impact of the higher temperature on the second step of splicing in the *Δsub2 Δmud2* extract is hard to assess in this experiment. In all three extracts one of the products, excised lariat, is detectable at the 5 minutes time point. However, in the *Δsub2 Δmud2* extract this band is fainter than in the other extracts at this timepoint, possibly suggesting a lower efficiency of the second step. The other product of the second step, the mRNA, is first detectable at later time points, 10-15 minutes in the *Δsub2 Δmud2* extract. Unfortunately, degradation of the pre-mRNA and mRNA in both *the SUB2 Δmud2* extract and the wildtype extract obscures the detection of the mRNA product for accurate comparison.

Given that the growth of the *Δsub2 Δmud2* strain falls off with increased temperature, the apparent similarity in splicing activity among these three extracts was

not anticipated. It is possible that the lack of detectable splicing phenotype in *the Δsub2 Δmud2* extracts at 33°C merely reflects the requirement for higher temperature (eg, 37°C) to observe a ts- defect in splicing activity in vitro in these extracts. However, it is also possible that this lack of significant defect in splicing activity in the *Δsub2 Δmud2* at high temperature reveals that Sub2 function may be required for an essential function in vivo that is not rate limiting at high temperature in vitro. Results from analysis of the spliceosome assembly phenotype at this temperature are consistent with the former possibililty (see below). Parallel in vitro and in vivo splicing activity and assembly assays at higher temperatures will be required to unambiguously confirm these results.

At 16°C, splicing activity of both the *Δsub2 Δmud2* extract and the *SUB2 Δmud2* extract was reduced in comparison to the wildtype extract (Figure 14). Splicing products from either step are barely detectable in the *Δsub2 Δmud2* extract. At 15-20 minutes, a faint amount of lariat intermediate and excised lariat are detected in the *Δsub2 Δmud2* extract. The amount of these intermediates at these last two timepoints in the timecourse is less than can be detected in the wildtype extract at the 5 minute timepoint, suggesting a signifcant decrease in splicing efficiency at this temperature in the *Δsub2 Δmud2* extract. Although the last timepoint for the timecourse of the *SUB2*Δmud2 extract is missing, there also appears to be a slowing of splicing activity in this extract compared to the wildtype extract. The amount of splicing intermediates and products at the last timepoint obtained (15 minutes) in the *SUB2Δmud2* extract are only slightly greater than those detected in the 5 minute timepoint in the wildtype extract. This result was surprising, given that MUD2 is a non-essential gene. However, detection of a cs- defect associated with deletion of MUD2 has been reported recently (Rutz and Seraphin 2000). This cs-

growth defect of a Δmud2 strain has been suggested to reflect a function for Mud2 in pre-mRNA retention. However, these splicing activity results with the *SUB2 Δmud2* strain suggest the cs- defect previously detected in a Δmud2 strain could be due to a defect in a splicing function. At any rate, the further depression of splicing activity in *the Δsub2 Δmud2* extract suggests that loss of function of Sub2 in the cold has a significant impact on splicing activity at this temperature as well.

2. *Analysis of spliceosome assembly at different temperatures*

The spliceosome assembly phenotype of these extracts was also assessed by native gel electrophoresis (Cheng and Abelson 1987). At room temperature (Figure 15), the assembly profile of the *SUB2 Δmud2* samples was identical to the wildtype samples (data not shown). Consistent with the splicing activity results at room temperature, in the *Δsub2 Δmud2* extract the kinetics of the appearance of the PS (referred to as "complex B" in this gel system) and the splicesome (referred to as "complex A" in this gel system) were slowed. Additionally, the amounts of these splicing complexes were reduced relative to that detectable in the wildtype extract. Also, a larger fraction of the radiolabeled substrate in the *Δsub2 Δmud2* extract persisted in the heterogeneous (H) complex region of the gel than can be seen in the wildtype extract. While the functional significance of the H complex is unclear, these data raise the possibility that the slow growth phenotype and decreased splicing efficiency in the *Δsub2 Δmud2* extract are likely due to a decrease in PS formation or stability in the absence of Sub2 and Mud2.

At higher temperatures, the assembly profile of the *SUB2 Δmud2* samples was again identical to the wildtype samples (data not shown). At the earliest timepoint

examined in both extracts, the bulk of the radiolabeled RNA substrate was present in the spliceosomal A complex on the native gel (Figure 16, 5 minute timepoint and data not shown). In contrast, in the *Δsub2 Δmud2* extract, the bulk of the radiolabeled transcript was present in the prespliceosome complex B. The migration of this complex does appear to shift higher in the gel over time, consistent with the possibility that it is being converted to the spliceosomal complex A. However, a more discrete band corresponding to complex A is typically detected. Because there is so much complex B in these lanes, it is difficult to asses if the altered migration of this PS-like complex indeed reflects conversion to complex A, or merely a smearing of the band corresponding to complex B. If complex A formation is indeed occurring in the *Δsub2 Δmud2* extract, the kinetics of this process are slowed relative to the kinetics in the wildtype extract, despite the fact that surprisingly large amounts of PS complex are detected under these conditions. Although there is still transcript present in the region of the gel corresponding to the H complex in the *Δsub2 Δmud2* extract, it appears to have chased into PS complex much more efficiently than at room temperature. However, an additional unidentified complex (*), which migrates just below the PS complex can be detected in both extracts. This complex appears to persist in the *Δsub2 Δmud2* extract compared to the wildtype extract. Whether the detection of this complex is reproducible and if so, what factors are present in this complex remains to be addressed. In summary, this preliminary analysis of spliceosome assembly at 33°C demonstrates that a PS or a PS-like complex accumulates during spliceosome assembly in the *Δsub2 Δmud2* extract at 33°C. These observations raise the possibility that at high temperature, the growth defect of the *Δsub2 Δmud2* strain may be due to the accumulation of non-functional or stalled PS complexes.

At 16°C, formation of both PS complex B or spliceosomal complex A is barely detectable in the *Δsub2 Δmud2* extract over this timecourse of 30 minutes (Figure 17). A very small amount of PS complex B can be detected at the 30 minutes time point in the *Δsub2 Δmud2* extract, corresponding to only slightly more than can be detected at the 5 minutes timepoint in the wildtype extracts. Almost all the radiolabeled transcript migrates as a large smear in the H complex region of the gel. Another distinct difference is the stability of an additional complex (*) which migrates below the PS complex and is detected in all three extracts. In the *Δsub2 Δmud2* extract extract, this complex appears to disappear over time, whereas it persists in the other extracts.

In contrast, in wildtype extracts, a significant portion of the radiolabeled transcript appears to be incorporated into spliceosomal complexes at 16°C. PS complex B can be clearly detected after 10 minutes. While the kinetics of spliceosomal complex A formation are difficult to assess with this gel, due to the smearing migration of PS complex B, there does appear to be a higher molecular weight complex migrating just below the wells in this extract that may correspond to complex A. Thus, it is likely that the kinetics of the appearance of spliceosomal complexes formed at this temperature are much slower than at other temperatures even in wildtype extracts. Longer incubations of transcript in *Δsub2 Δmud2* extract may be required to obtain more insight on the defect in spliceosomal complex formation in the *Δsub2 Δmud2* extracts.

Interestingly, the *SUB2 Δmud2* extract also appears to have a significant defect in both PS complex formation and spliceosomal complex A formation. The defect in this extract is not as severe as the *Δsub2 Δmud2* extract; however, it is quite significant in comparison to the wildtype extract. Like the *Δsub2 Δmud2* extract, a large fraction of the

transcript present in this extract also migrates as a large smear in the H complex region of the gel. Additionally, the (*) complex is also present, and persists over time. Overall, this extract appears to have a phenotype consistent with a defect in PS formation in the cold. This result was surprising, although as mentioned above, previous data have implicated a cs- growth defect detectable in certain strain backgrounds in which MUD2 is deleted (Rutz and Seraphin 2000). Moreover, loss of BBP and MUD2 have been implicated in affecting the efficiency of PS formation in vitro (Rutz and Seraphin 1999). Finally, the splicing substrate used in this experiment does not efficiently form CC2 (Ruby and Abelson 1988). This raises the possibility that this splicing and assembly phenotype may not have been detected previously because most in vitro analyses of Δmud2 extracts have been performed with transcripts that form both CC1 and CC2 relatively efficiently (Legrain et al. 1988; Séraphin et al. 1988; Séraphin and Rosbash 1991b). Ultimately, these results will require confirmation, but these preliminary observations raise the possibility that splicing activity and spliceosome assembly in the *SUB2 Δmud2* extract in this strain background is severely affected in the cold. The additional loss of Sub2 function appears to further decrease the splicing efficiency and PS formation, as seen with the *Δsub2 Δmud2* extract.

In summary, the preliminary analysis of the splicing efficiency and assembly phenotypes in the *Δsub2 Δmud2* extract appear consistent with our predictions for a pathway strongly influenced by temperature, in a pattern that may reflect a competitive binding interaction at the BPS between BBP and the U2 snRNP. At low temperatures, the splicing activity and spliceosome assembly phenotypes are drastically reduced prior to PS formation. These results are consistent with our expectation that low temperatures

would stabilize and result in a defect in PS formation in the absence of Sub2. Permissive temperature splicing activity and assembly results in an intermediate phenotype. Splicing activity kinetics are slowed, and spliceosome assembly proceeds, but the kinetics of the appearance of these spliceosomal complexes are slowed relative to wildtype extracts, and the amount of the complexes detected in the *Δsub2 Δmud2* extract are also lower than observed in a wildtype extract. These observations are consistent with an intermediate effect of the temperature on the binding of BBP in the absence of Sub2 and Mud2. At this temperature, BBP binding may be sufficient to allow for relatively stable CC2 complex formation, but sufficiently destabilized to allow for U2 snRNP addition in the absence of Sub2. In contrast, at high temperatures, splicing efficiency is increased and PS formation is not slowed, perhaps due to the destabilizing effect of increased temperature on the BBP-BPS interaction. However, the PS complexes formed in the *Δsub2 Δmud2* extract at high temperature accumulate, and their conversion to spliceosome complexes may be slowed. Thus, although U2 snRNP can add more readily to the BPS under these conditions, it may do so prematurely or less accurately in the *Δsub2 Δmud2* extract, resulting in formation of less productive or less functional PS complexes.

DISCUSSION

The experiments described in this chapter were performed to assess the model for Sub2 function in pre-mRNA splicing (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). The detection of distinct genetic interactions between the newly isolated conditional alleles of BBP (Rutz and Seraphin 2000) and the previously

characterized *sub2-1*, *sub2-5* and the *sub2* null alleles (Kistler and Guthrie 2001) support the hypothesis that Sub2 functionally interacts with BBP during pre-mRNA splicing in yeast. Observation of two different types of allele-specific interactions between *sub2-5* and *sub2-1* and two different classes of *msl5* alleles also further supports and extends our hypothesis that these *sub2* alleles are defective for different functions that the wildtype Sub2 performs in vivo. Detection of genetic interactions between the *sub2* alleles and the PS *prps* also are consistent with our model for Sub2 function, although the unexpectedly mild genetic interactions between these factors raises a number of questions about the nature of the functional role that Sub2 plays during PS formation. Finally, preliminary results from in vitro analysis of the splicing activity and assembly phenotypes in extracts derived from the *Δsub2 Δmud2* strains conform to our expectations based on our model for a Sub2-Mud2 independent splicing pathway. The implications of the results of each of these experiments are described in detail below. A refined model for Sub2 function is also proposed.

Implications of allele-specific interactions of sub2-1 with msl5-5 and msl5-2

Based on in vitro and in vivo analysis, the ts- *msl5* alleles have been proposed to function in splicing at the level of pre-mRNA retention in the nucleus (Rutz and Seraphin 2000). The authors suggest that this may be the general function of BBP in addition to a previously detected influence on the kinetics of later stages of spliceosome assembly (Rutz and Seraphin 1999; Rutz and Seraphin 2000). Consistent with the latter possible function, the *msl5-5* allele affected both CC2 formation and subsequent PS formation in vitro at low temperature and has only a mild impact on pre-mRNA retention in vivo

(Rutz and Seraphin 2000). Thus, it is likely that the two different classes of *msl5* alleles exhibit different levels of defects with regard to pre-mRNA retention and pre-mRNA splicing function of BBP.

It may be particularly telling that the *sub2-1* allele, which has been demonstrated to play a role in splicing in vitro, strongly interacts with only with the *msl5-5* allele, while the *sub2-5* allele, which has no detectable in vitro splicing phenotype, does not. The detection of significant genetic interactions between *sub2-1* and *msl5-5* is consistent with our previous results implicating a defect in a splicing function for the *sub2-1* mutant (Kistler and Guthrie 2001). Moreover, the ability of the *msl5-5* allele to bypass the requirement for Sub2 function in vivo further implicates this allele of BBP to a splicing function linked to Sub2 function. These genetic interactions mirror those we detected between *SUB2* and the null allele of MUD2 (Kistler and Guthrie 2001), and further support our hypothesis that Sub2 may function to promote the removal of both Mud2 and BBP from the branchpoint region of the pre-mRNA substrate.

Sequence analysis of the *msl5-5* mutant provides further support for this possibility. Although the *msl5-5* allele we examined in our genetic studies contains 13 mutations, the only mutations required to confer the cs- growth defect reside in the KH domain and the STAR-QUA2 domains of the *MSL5* sequence. These 2 essential mutations fall in regions of these domains highly conserved among all the BBP homologs from yeast to humans (Abovich and Rosbash 1997; Rain et al. 1998; Rutz and Seraphin 2000) (Berglund et al. 1998b), and have been implicated to play a role in RNA binding and specificity in yeast BBP (Berglund et al. 1997; Berglund et al. 1998b; Rain et al. 1998; Rutz and Seraphin 2000). The other 11 mutations fall in regions of the *MSL5* ORF

that are less phylogenetically conserved. Therefore it is likely that the defects conferred by *msl5-5* result from decreased binding activity and binding specificity of BBP with the BPS. Thus, the ability of this *msl5-5* allele to bypass the requirement for Sub2 function in vivo seems highly consistent with our hypothesis that Sub2 may function to promote the removal of BBP as well as MUD2 from the BPS during spliceosome assembly.

The synthetic lethal interaction detected between *sub2-1* and *msl5-2* was also striking. This synthetic lethal interaction could reflect a bona fide allele-specific interaction that might provide further insight into the functions of both BBP and Sub2 during splicing. However, the allele used in our studies was actually the original *msl5-2* isolate containing 17 mutations in conserved and non-conserved regions through the entire BBP ORF (Rutz and Seraphin 2000). This allele was the most severe of the ts- BBP alleles isolated. This raises the possibility that the synthetic interaction we detect between the ts- *sub2-1* and this ts- allele of *msl5-2* may simply reflect an additive effect of combining these two ts- alleles. We have recently received the *msl5-2* allele containing the three minimal mutations required to confer the ts- growth defect. These mutations are found in conserved residues in two distinct functional domains of BBP, the U2AF65/Mud2-interacting domain and the KH domain (Rutz and Seraphin 2000). Repeating the analysis of the *sub2-1* and *msl5-2* growth phenotype with this version of the allele will likely clarify the genetic interaction and possible functional link between these two mutants.

Implications of allele specfic interactions of sub2-5 with msl5-3 and msl5-9

Although no clear function has been identified for the *sub2-5* allele through vitro splicing assays, it appears to have a significant impact on the growth of the *msl5-3* and *msl5-9* alleles, while *sub2-1* has only a minor effect on the phenotypes of these alleles. Interestingly, the *msl5-3* and *msl5-9* alleles also do not have a significant impact on splicing activity in vitro (Rutz and Seraphin 2000). The *msl5-3* and *msl5-9* extracts are clearly defective in formation of the ATP-independent CC2 complex; however, this defect does not appear to affect the formation of the PS complex, or in vitro splicing activity in general (Rutz and Seraphin 2000). In contrast, in vivo experiments demonstrate that the *msl5-3* and *msl5-9* mutants are weakly defective for splicing of pre-mRNAs containing splice site mutations, and severely defective in nuclear retention of pre-mRNA (Rutz and Seraphin 2000). Thus, it is possible that the detection of significant genetic interactions between *sub2-5* and *these msl5* alleles may explain the previous failure to detect a splicing defect in vitro with this allele of *SUB2* (Kistler and Guthrie 2001). It may be that the genetic interactions between *sub2-5* and BBP and MUD2 reflect a distinct defect in a very early function of Sub2, which affects pre-mRNA retention or stability in the nucleus rather than in the splicing reaction per se.

Implications of genetic interactions between sub2 and PS Prps

The results of our tests for genetic interactions between *SUB2* and factors required for PS formation were also consistent with our hypothesis for the function of Sub2 in splicing. Exacerbation of the growth phenotypes conferred by the PS Prps when combined with the *sub2* alleles was observed in our directed analysis of *SUB2* genetic

interactions. The growth defects of *prp9-1*, *prp11-1*, *prp21-1*, and both alleles of the DEAD box ATPase *PRP5* were mildly exacerbated at both 20°C and 23°C by the presence of the *sub2* alleles. However, the mildness of this phenotype was somewhat surprising, given our hypothesis that Sub2 functions in an ATP-dependent step to promote the formation of a productive PS by removal of Mud2 and BBP from the BPS. It may be that the mild exacerbation of growth defects reflects the possibility that the functional interaction between Sub2 and the U2 snRNP is less direct than that between Sub2 and its putative targets, Mud2 and BBP.

The mildness of the *sub2*-PS *prp* interactions also raises the possibility that the hypothesized function that Sub2 plays may not actually be fully required under all conditions for PS formation. This possibility is supported by the observation that deletion of *CUS2* allows for the formation of the PS in the absence of ATP in vitro (Perriman and Ares 2000). This Δ*cus2* PS is functional; upon addition of ATP it can be chased to spliceosome complexes (Perriman and Ares 2000). Thus, in this context our hypothesized requirement for an ATP-dependent removal of BBP and Mud2 from the BPS appears to have been bypassed in vitro. Consistent with this possibility, preliminary data suggests that deletion of *CUS2* can bypass the requirement for Sub2 function at low temperature. It has been proposed that the loss of Cus2 function allows the U2 snRNP to adopt a conformation that is capable of adding to naked BPS, or capable of competing for BPS binding with BBP and Mud2 in the absence of ATP (Perriman and Ares 2000). Indeed, mutations in U2 snRNA predicted to alter the structure of the U2 snRNA phenocopy the effect of Δ*cus2*, in that they also allow for PS formation in the absence of ATP (Perriman and Ares 2000).

However, although ATP is not required for PS formation in the Δ*cus2* extract, the non-hydrolyzable ATP analog AMP-PNP depresses PS formation, and addition of ATP has a stimulatory effect on the kinetics and extent of PS formation in these extracts (Perriman and Ares 2000). Although the ATPase function of the spliceosomal DEAD-box ATPase Prp5 likely contributes to these ATP-dependent effects on the Δ*cus2* PS complex, Sub2 may also influence the formation of the Δ*cus2* PS in the presence of ATP. Further analysis of the genetic interactions between the *sub2* alleles and available *CUS2* alleles may shed additional light on this possible interplay of functions between these two factors.

An alternative explanation for the mild genetic interactions between *SUB2* and PS Prps may also reflect the possibility that a synthetic lethal interaction is not necessarily clearly predicted, based on our hypothesis for the function of the PS Prps and Sub2 during splicing. We had expected a strong synthetic lethal interaction, especially in the context where two ATPases defective in independent functions which both contribute to PS formation were combined. However, it may be that the PS prp mutant phenotypes are epistatic to the *sub2* mutant phenotypes. For example, if Prp5 cannot function to modulate the U2 snRNA in the U2 snRNP to enable it to basepair with the BPS, the availability of the BPS is likely a downstream event irrelevant in terms of its effect on the growth defects conferred by the PS Prps.

Finally, it is also possible that the mild exacerbation of the PS *prp* mutant phenotype by the *sub2* alleles may imply that the hypothesis for a functional role for Sub2 in promoting the formation of a productive PS complex is incorrect. It may be that Sub2 actually functions at a later step in splicing, after PS formation. This possibility is

consistent with the accumulation of PS-like complexes observed in heat-inactivated *sub2-1* and *sub2-201* extracts (Kistler and Guthrie 2001; Libri et al. 2001). Biochemical experiments demonstrate that the activity defect conferred by both *sub2-1* and *sub2-201* can be chased (Kistler and Guthrie 2001; Libri et al. 2001). However, the kinetics and efficiency of the chase activity were not extensively examined in either of these experiments. Moreover, the assembly phenotype was not addressed in parallel in these chase experiments. Thus, it remains to be rigorously determined if these alleles are defective for a Sub2 function that influences the formation of a productive PS (Kistler and Guthrie 2001), rather than a defect in Sub2 function after PS formation. Further detailed biochemical analysis of the kinetics of the splicing activity and PS complex chase activity in these extracts and the *Δsub2 Δmud2* extracts will likely prove helpful in distinguishing these two different hypotheses for Sub2 function during splicing.

Implications of splicing activity and assembly phenotypes in Δsub2Δmud2 extracts

Biochemical analysis of the splicing activity and spliceosome assembly phenotypes in extracts derived from a *Δsub2 Δmud2* strain provided an additional opportunity to test our models for Sub2 function in splicing. In general, the splicing and assembly phenotypes detected in vitro in this extract were consistent with our expectations for a Sub2-Mud2 independent splicing pathway that is strongly influenced by possible changes in the binding interaction between BBP and the BPS due to temperature. Direct evidence for such hypothesized changes in the association of BBP with the BPS of a pre-mRNA substrate in these extracts would bolster such conclusions considerably. Analysis of the in vitro phenotype of this extract on the RP51A transcript,

on which both PS formation and CC formation can be assessed, would also provide an independent, and perhaps more informative, means to verify this interpretation of the impact of temperature on the Sub2-Mud2 independent splicing pathway.

A refined model for Sub2 function in pre-mRNA splicing

On the whole, the results obtained from the experiments described in this chapter may have moved our understanding of the function of Sub2 during pre-mRNA splicing a step forward. Although a significant amount of follow-up biochemistry will be required to confirm the interpretations of the preliminary genetic and biochemical results described here, these analyses strongly indicate that Sub2 does indeed play multiple roles during pre-mRNA splicing. A refined model based on the results presented here and recent work on an analogous series of interactions at the 5'ss is presented in Figure 18 and 19.

I. A role for Sub2 in regulating BPS fidelity through RNA transport?

While our understanding of the specific mechanistic function that Sub2 plays during splicing remains unclear, our results from genetic analysis implicate a significant interaction between Sub2, Mud2, and BBP at an early stage of BPS recognition during pre-mRNA splicing. Recent work on BBP has raised the possibility that efficient BPS recognition may be an important decision point in the RNA processing pathway of a nascent pre-mRNA molecule. In the absence of fully functional BBP, the leakage of pre-mRNA molecules to the cytoplasm is significantly elevated (Rutz and Seraphin 2000). The strong genetic interactions between the *sub2-5* allele and *msl5* alleles defective for

pre-mRNA retention, suggest that Sub2 may also be involved in this early decision point during pre-mRNA processing. Thus the hypothesized early, ATP-independent Sub2 function in collaboration with BBP and Mud2 to stabilize the CC2 complex on the pre-mRNA substrate, may serve to both retain a pre-mRNA transcript in the nucleus and target it to the splicing pathway. It is possible that such a function for Sub2 may play a role in the fidelity of recognition of the BPS by regulating a discard pathway that occurs at the level of RNA transport and/or nuclear turnover (Figure 18). In this scenario, Sub2 may effectively act as a "timer" in a kinetic proofreading role similar to that proposed for Prp16 and the conformational change of the lariat intermediate at the second step of splicing. However, in this case, Sub2 is effectively monitoring the stability of the BBP-Mud2-BPS interaction in order for forward progression of splicing, rather than a conformational change in the substrate.

II. Striking parallels between early recognition events at the 5'ss and BPS

The genetic and biochemical results obtained through these experiments also suggest that the recognition events at the BPS may share a number of parallels to the switch of RNA-RNA interactions required for accurate recognition of the 5'ss during pre-mRNA splicing. In fact, it is possible to make a direct analogy between the recognition events at these two splice sites (Figure 19). At the 5'ss, U1 interacts in an ATP-independent, sequence specific manner with the nucleotides of the 5'ss consensus sequence (Zhuang and Weiner 1986; Séraphin et al. 1988; Siliciano and Guthrie 1988; Rosbash and Séraphin 1991). This interaction is stabilized by an accessory protein component of the U1 snRNP, U1C (Tang et al. 1997). In an ATP-dependent step, a

spliceosomal DEAD box protein, Prp28 is required to catalyze the exchange of the U1 basepairing interaction with the 5'ss for a U6 basepairing interaction with the 5'ss (Staley and Guthrie 1999). Prp28 is likely associated with the tri-snRNP, and thus perhaps targetted to act at the 5'ss through the association of the tri-snRNP with the pre-mRNA substrate (Staley and Guthrie 1999). This region of the U6 snRNA is not likely to be basepaired to itself or any other snRNA in the triple snRNP (Brow and Guthrie 1988; Madhani and Guthrie 1994). However, progression of this tri-snRNP associated with the 5'ss to a functional spliceosomal complex requires disruption of the extensive basepairing interaction between U4 and U6 in the tri-snRNP (Konarska and Sharp 1986; Cheng and Abelson 1987; Konarska and Sharp 1987; Madhani and Guthrie 1992; Madhani and Guthrie 1994). Another tri-snRNP associated DExH-box ATPase, Brr2 has been implicated in this function (Raghunathan and Guthrie 1998a).

At the BPS, BBP interacts in a similar, ATP-independent, sequence specific manner with the nucleotides of the BPS consensus (Berglund et al. 1997). This sequence-specific interaction is also stabilized by another protein, Mud2 (Berglund et al. 1997; Berglund et al. 1998a). From our genetic analysis and independent biochemical analysis, it appears that Sub2 also may play a stabilizing role in the initial stages of its interactions with these factors bound at the BPS in the absence of ATP (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). In an ATP-dependent step, Sub2 may be involved in promoting the exchange of the BBP-Mud2 complex bound to the BPS for the U2 snRNA basepairing interaction at the BPS (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). Similar to U6 in the tri-snRNP, it is likely that the region of U2 snRNA which basepairs with the BPS is not accessible. Again, although

this region is predicted to be single stranded in the U2 snRNA (Guthrie and Patterson 1988), remodeling of the U2 snRNP by a DEAD box ATPase is necessary for this step of splicing in an otherwise wildtype strain. In this case, the spliceosomal ATPase Prp5 is required for PS formation (Ruby et al. 1993) and has been shown to influence the accessibility of the BPS interacting region of the U2 snRNA in vitro (Dalbadie-McFarland and Abelson 1990; O'Day et al. 1996; Wiest et al. 1996). Thus, in recognition events at the BPS, Prp5 may affect the U2 snRNA in a manner analogous to the way that Brr2 affects the triple snRNP at the 5'ss.

Although the switch of U1 and U6 at the 5'ss is an active process demonstrated to require Prp28 and ATP (Staley and Guthrie 1999), the affinities of different factors which interact with the 5'ss influence this exchange. Hyperstabilization of the U1-5'ss can be overcome by either loss of function of U1C (ref Chen et al, 2001), or extension of the U6-5'ss basepairing interaction (Staley and Guthrie 1999). Hyperstabilization of this interaction is also exacerbated by defects in function of Prp28 (Staley and Guthrie 1999). As previously mentioned, loss of function of Prp28 can be bypassed by mutations in U1C which decrease its ability to interact with, and thus stabilize, the U1-5'ss interaction (Chen et al. 2001). Additionally, reduction in the basepairing interaction of U1 with the 5'ss can suppress the growth defects conferred by a mutant allele of Prp28 (Staley and Guthrie 1999) (Chen et al. 2001). It is likely that this balance of affinities provides a mechanism by which the 5'ss can be inspected multiple times during spliceosome assembly, thus enhancing the accuracy of recognition and splicing fidelity.

A similar balance of affinities among the components that interact with the BPS may be inferred from our genetic analysis of interactions between Sub2 and the

analogous BPS factors. Reminiscent of the genetic interactions detected between mutant alleles of U1C and *PRP28*, the null allele of MUD2, which likely destabilizes the interaction of BBP-BPS interaction, can suppress the growth defect of the *sub2-1* allele (Kistler and Guthrie 2001). Consistent with this possibility, the *msl5-5* allele of BBP, which likely destabilizes the BBP-BPS interaction, also suppresses the growth defect of the *sub2-1* allele. Moreover, as observed with U1C and Prp28, both of these MUD2 and BBP mutants can bypass the requirement for Sub2 function in vivo.

In contrast, mutations in Prp5, which may decrease the accessibility of the U2 snRNA BPS interacting region (O'Day et al. 1996; Wiest et al. 1996), exacerbate the cold sensitive growth defects of the *sub2* mutants. This interaction may reflect a relative stabilization of the BBP-BPS interaction, reminiscent of the exacerbation of the growth defect of a *prp28* mutant due to increased basepairing between U1-5'ss interaction. Disruption of *CUS2*, a non-essential splicing factor that affects the U2 snRNA structure (Yan et al. 1998), appears to have the opposite effect, in that it allows for growth in the absence of Sub2 in the cold. As described above, the absence of Cus2 allows for U2 association with the BPS in the absence of ATP (Perriman and Ares 2000). It is possible that the altered conformation adopted by U2 in the absence of Cus2 effectively increases the affinity of the U2 snRNP-BPS interaction such that it can compete for BPS binding with BBP and Mud2 more efficiently in the absence of ATP or Sub2. A similar balance of affinities was demonstrated between U1 and U6 at the 5'ss (Staley and Guthrie 1999). In the context of a hyperstabilized U1-5'ss basepairing interaction, increasing the basepairing potential of U6 was observed to suppress the splicing defects conferred by U1-5'ss hyperstabilization.

These possible similarities between early recognition of the 5'ss and BPS that occur before the first step of splicing are particularly striking. Taken together, these parallels further bolster the notion that the requirement for ATP during pre-mRNA reflects a mechanism through which the short splice site consensus sequences of introns can be recognized multiple times before assembly and activation of the catalytic core of the spliceosome. It is likely that evolution of a requirement for ATP during spliceosome assembly and activation was advantageous. Such a requirement allows for the possibility of multiple opportunities for fidelity maintenance during the process of pre-mRNA splicing as well as the potential for rapid post-transcriptional regulation of gene expression.

Future directions: addressing remaining unanswered questions on the function of Sub2

As appealing as this analogy may be, several questions remain with regard to the specific function of Sub2. How Sub2 may potentially function in pre-mRNA retention and splicing in collaboration with BBP and Mud2 remains an open question ripe for detailed analysis. The *Δsub2 Δmud2* extracts offer a potentially powerful system in which to directly test the impact of mutations in the different helicase domains of Sub2 on the splicing activity and spliceosome assembly phenotypes of these extracts. Directed mutations in the conserved motifs of the helicase domain of Sub2 could be generated. These mutant versions of Sub2 could be tested for phenotypes in yeast, and directly purified from yeast or produced recombinantly in bacteria and purified. Such proteins could be used for add-back and complementation experiments in the *Δsub2 Δmud2* extracts, as well as other minimal assay systems described below.

Further inroads into these questions remaining related to Sub2 function may also be made through cytological and biochemical analysis of the *sub2* alleles in both wildtype and well as the *msl5* mutant strain backgrounds. Examination of RNA localization by in situ hybridization studies of poly A+ RNA would likely address the impact of *sub2* on RNA transport in the presence and absence of the different alleles of BBP. Additionally, the activity of the pre-mRNA retention reporters utilized in the analysis of the *msl5* mutants could also be studied in the *sub2* strains to specifically assess the cytoplasmic leakage of pre-mRNA in *sub2* mutants on their own or in combination with the different alleles of BBP. Primer extension analysis of these reporters could be carried out in parallel with such studies to test the possibility that *sub2* may be act as a trigger for a discard pathway involving either nuclear or cytoplasmic degradation.

Analysis of the protein-protein interactions and protein-RNA interactions of BBP, Mud2, and most importantly, Sub2, in wildtype and mutant *sub2* strain backgrounds may also uncover important clues to the alterations of interactions among these factors in the context of different *sub2* alleles. Such studies might uncover unanticipated protein-protein or protein-RNA interactions that might further elucidate the functions of Sub2 in splicing, pre-mRNA retention, or possible connections with potential discard pathways.

Affinity isolation of CC2 complexes from *sub2* mutant extracts and analysis of their protein components, stability, and the ability of purified Sub2 protein to bind or disrupt these complexes would provide a direct test of our hypothesized functions for Sub2. Similar studies have been performed with success in the case of the DEAD-box ATPase Mot1, which was demonstrated to bind to TBP-DNA complexes in the absence

of ATP, and disrupt these interactions in the presence of ATP (Auble et al. 1994; Auble et al. 1997).

Biochemical analysis of the splicing activity and spliceosome assembly phenotypes in the exacerbated *sub2*-PS *prp* double mutants, as well as the suppressed *sub2-msl5* mutants may also provide more detailed information regarding the specific functional interplay of these factors. Analysis of directed genetic interactions between the *sub2-1* allele and U2 mutants defective for PS formation or a less biased screen against a bank of U2 mutants might also prove useful in testing our understanding of the spliceosome assembly phenotype of this allele of *SUB2*. Further analysis of the genetic interactions of the *sub2* alleles with the *CUS2* null allele may also prove useful in evaluating our current model for Sub2 function.

Further directed and open-ended genetic studies with the *sub2-1* and *sub2-5* mutants might also provide additional inroads to understanding the function(s) of Sub2. Directed genetic analysis with *sub2-1* and factors known to be involved with triple snRNP dynamics would directly address the untested possibility that Sub2 might have additional functions in splicing after PS formation. It would be particularly interesting to examine the genetic interactions with *PRP24* in this regard. *PRP24* was identified independently in two genetic screens. The original temperature sensitive isolate of *PRP24, prp24-1* is defective for splicing at the non-permissive temperature in vivo (Vijayraghavan et al. 1989). Additional alleles of *PRP24* were identified as suppressors of a cs mutation in U4 snRNA which destabilizes the U4/U6 di-snRNP (Shannon and Guthrie 1991). Based on a series of in vitro and vivo studies, *PRP24* has implicated in annealing the U4 and U6 snRNA components of the spliceosome (Strauss and Guthrie

1991; Ghetti et al. 1995; Raghunathan and Guthrie 1998b). The U4-U6 annealing activity of Prp24 is required to regenerate a functional U5.U4/U6 particle for multiple rounds of splicing in vitro (Raghunathan and Guthrie 1998b). Interestingly, an allele of *PRP21*, *prp21-2* was identified as an allele-specific extragenic suppressor of the growth defect conferred by the *prp24-1* mutant (Vaidya et al. 1996). This allele of *prp21-2* appears to suppress both the splicing defect and the decrease in U6 snRNA levels observed in the *prp24-1* mutant at the non-permissive temperature (Vaidya et al. 1996). It has been hypothesized that the *prp21-2* mutant may suppress the defects conferred by *prp24-1* through an indirect effect, likely via an impact on the U2-U6 interactions required for splicing activity that are mutually exclusive with U4-U6 interactions in the triple snRNP (Vaidya et al. 1996). Examination of the genetic interactions between *sub2* mutants and available *prp24* alleles, as well as the novel *prp21-2* allele may elucidate a function for Sub2 in steps of spliceosome assembly where functional links between the U2 snRNP and tri-sRNP have ben observed.

Likewise, analysis of directed genetic interactions with Brr2 might also yield significant defects when combined with *sub2* mutants. An allele of *BRR2* was isolated independently on the basis of its synthetic lethal phenotype with mutations in stem-loop Ia of the U2 snRNA, suggesting that Brr2 may be involved in the exchange of U4 interactions for U2 interactions with U6 during splicing (Xu et al. 1996). Defects in coordination of such interactions between the triple snRNP and U2 snRNP during spliceosome assembly might lead to a block in tri-snRNP association or accumulation of PS complexes as we observe in vitro with *sub2* mutants. Thus analysis of the interactions between *sub2* alleles and the different alleles of *BRR2*, in particular the *slt22-1* allele (Xu

et al. 1996), may also provide insight into a possible function for Sub2 after PS formation.

Similar studies with *PRP28*, another DEAD box ATPase linked to both the triple snRNP and U1 (Strauss and Guthrie 1991; Strauss 1992; Strauss and Guthrie 1994; Staley and Guthrie 1999)may also provide a useful venue for directed genetic analysis with *sub2* mutants. Sub2 has been linked to the U1 snRNP through its ability to suppress a cold sensitive growth defect due to deletion and tagging of two U1 snRNP components, NAM8 and PRP40, respectively (Libri et al. 2001). Although this link to the U1 snRNP may solely reflect the function of Sub2 at early stages of splicing, before PS formation, it may also reveal a connection to a function with Prp28 activity after PS formation. Thus, directed genetic analysis of the interactions of *PRP28* and *SUB2* may also provide further insight into the defect in PS progression detected in the *sub2-1* and *Δsub2 Δmud2* extracts at high temperature.

Based on the link to pre-mRNA retention that we detect for *sub2* through the allele-specific interactions we observed between *sub2-5* and *msl5-3*, directed analysis between *sub2-5* and mutants involved in the cytoplasmic non-sense mediated decay pathway may also be a fruitful endeavor for directed genetic analysis. Deletion of the non-essential UPF1 gene is of particular interest, since this factor has been demonstrated to be involved in degrading pre-mRNA in the cytoplasm (He et al. 1993; Long et al. 1995). Moreover, deletion of UPF1 is lethal in combination with the *msl5-3* and *msl5-9* alleles (Rutz and Seraphin 2000). Since *sub2-5* suppresses these *msl5* alleles, it would be of interest to determine the impact of the null allele of UPF1 on the phenotype of the *sub2-5* allele.

Directed analysis of the phenotype of mutant components of the exosome (Mitchell et al. 1997), which has been implicated in RNA processing events in the nucleus (Decker 1998; Allmang et al. 1999) in the nucleus would be another avenue for investigating the hypothesized link between Sub2 and a nuclear discard pathway (Mitchell et al. 1997; Decker 1998). An ultimate prediction of this model is that the fidelity of BPS recognition might be relaxed in the context where the proposed link between Sub2 and a discard pathway. This could be easily assessed by comparing the copper resistance and splicing activity of ACT-CUP splicing reporters bearing BPS mutants in such *sub2-5*-discard-defective double mutants to *ACT-CUP* activity and splicing in single mutant strains.

Identification of a possible discard pathway linked to Sub2 function might also be identified through open-ended genetic studies with *sub2* mutants. In particular, synthetic lethal analysis may prove successful in identification of a degradation pathway linked to Sub2 function. Synthetic lethal screens with both the *sub2-1* and *sub2-5* alleles would likely also serve as a fruitful, less biased method to clarify the different pathways that Sub2 may be directly involved in or functionally linked to in the cell. A series of assays related to splicing, RNA transport, pre-mRNA retention, RNA stability and splicing fidelity could be employed in such screens to rapidly classify any synthetic lethal mutants obtained.

MATERIALS AND METHODS

Yeast strains used in this study

yAK265 *MATα Δsub2::HIS3 ade2 ura3 leu2 his3 trp1*/pAK269

yJP302	*MATa Δmsl5::KAN ade2 ura3 leu2 his3 trp1 Δcup1*/pBS1703
yAK653	yAK265 x yJP302
yAK657	*MATα Δsub2::HIS3 Δmsl5::KAN ade2 ura3 leu2 his3 trp1 Δcup1/* pAK363, pAK269
SS330	*MATa PRP⁺ ade2-101 his3Δ-200 ura3-52 tyr1*
SRY5-3b	*MATα prp5-3 ade2-101 his3Δ-200 leu2-3,112 ura3-52 tyr1*
YAK373	*MATa prp5-3 ade2-101 his3Δ-200 leu2-3,112 ura3-52 tyr1*
SRY9-1c	*MATa prp9-1 ade2-101 his3Δ-200 his4 leu2 ura3-52 tyr1*
SRY11-1a	*MATα prp11-1 ade2-101 his3Δ-200 ura3-52 tyr1*
YAK378	*MATα prp11-1 ade2-101 his3Δ-200 ura3-52 tyr1*
SRY21-1f	*MATα prp21-1 ade2-101 his3Δ-200 leu2 ura3-52 tyr1*
SRY5-1c	*MATa prp5-1 ade2-101 his3Δ-200 his4 leu2 trp ura3-52 lys2 tyr1*
YAK293	*MATa Δsub2::HIS3 ade2 his3 leu2 trp1 ura3/*pAK269
yAK385	*prp5-3/prp5-3* diploid derived from SRY5-3 x yAK373
yAK388	*prp11-1/prp11-1* diploid derived from SRY11-1a x yAK378
yAK400	*prp21-1/+* diploid derived from SRY21-1f x SS330
yAK478	*MATa PRP21⁺ Δsub2::KAN ade2-101 his3Δ-200 ura3-52 tyr1*/pAK269
yAK397	*prp9-1/+* diploid derived from SRY9-1c x yAK478
yAK465	*prp5-1/+* diploid derived from yAK382 x yAK478
yAK463	*MATa prp5-3 Δsub2::KAN ade2-101 his3Δ-200 leu2-3,112 ura3-52 tyr1/* pAK269
yAK495	*MATα prp11-1 Δsub2::KAN ade2-101 his3Δ-200 ura3-52 tyr1*/pAK269
yAK479	*MATa prp21-1 Δsub2::KAN ade2-101 his3Δ-200 ura3-52 tyr1*/pAK269

yAK499	*MATa prp9-1 Δsub2::KAN ade2-101 his3Δ-200 ura3-52 tyr1*/pAK269
yAK550	*MATα prp5-1 Δsub2::KAN ade2-101 his3Δ-200 ura3-52 tyr1*/pAK269
yAK554	*MATa PRP5⁺ Δsub2::KAN ade2-101 his3Δ-200 ura3-52 tyr1*/pAK269
yAK682	*MATa cus2::HIS3 ade2 ura3 leu2 his3 trp1*
yAK674	*MATα Δsub2::TRP1 Δmud2::KAN ade2 ura3 his3 leu2 trp1*/pAK269
yAK678	yAK682 x yAK674
yAK719	*MATa Δsub2::TRP1 cus2::HIS3 ade2 ura3 leu2 his3 trp1*/pAK269
yAK483	*MATa Δsub2::HIS3 Δmud2::KAN ura3 leu2 his3 trp1*
yAK313	*MATa Δsub2::HIS3 ura3 leu2 his3 trp1*/pAK297
yAK662	*MATa Δsub2::HIS3 Δmud2::KAN ura3 leu2 his3 trp1*/pAK297
yAK767	*MATa cus2::HIS3 Δmud2::KAN ura3 leu2 his3 trp1*
yAK768	*MATa cus2::HIS3 Δmud2::KAN Δsub2::TRP1 ura3 leu2 his3 trp1*/pAK269

yJP302 was a generous gift provided by J. Pan. SRY strains were provided by J. Staley and I. Barta, and are described in (Ruby et al. 1993). To investigate the genetic interactions between *SUB2* and BBP, a *sub2-msl5* plasmid shuffle strain was generated by crossing yAK265 carrying with yJP302 to generate the diploid strain yAK653. The plasmid shuffle strain yAK657 was generated by random sporulation of yAK653 onto -HIS-URA media and screened for KAN^r haploids. To test if the conditional *msl5* alleles could bypass the requirement for Sub2 function in vivo, yAK657 was transformed with the *MSL5 LEU2* CEN plasmids provided by B. Seraphin (see below), then streaked to 5-FOA at the temperatures indicated in Table 2 to lose both the *SUB2* and *MSL5* wildtype

URA plasmids. Bypass suppression by the *msl5-5* allele was confirmed by transforming yAK657 with pBS1814 or pBS2082 and one the following additional *TRP* plasmids: pRS314, pJP127, or pAK385. These transformants were streaked to 5-FOA-TRP media at 23°C to lose both the *SUB2* and *MSL5* wildtype *URA* plasmids. To test for genetic interactions between the *sub2* alleles and the BBP alleles, yAK657 strains containing the BBP alleles were transformed with pAK385 (*SUB2* in pRS314), pAK389 (*sub2-1* in pRS314), and pAK401 (*sub2-5* in pRS314). These transformants were then streaked to 5-FOA media at various temperatures indicated in Table 1. Strains which were viable were streaked on YEPD at the temperatures indicated in Table 1 in parallel with strains containing only one of the mutants to assess the impact of combining these different alleles of *SUB2* and BBP in one strain.

MATa versions of SRY5-3 and SRY11-1 were generated through mating locus swap using pSC9 (see below) by two-step gene replacement (S. Chu and I. Herskowitz, personal communication). Diploid strains were generated by the indicated strains. To assess the growth impact of combining *sub2* and *prp* mutants defective for PS formation *sub2-prp* plasmid shuffle strains were generated by transforming these diploids with the 2kb SpeI-Xho fragment of pAK348 to generate a chromosomal *Δsub2::KANMX* allele (Longtine et al. 1998). The *Δsub2::KANMX* allele was confirmed through whole cell PCR of the KAN^r transformants. The resulting strains were transformed with pAK269, and sporulated. Haploid *URA+* strains were screened for KAN^r, and the presence of *Δsub2::KANMX* in the chromosome was confirmed by whole cell PCR. The resulting haploid *sub2-prp* shuffle strains indicated below were transformed with pAK354 (*SUB2* in pRS313), pAK356 (*sub2-1* in pRS313), or pAK355 (*sub2-5* in pRS313). These

transformants were streaked to 5FOA at 23°C media to lose pAK269. The growth of the resulting double mutant strains was assessed by streaking side-by-side on YEPD plates as shown in Figure 5-10.

A *sub2-cus2* shuffle strain was generated to assess the impact of *cus2::HIS3* on the lethality of the *Δsub2* null allele, and the *sub2* conditional alleles. This strain was generated by transforming the PvuII fragment of the *cus2::HIS3* plasmid into a wildtype W303 strain to generate yAK682. This strain was crossed to yAK674 to generate the diploid strain yAK678. A haploid *cus2::HIS3 Δsub2::TRP* strain was isolated by random sporulation on SD–HIS-URA media, followed by replica-plating to YEPD+KANAMYCIN, SD-TRP, and 5-FAA media. Additional haploid double mutant and triple mutant strains yAK767, yAK768 were also identified in this secondary screen, and are shown in Figure 11. These strains and yAK293 and yAK313 were assayed for growth in the absence of *SUB2* by streaking on 5-FOA media as shown in Figure 11.

Plasmids used in this study

pRS313 (*HIS3 CEN* vector)

pRS314 (*TRP1 CEN* vector)

pRS315 (*LEU2 CEN* vector)

pRS316 (*URA3 CEN* vector)

pAK269 (*SUB2* in pRS316)

pAK297 (*SUB2* in pRS315)

pAK322 (*sub2-1* in pRS315)

pAK320 (*sub2-5* in pRS315)

pAK354 (*SUB2* in pRS313)

pAK356 (*sub2-1* in pRS313)

pAK355 (*sub2-5* in pRS313)

pAK385 (*SUB2* in pRS314)

pAK389 (*sub2-1* in pRS314)

pAK401 (*sub2-5* in pRS314)

pAK348 (*Δsub2::KAN* in pCR2.1 [Invitrogen])

pJP127 (*MSL5 2μ TRP*)

pBS1703 (*MSL5* in pRS416)

pBS1811 (*msl5-2* in pRS415)

pBS1812 (*msl5-3* in pRS415)

pBS1814 (*msl5-5* in pRS415)

pBS1817 (*msl5-9* in pRS415)

pBS 2082 (*MSL5* in pRS415)

pMA *cus2::HIS3* in pTZ19R

pSC9 (*MATa1* HindIII fragment in pRS306)

pFA6a (*KANMX* Marker cassette)

pJP127 was generously provided by J. Pan. pBS plasmids were provided by B. Seraphin, and are described in (Rutz and Seraphin 2000). pMA *cus2::HIS3* was provided by M. Ares, and is described in (Yan et al. 1998). pRS vector plasmids were provided by P. Hieter and are described in (Sikorski and Hieter 1989). pSC9 is an integrating plasmid

(pRS306) containing the HindIII fragment of the MATa1 locus that was provided by S. Chu. pFA6a is described in (Longtine et al. 1998) and was provided by P. Preker.

pAK plasmids were generated in this study. pAK354, pAK356, and pAK355 were generated by subcloning the 2kb ApaI-XbaI fragment from pAK297, pAK320 (Kistler and Guthrie 2001), and pAK322 respectively, into pRS313 cleaved with ApaI and XbaI. pAK385, pAK389, and pAK401 were generated by subcloning the 2kb ApaI-NotI fragment from pAK354, pAK356, and pAK355 respectively, into pRS314 cleaved with ApaI and NotI. pAK348 contains 280bp of *SUB2* 5'UTR, the *KANMX* coding sequence derived from pFA6a (Longtine et al. 1998), and 380bp of *SUB2* 3'UTR in the TOPO T/A pCR2.1 cloning vector (Stratagene). The 5'UTR fragment was generated by PCR of oAK297 using oligo primers oAK146 and oAK188. The central *KANMX* fragment was generated by PCR of pFA6a using oligo primers oAK186 and oAK187. The 3'UTR fragment was generated by PCR of pAK297 using oligo primers oAK189 and oAK137. After purification of the PCR products derived from each of these PCR reactions, these three PCR products were amplified together in a PCR reaction with oligo primers oAK146 and oAK137. The resulting 2kb PCR product was subcloned into the pCR2.1 vector (Invitrogen).

Oligonucleotides used in this study

oAK137 (*SUB2* 3'UTR) GAAAATTTCAAGATTTTTTCCAAAT

oAK146 (*SUB2* 5'UTR) AAAATAGTCGACAACACGCCGAATAAAC

oAK186 (*sub2* SFH Longtine R1)

TCTTTATATAATCTATATAAAAACGTATCTTTTTTCCTTTAGAATTCGAGCTCG

TTTAAAC

oAK187 (*sub2* SFH Longtine F2)

TTAAAAAAAGCTCGTTCATTTTTTTAAAAACAGCAACGAATGCGGATCCCCG

GGTTAATTAA

oAK188 (LFH Longtine F2 antisense)

TTAATTAACCCGGGGATCCGCATCGTTGCTGTTTTTAAAAAAATGA

oAK189 (LFH Longtine R1 antisense)

GTTTAAACGAGCTCGAATTCAGGAAAAAAGATACGTTTTTATATAG

In vitro splicing and spliceosome assembly assays

Splicing extracts derived from yAK483 (*Δsub2 Δmud2* strain), yAK662 (*SUB2 Δmud2* strain), and yAK313 (*SUB2 MUD2* strain) were prepared (Ansari and Schwer 1995; Umen and Guthrie 1995). Splicing assays were carried out under standard buffer conditions at 16°C, room temperature (approximately 23°C), and 33°C, as previously described (Kistler and Guthrie 2001). Spliceosome assembly from the timepoints tested in the activity assays was examined in parallel by native gel analysis (Cheng and Abelson 1987).

Figure 1. Growth of *sub2-msl5* double mutants at 25°C. Wildtype *MSL5* and *msl5* mutant alleles in a wildtype *SUB2* strain (top), the *sub2-1* strain (middle), and *sub2-5* strain (bottom) grown on YEPD plates for 3 days.

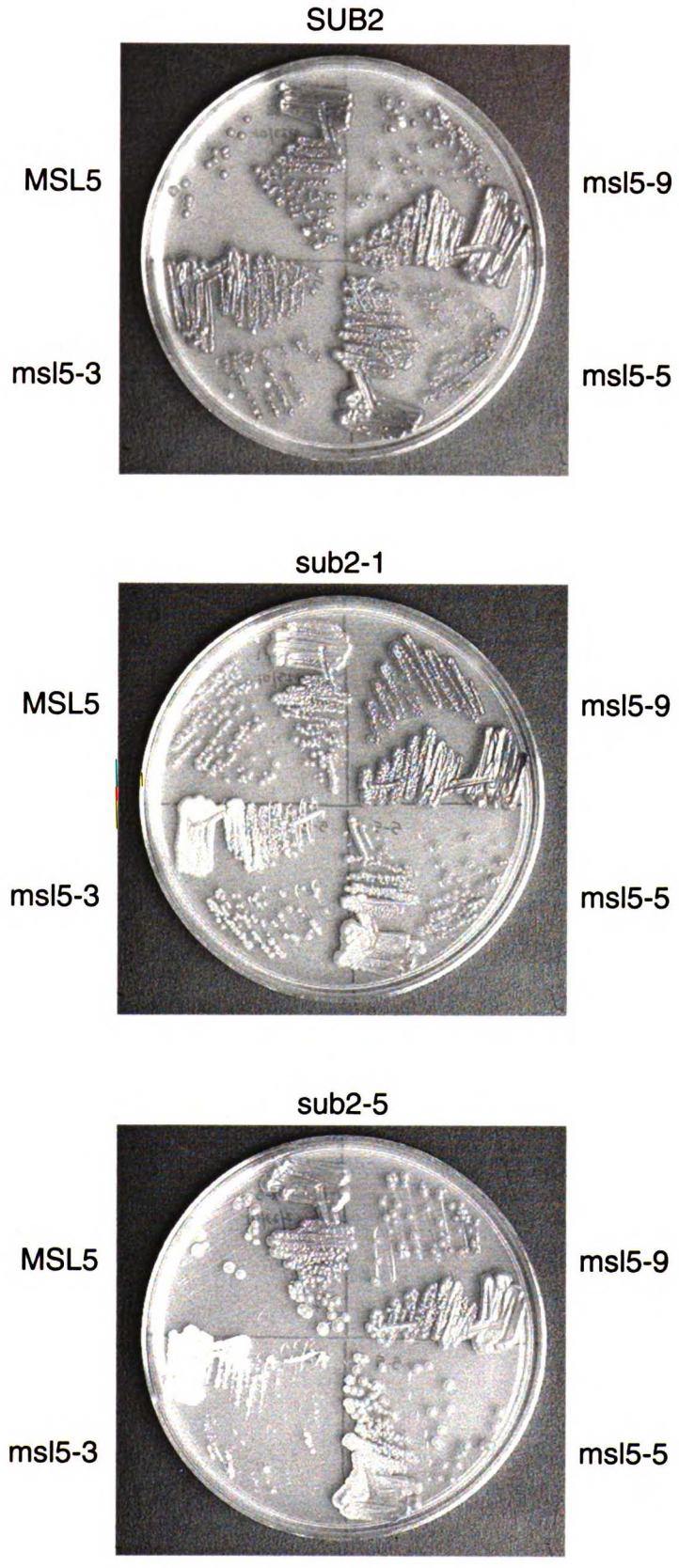

SUB2
MSL5
msl5-9
msl5-3
msl5-5
sub2-1
MSL5
msl5-9
msl5-3
msl5-5
sub2-5
MSL5
msl5-9
msl5-3
msl5-5

Figure 2. Growth of *sub2-msl5* double mutants at 37°C. Wildtype *MSL5* and *msl5* mutant alleles in a wildtype *SUB2* strain (top), the *sub2-1* strain (middle), and *sub2-5* strain (bottom) grown on YEPD plates for 3 days.

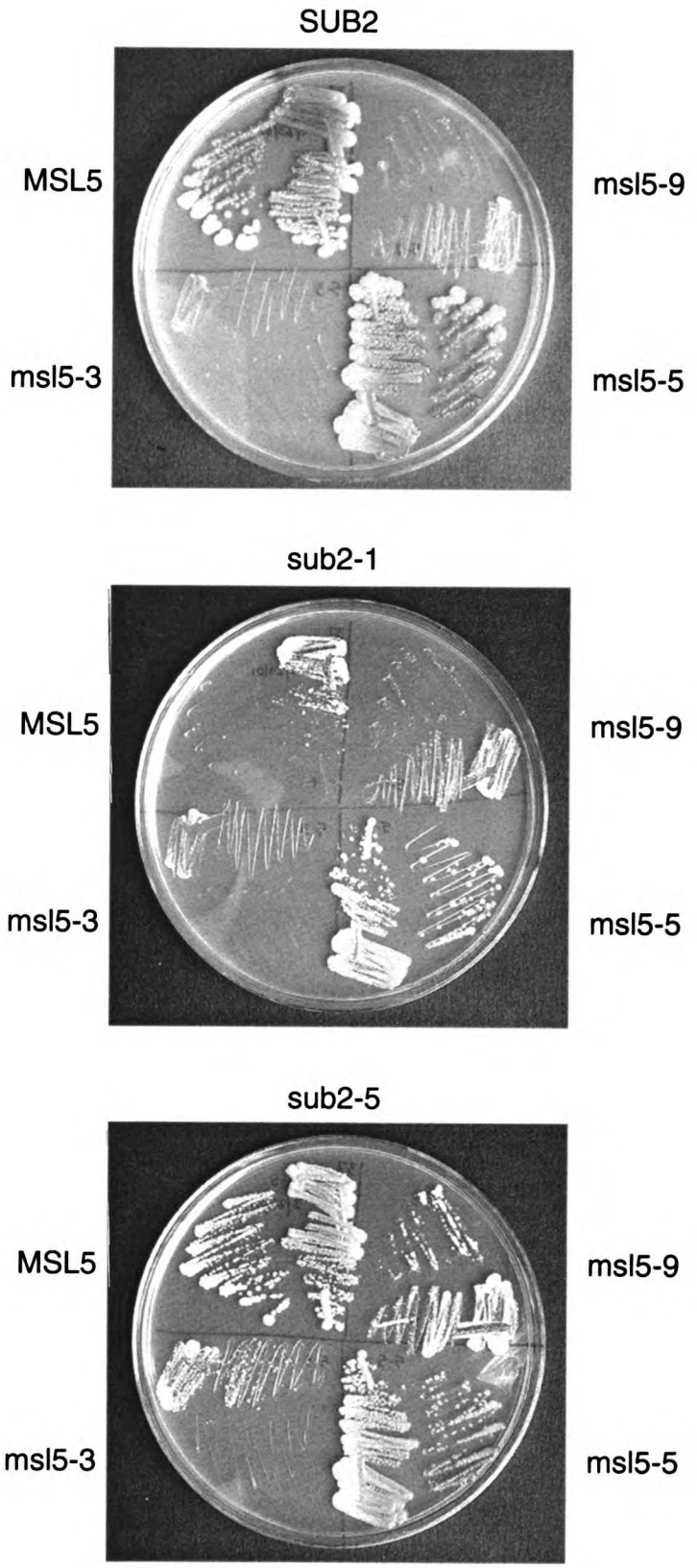

SUB2
MSL5
msl5-9
msl5-3
msl5-5
sub2-1
MSL5
msl5-9
msl5-3
msl5-5
sub2-5
MSL5
msl5-9
msl5-3
msl5-5

Figure 3. Bypass suppression of *Δsub2* by *msl5-5* at 25°C. Growth of yAK657 (*Δsub2 Δmsl5/pSUB2 URA* and *pMSL5-URA*) containing an additional *LEU* vector plasmid, wildtype *MSL5-LEU*, or the conditional BBP alleles (*msl5-2*, *msl5-3*, *msl5-5*, and *msl5-9*) on 5-FOA-LEU media at 25°C.

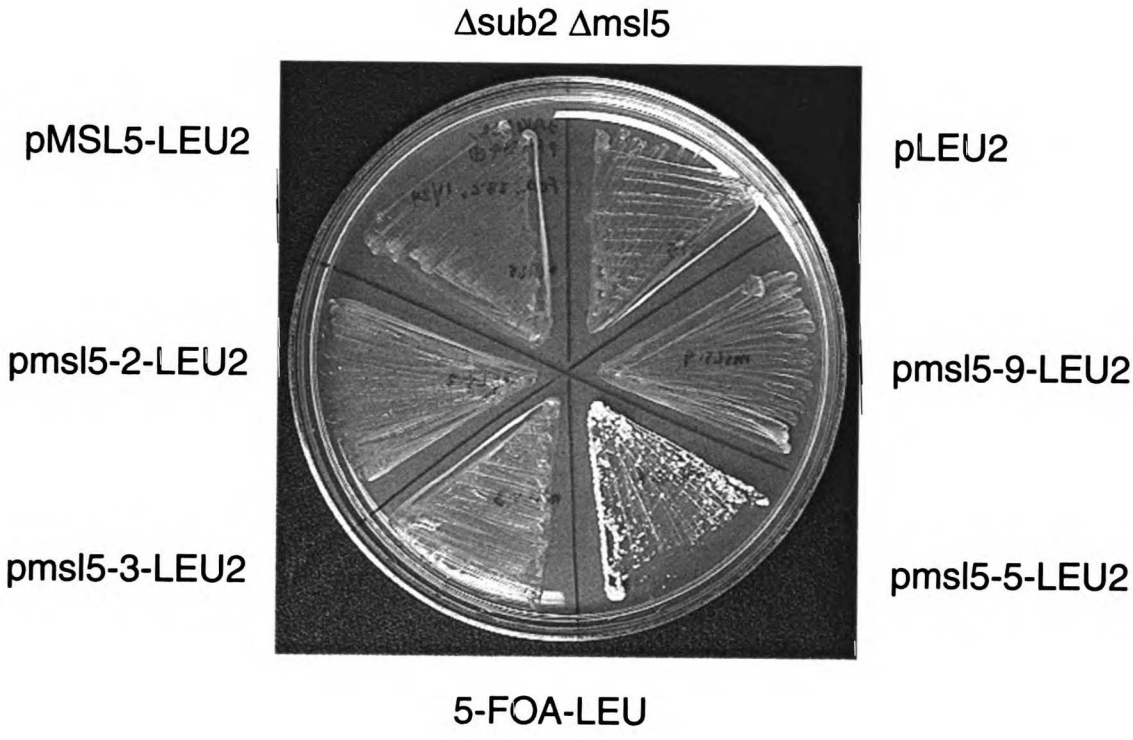

Δsub2 Δmsl5
pMSL5-LEU2
pLEU2
pmsl5-2-LEU2
pmsl5-9-LEU2
pmsl5-3-LEU2
pmsl5-5-LEU2
5-FOA-LEU

Figure 4. Confirmation of the *msl5-5* bypass suppression. Growth of yAK657 (*Δsub2 Δmsl5/pSUB2 URA* and *pMSL5-URA*) containing either *MSL5-LEU* (left side) or *msl5-5-LEU* (right side) in combination with *pSUB2-TRP1*, *pMSL5-TRP1*, or *TRP* vector, on 5FOA-*TRP* at 25°C.

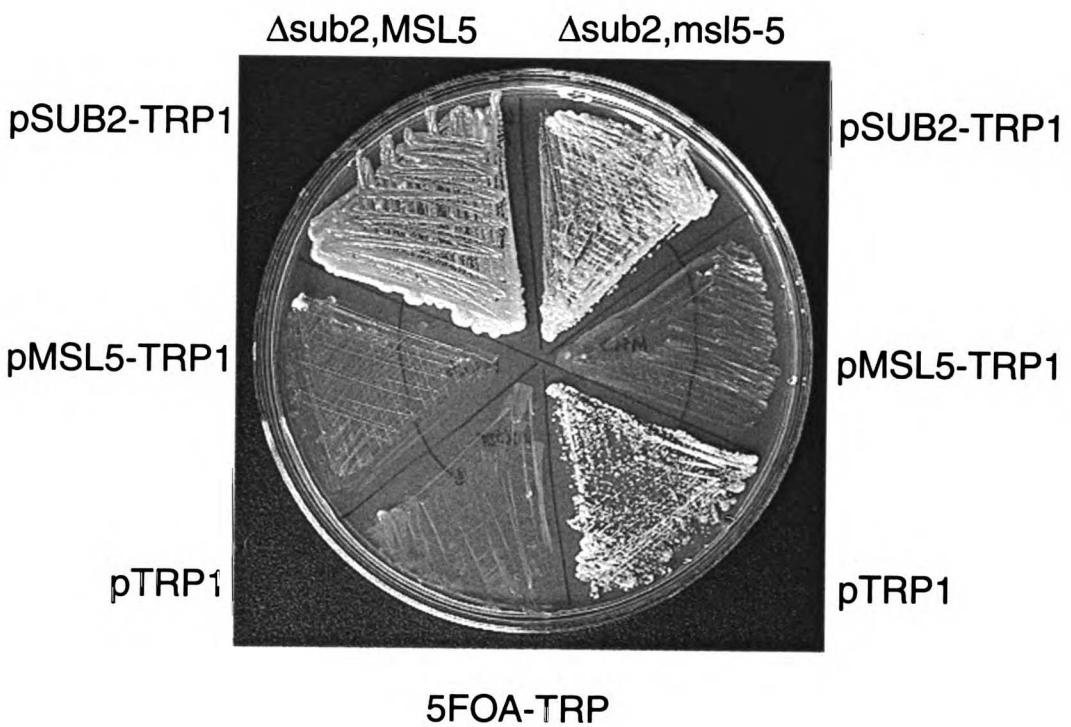

Δsub2,MSL5
Δsub2,msl5-5
pSUB2-TRP1
pSUB2-TRP1
pMSL5-TRP1
pMSL5-TRP1
pTRP1
pTRP1
5FOA-TRP

Figure 5. Growth impact of *sub2* on the PS *PRPs* at 20°C. Side-by-side comparison of the growth of WT *SUB2*, *sub2-1*, and *sub2-5* in WT *PRP*, *prp5-3*, *prp9-1*, *prp11-1*, and *prp21-1* strain backgrounds. A key to the identification of each streak diagrammed. Two independent transformants of each strain were assayed.

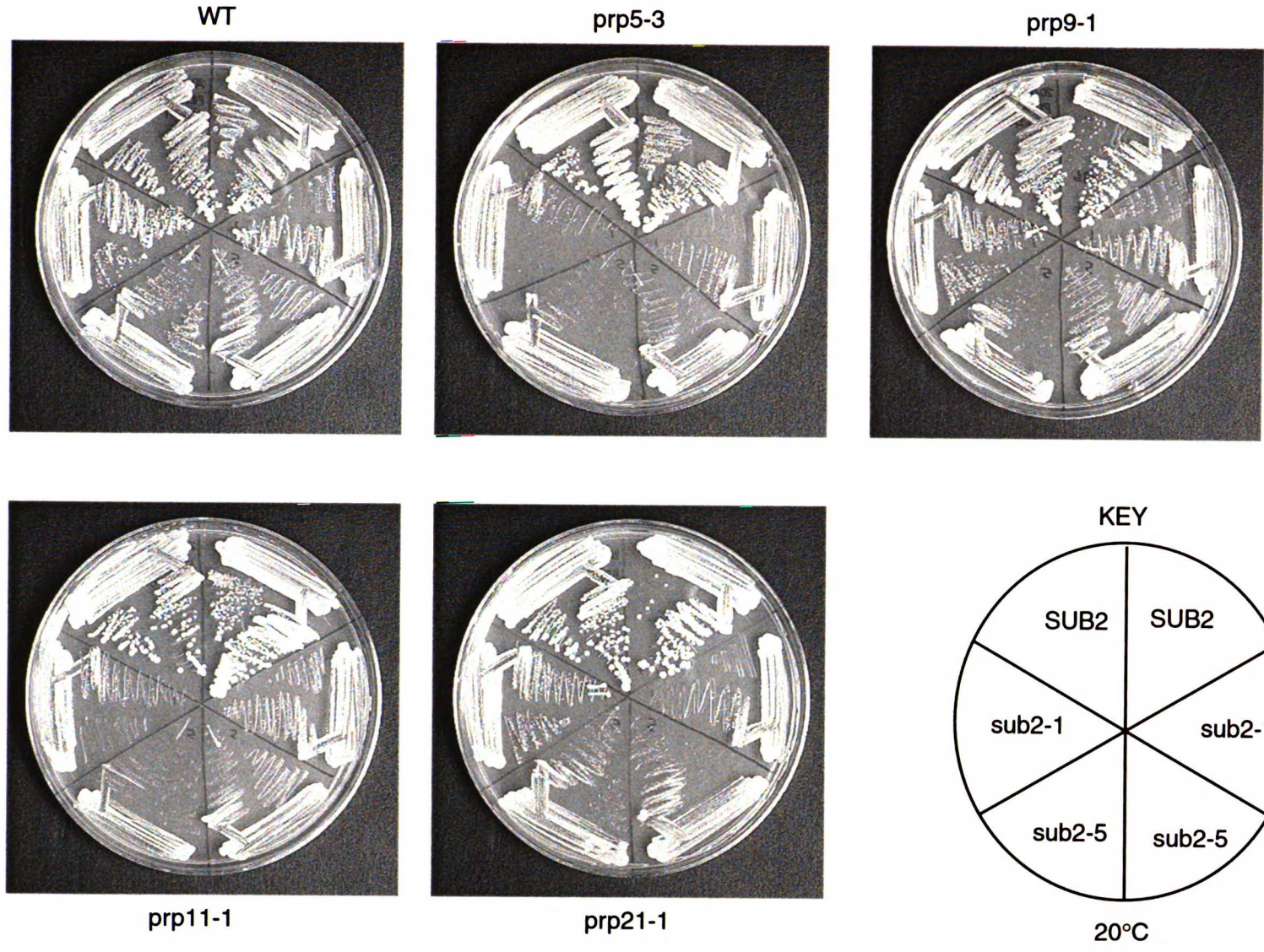

WT
prp5-3
prp9-1
prp11-1
prp21-1
KEY
SUB2
SUB2
sub2-1
sub2-1
sub2-5
sub2-5
20°C

Figure 6. Growth impact of *sub2* on the PS *PRPs* at 20°C, continued: side-by-side comparison of the growth of WT *SUB2*, *sub2-1*, and *sub2-5* in WT *PRP* and *prp5-1* strain backgrounds. A key to the identification of each streak diagrammed. Two independent transformants of each strain were assayed.

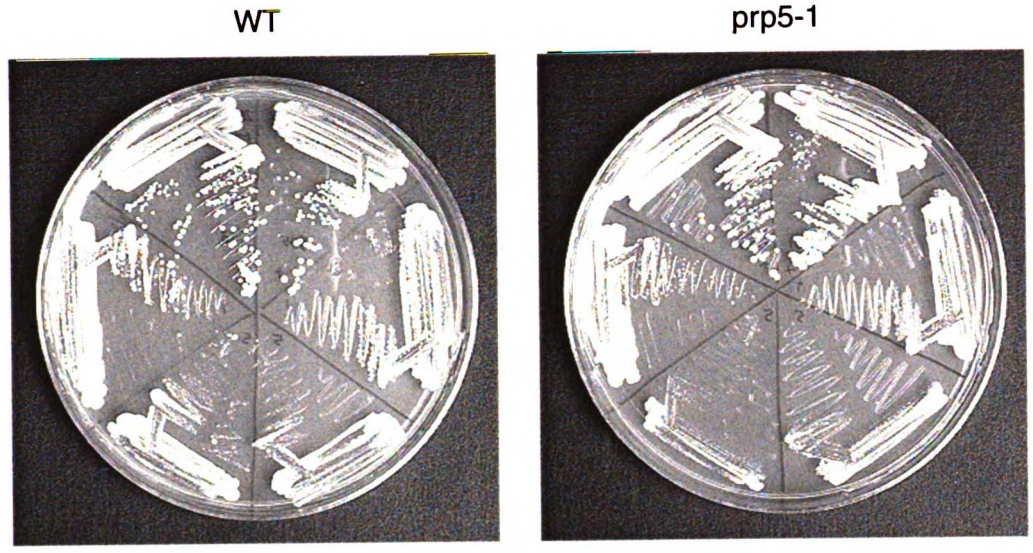

WT
prp5-1
KEY
SUB2
SUB2
sub2-1
sub2-1
sub2-5
sub2-5
(two independent tfs)
20°C

Figure 7. Growth impact of *sub2* on the PS *PRPs* at 23°C. Side-by-side comparison of the growth of WT *SUB2*, *sub2-1*, and *sub2-5* in WT *PRP*, *prp5-3*, *prp9-1*, *prp11-1*, and *prp21-1* strain backgrounds. A key to the identification of each streak diagrammed. Two independent transformants of each strain were assayed.

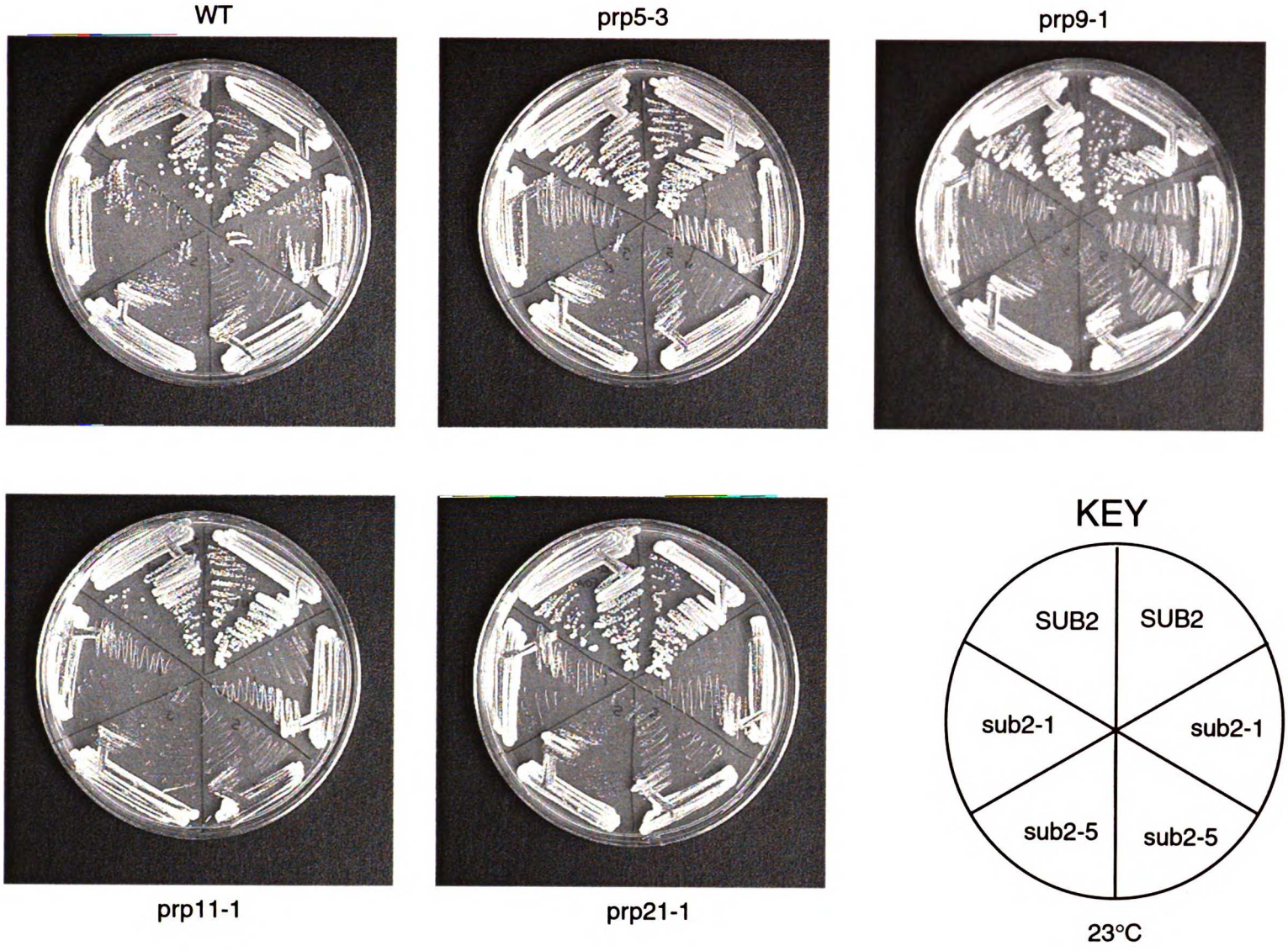

WT
prp5-3
prp9-1
prp11-1
prp21-1
KEY
SUB2
SUB2
sub2-1
sub2-1
sub2-5
sub2-5
23°C

Figure 8. Growth impact of *sub2* on the PS *PRPs* at 23°C, continued: side-by-side comparison of the growth of WT *SUB2*, *sub2-1*, and *sub2-5* in WT *PRP* and *prp5-1* strain backgrounds. A key to the identification of each streak diagrammed. Two independent transformants of each strain were assayed.

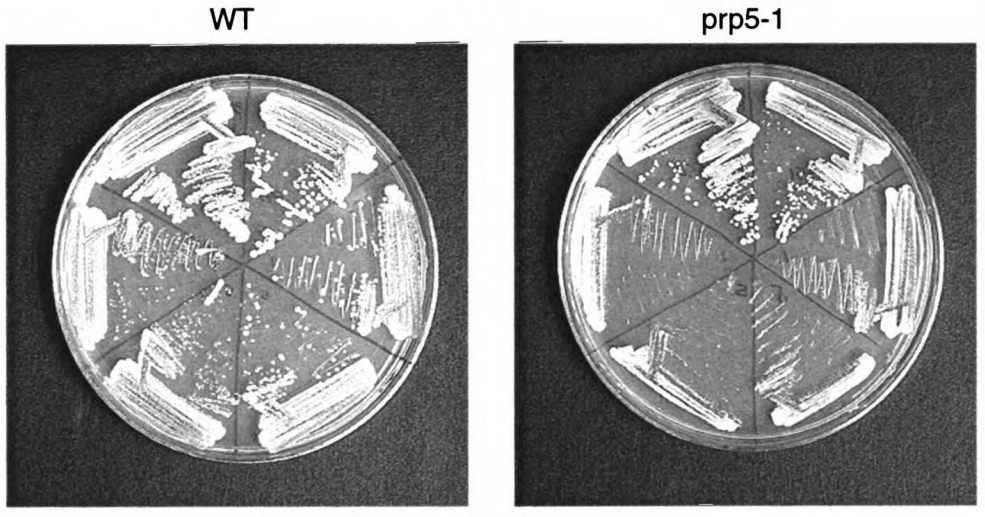

WT
prp5-1
KEY
SUB2
SUB2
sub2-1
sub2-1
sub2-5
sub2-5
(two independent tfs)
23°C

Figure 9. Growth impact of *sub2* on the PS *PRPs* at 25°C. Side-by-side comparison of the growth of WT *SUB2*, *sub2-1*, and *sub2-5* in WT *PRP*, *prp5-3*, *prp9-1*, *prp11-1*, and *prp21-1* strain backgrounds. A key to the identification of each streak diagrammed. Two independent transformants of each strain were assayed.

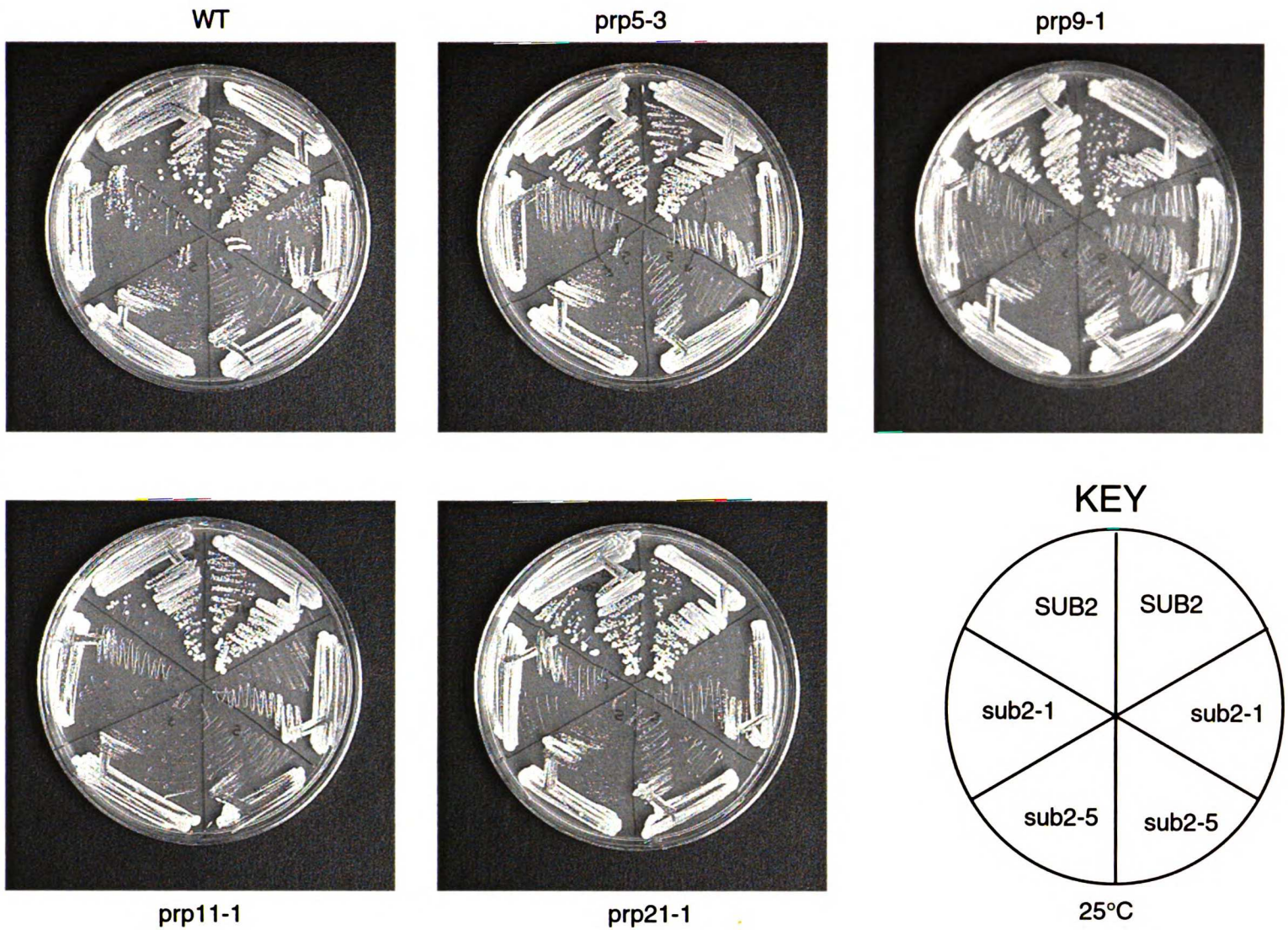

WT
prp5-3
prp9-1
prp11-1
prp21-1
KEY
SUB2
SUB2
sub2-1
sub2-1
sub2-5
sub2-5
25°C

Figure 10. Growth impact of *sub2* on the PS *PRPs* at 25°C, continued: side-by-side comparison of the growth of WT *SUB2*, *sub2-1*, and *sub2-5* in WT *PRP* and *prp5-1* strain backgrounds. A key to the identification of each streak diagrammed. Two independent transformants of each strain were assayed.

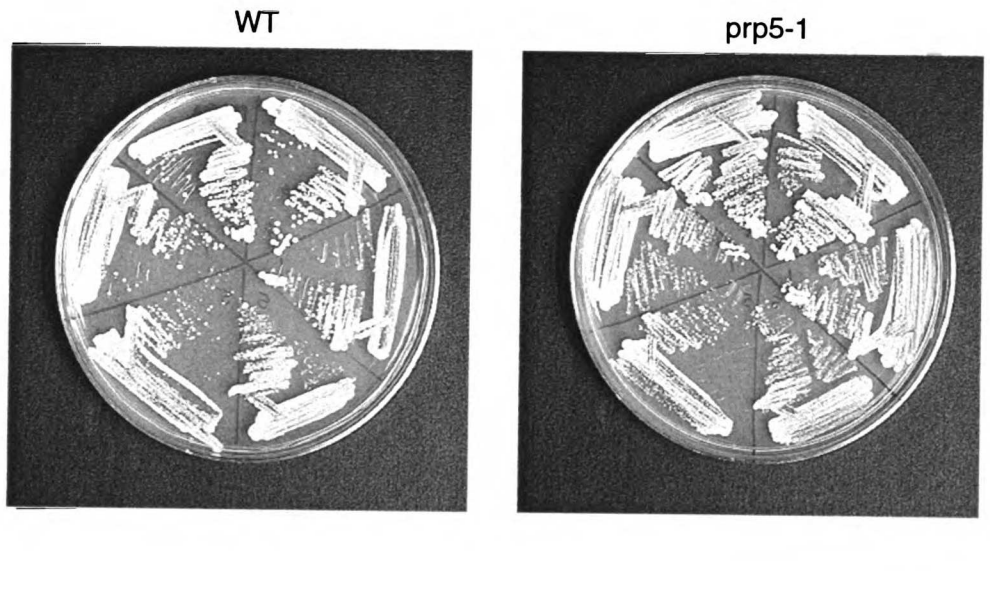

WT
prp5-1
KEY
SUB2
SUB2
sub2-1
sub2-1
sub2-5
sub2-5
(two independent tfs)
25°C

Figure 11. Bypass suppression of *Δsub2* by disruption of *CUS2* at 20°C. Growth of the *Δsub2 cus2::HIS3* strain and various controls on 5-FOA plates at various temperatures. Additional single, double and triple mutant strains are also shown for controls and growth comparison. A key to the identification of each streak is provided.

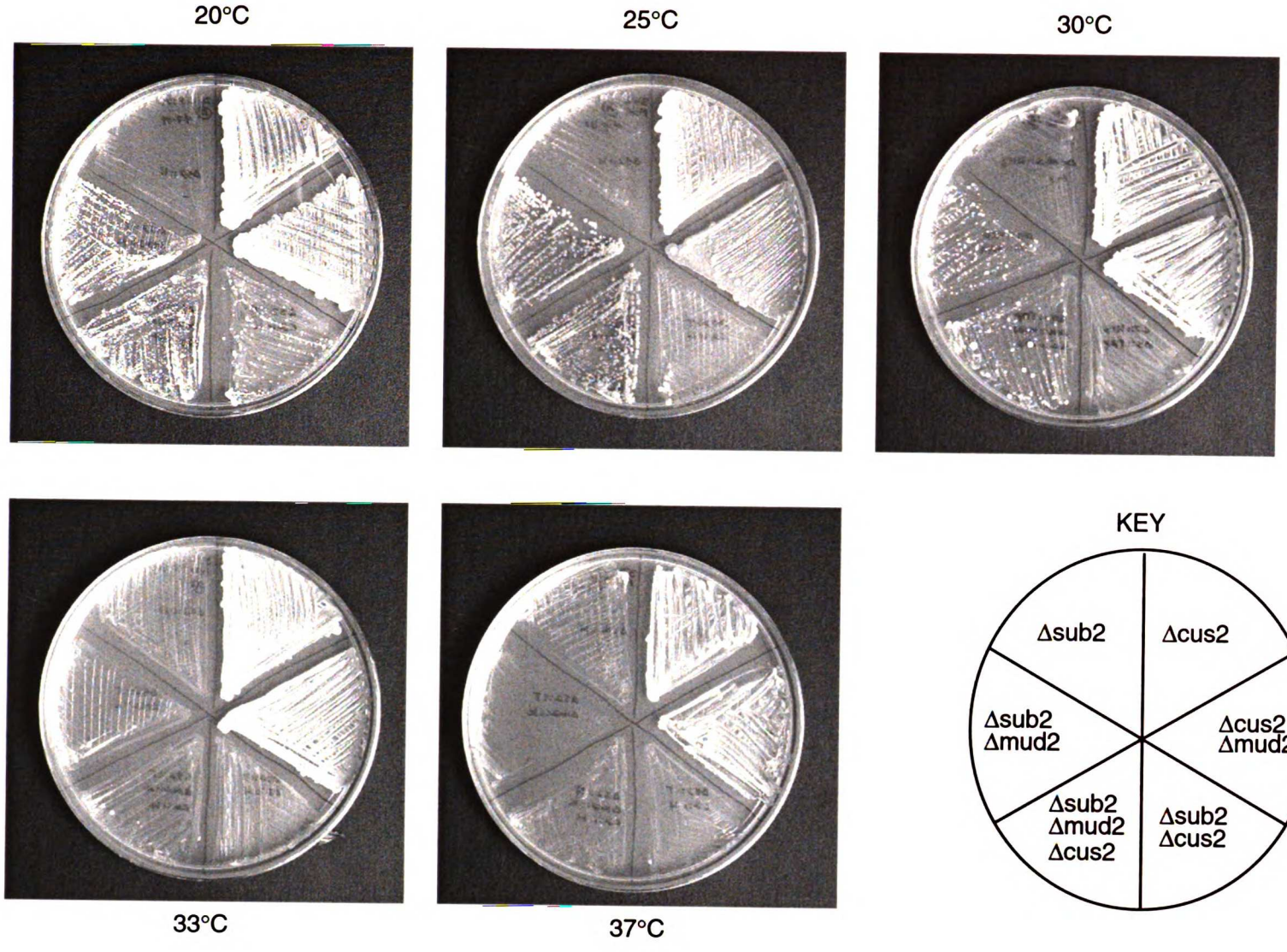

20°C
25°C
30°C
33°C
37°C
KEY
Δsub2
Δcus2
Δsub2
Δmud2
Δcus2
Δmud2
Δsub2
Δmud2
Δcus2
Δsub2
Δcus2

Figure 12. In vitro splicing activity of *Δsub2 Δmud2*, *SUB2 Δmud2*, and *SUB2 MUD2* extracts at room temperature. The migration of pre-mRNA, mRNA, free exon 1, and the lariat intermediate and excised lariat product are identified by the cartoons to the left of the gel.

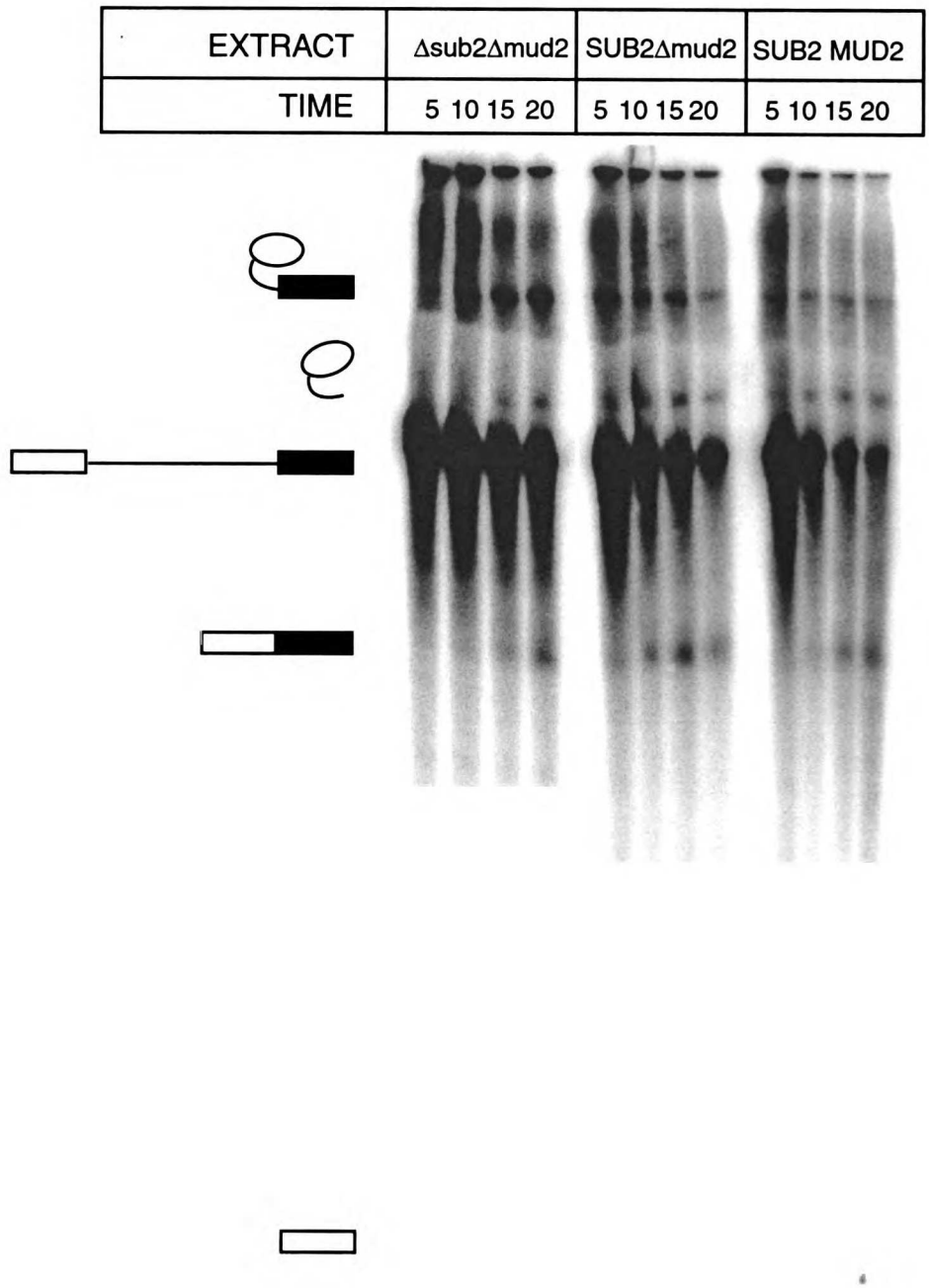

EXTRACT
Δsub2Δmud2
SUB2Δmud2
SUB2 MUD2
TIME
5 10 15 20
5 10 15 20
5 10 15 20

Figure 13. In vitro splicing activity of *Δsub2 Δmud2*, *SUB2 Δmud2*, and *SUB2 MUD2* extracts at 33°C. The migration of pre-mRNA, mRNA, free exon 1, and the lariat intermediate and excised lariat product are identified by the cartoons to the left of the gel.

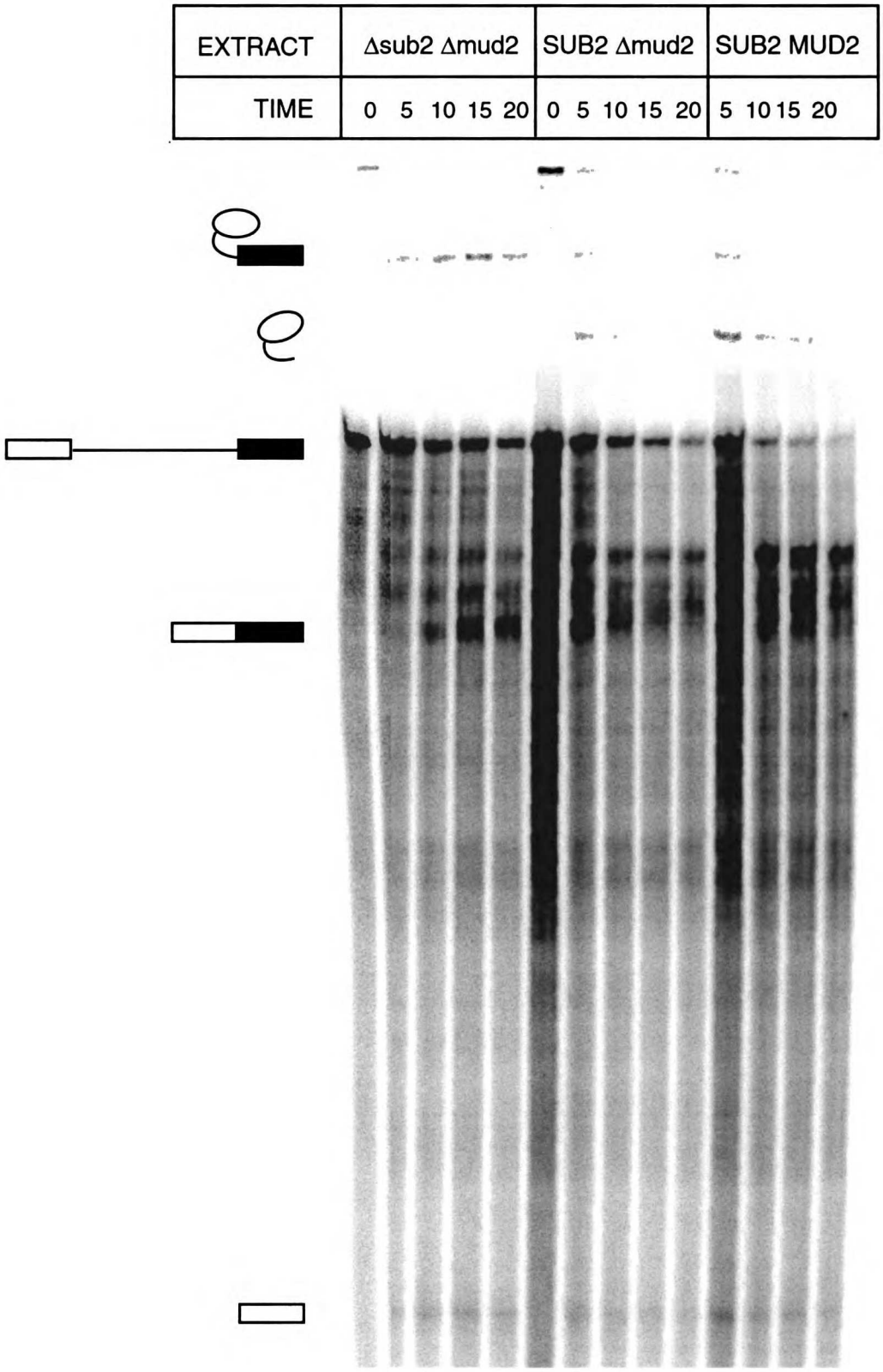

EXTRACT
Δsub2 Δmud2
SUB2 Δmud2
SUB2 MUD2
TIME
0 5 10 15 20
0 5 10 15 20
5 10 15 20

Figure 14. In vitro splicing activity of *Δsub2 Δmud2*, *SUB2 Δmud2*, and *SUB2 MUD2* extracts at 16°C. The migration of pre-mRNA, mRNA, free exon 1, and the lariat intermediate and excised lariat product are identified by the cartoons to the left of the gel.

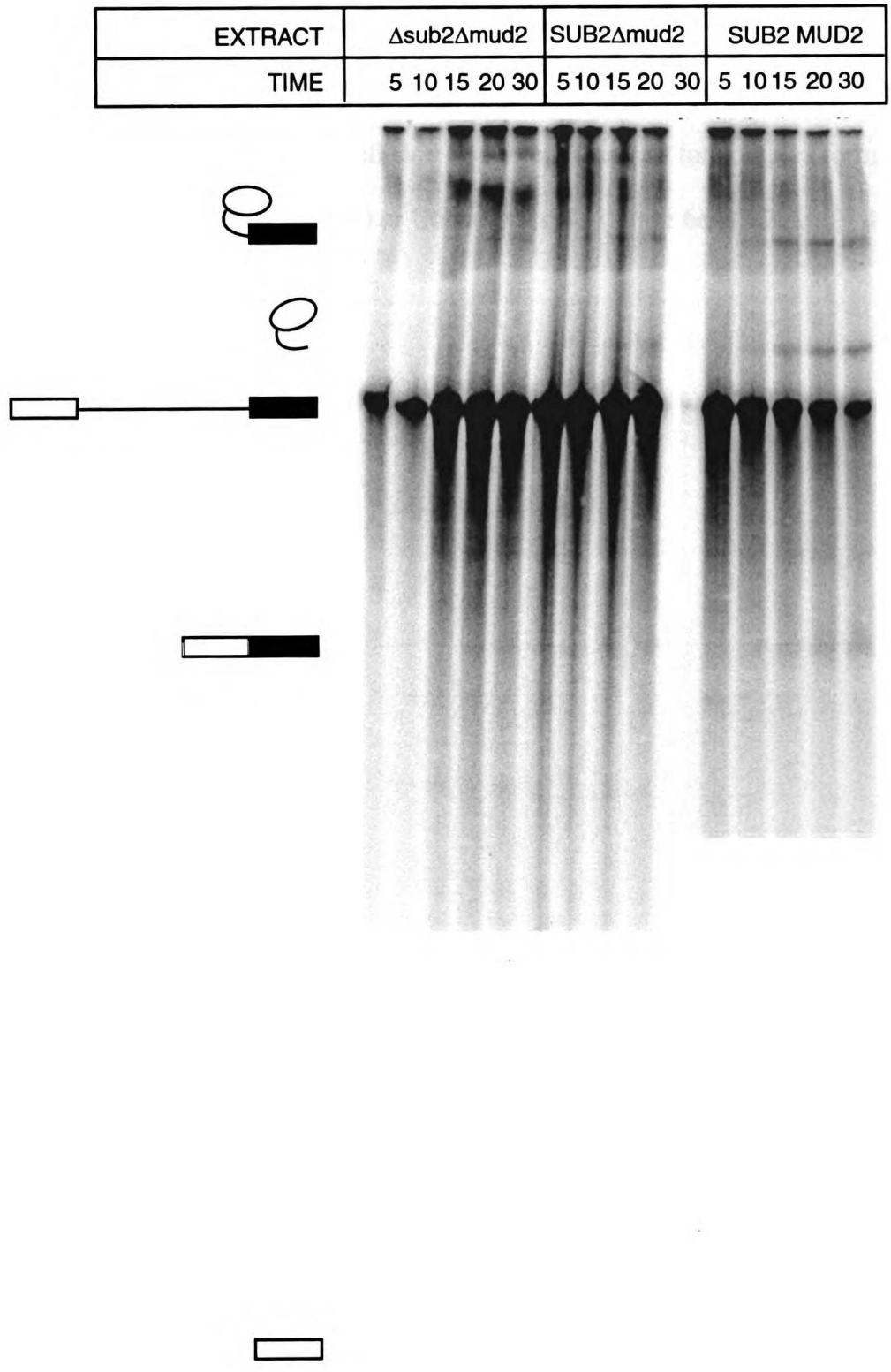

EXTRACT
Δsub2Δmud2
SUB2Δmud2
SUB2 MUD2
TIME
5 10 15 20 30
5 10 15 20 30
5 10 15 20 30

Figure 15. Native gel analysis of spliceosome assembly profile of *Δsub2 Δmud2* and *SUB2 MUD2* extracts at room temperature. The locations of the PS complex B, the spliceosome complex A, the heterogenous complex H, and an additional, unidentified complex (*) are identified on the left side of the gel. Controls for complex formation in extracts in which U2 snRNA (ΔU2) or U6 snRNA (ΔU6) have been RNAse H depleted are also included on the gel.

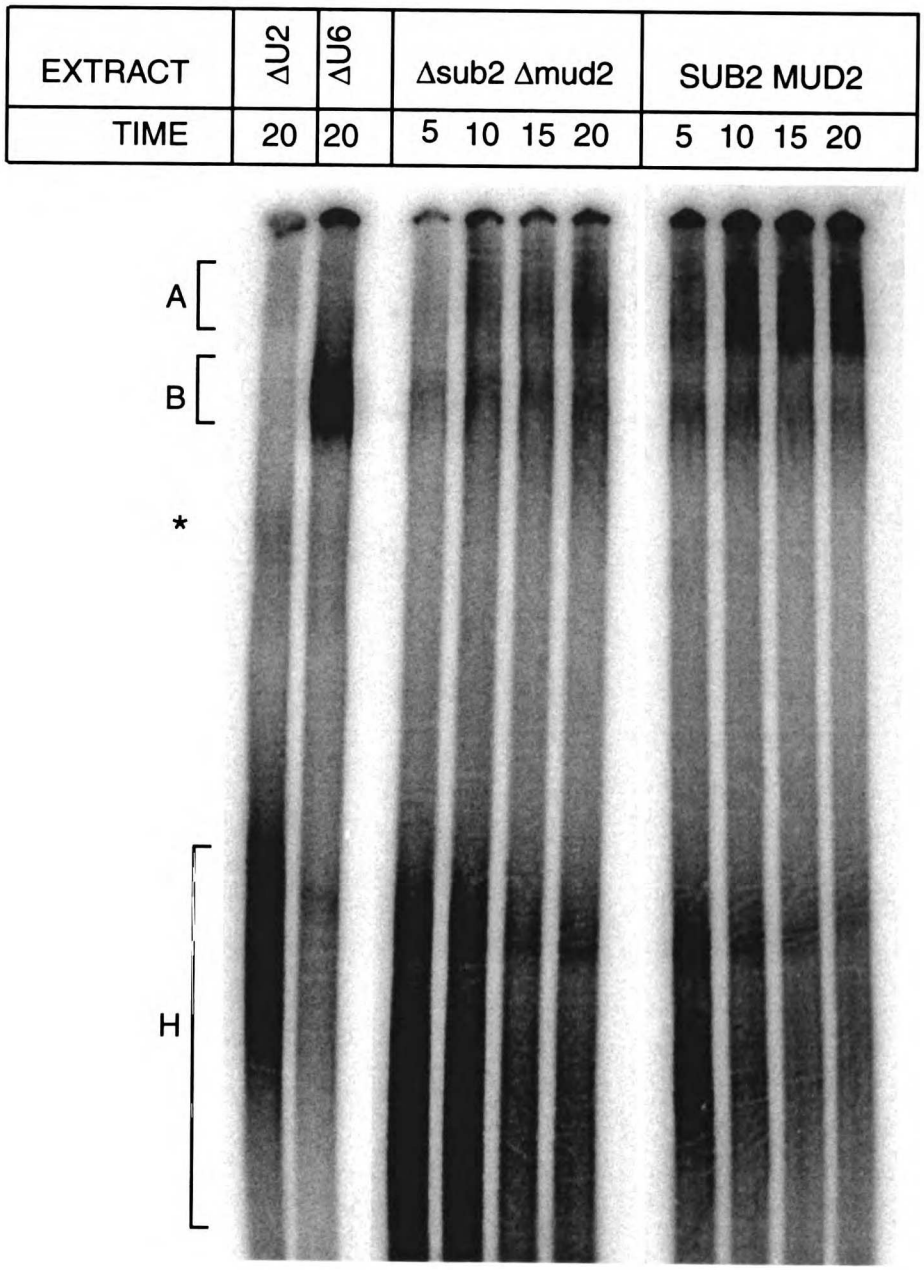

EXTRACT
ΔU2
ΔU6
Δsub2 Δmud2
SUB2 MUD2
TIME
20
20
5 10 15 20
5 10 15 20
A
B
*
H

Figure 16. Native gel analysis of spliceosome assembly profile of *Δsub2 Δmud2* and *SUB2 MUD2* extracts at 33°C. The locations of the PS complex B, the spliceosome complex A, the heterogenous complex H, and an additional, unidentified complex (*) are identified on the left side of the gel.

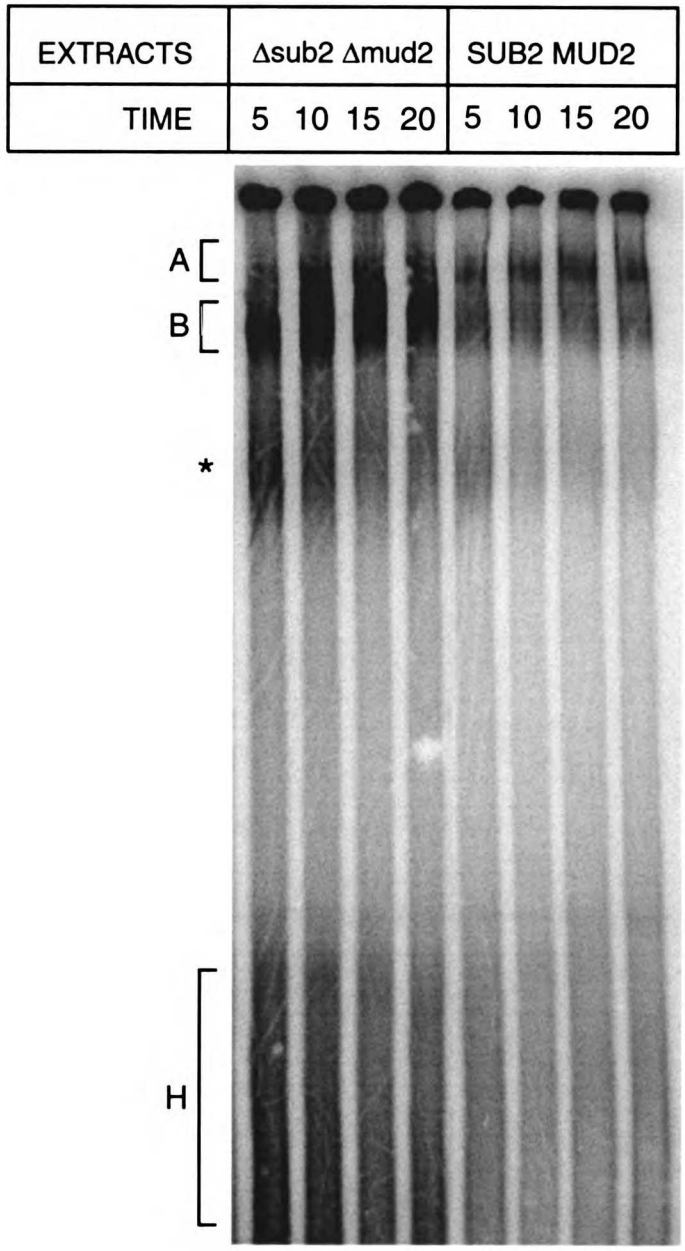

EXTRACTS
Δsub2 Δmud2
SUB2 MUD2
TIME
5 10 15 20
5 10 15 20
A
B
*
H

Figure 17. Native gel analysis of spliceosome assembly profile of *Δsub2 Δmud2*, *SUB2 Δmud2*, and *SUB2 MUD2* extracts at 16°C. The locations of the PS complex B, the spliceosome complex A, the heterogenous complex H, and an additional, unidentified complex (*) are identified on the left side of the gel. Controls for complex formation in extracts in which U2 snRNA (ΔU2) or U6 snRNA (ΔU6) have been RNAse H depleted are also included on the gel.

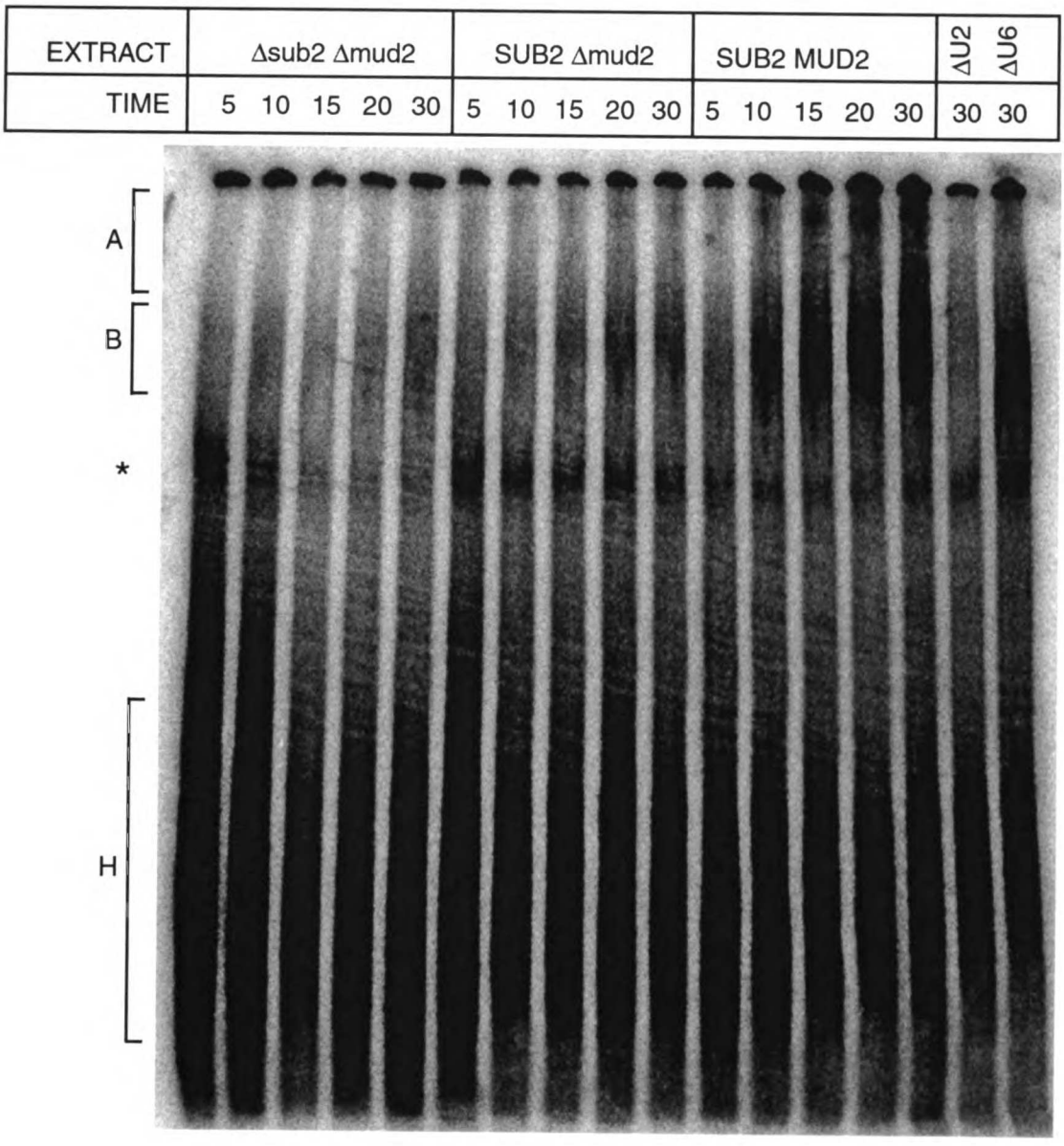

EXTRACT
Δsub2 Δmud2
SUB2 Δmud2
SUB2 MUD2
ΔU2
ΔU6
TIME
5 10 15 20 30
5 10 15 20 30
5 10 15 20 30
30 30
A
B
*
H

Figure 18. Model to illustrate two possible fates for pre-mRNA in the cell that Sub2 may regulate.

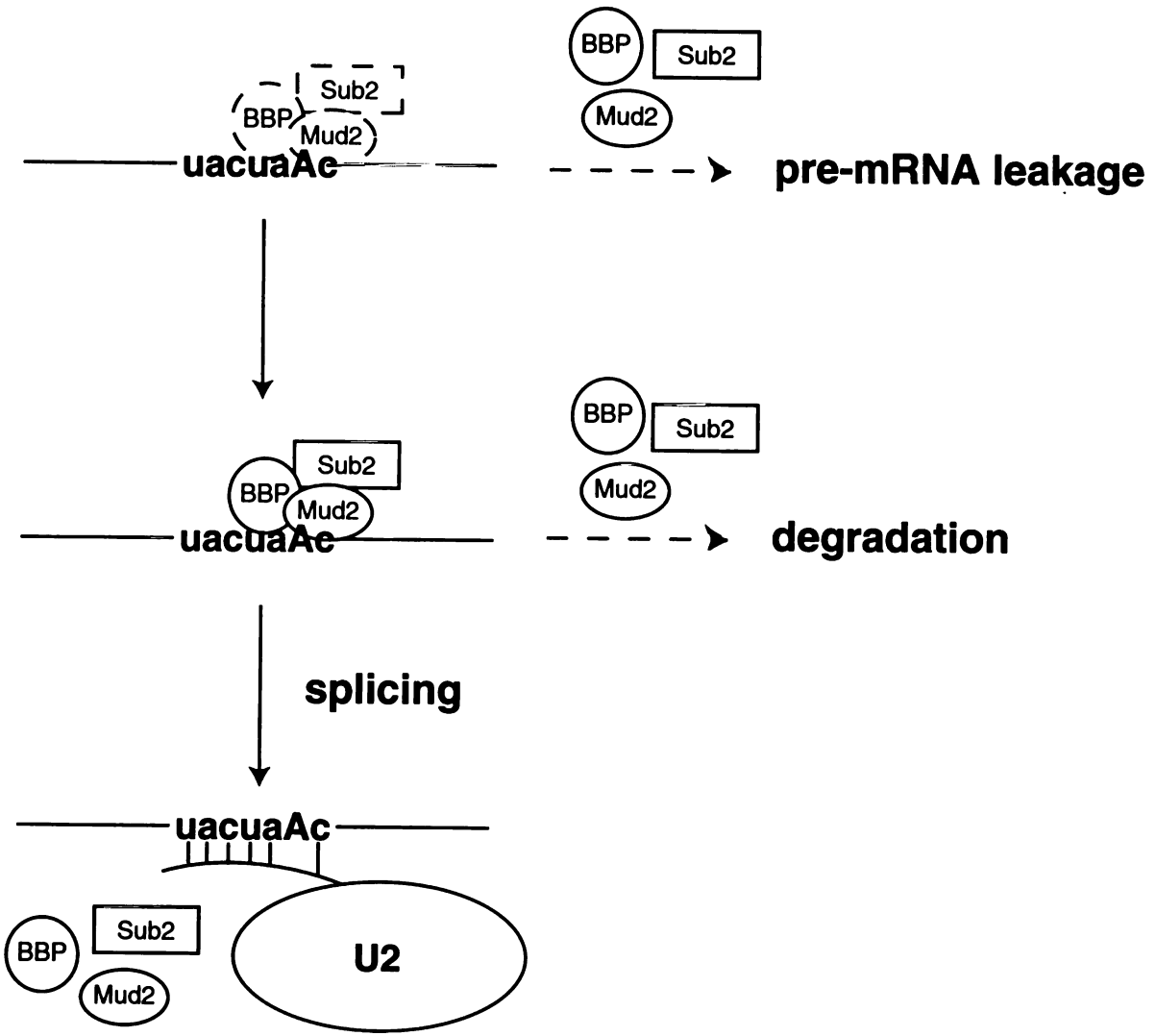

BBP
Sub2
Mud2
uacuaAc
pre-mRNA leakage
degradation
splicing
U2

Figure 19. Parallels between the exchange of factors required for early recognition at the 5'ss and the BPS. Analogous factors required for initial, sequence-specific binding at each of the consensus splice sites are colored in light blue. Factors that subsequently bind the consensus splice sites in an ATP-dependent manner are colored in dark blue. Factors that stabilize the ATP-independent interaction at each consensus splice site are colored pink. Factors which stabilize the non-interactive state of each of the ATP-dependent splice site binding factor are colored dark pink. ATPases that likely destabilize the ATP-independent interaction at each of the splice sites are colored light green. ATPases that likely lead to generation of the binding competent form of each of the ATP-dependent splice site binding factor are colored dark green.

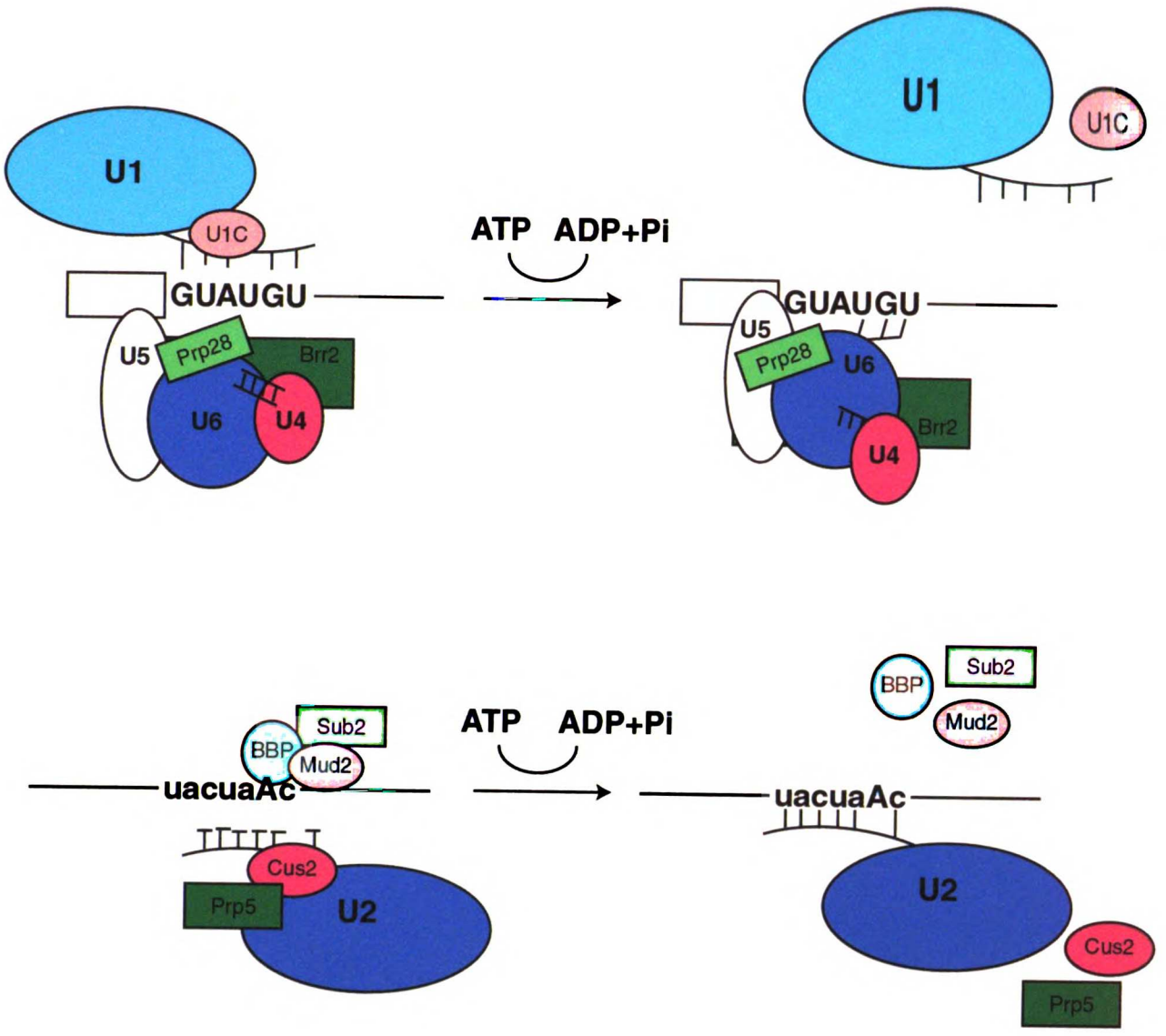

U1
U1C
GUAUGU
U5
Prp28
Brr2
U6
U4
ATP
ADP+Pi
U1
U1C
GUAUGU
U5
Prp28
U6
Brr2
U4
Sub2
BBP
Mud2
uacuaAc
Cus2
Prp5
U2
ATP
ADP+Pi
BBP
Sub2
Mud2
uacuaAc
U2
Cus2
Prp5

Table 1. Growth of *sub2 msl5* double mutants

Strain	16°C	20°C	23°C	25°C	30°C	33°C	37°C
SUB2, MSL5	+++	+++	+++	+++	+++	+++	+++
sub2-1, MSL5	+/-	+	++	++	++	+	+/-
sub2-5, MSL5	-	+/-	++	++	++	++	++
SUB2, msl5-2	+++	+++	+++	+++	-	-	-
sub2-1, msl5-2	-	-	-	-	-	-	-
sub2-5, msl5-2	-	-	++	++	-	-	-
SUB2, msl5-3	+++	+++	+++	+++	+/-	-	-
sub2-1, msl5-3	+/-	+	++	++	+	+/-	+/-
sub2-5, msl5-3	-	-	-	+/-	+	++	++
SUB2, msl5-5	-	+	+++	+++	+++	+++	+++
sub2-1, msl5-5	-	+	+++	+++	+++	+++	+++
sub2-5, msl5-5	-	-	++	++	++	++	++
SUB2, msl5-9	+++	+++	+++	+++	+++	+/-	-
sub2-1, msl5-9	+/-	+	++	++	++	+	+/-
sub2-5, msl5-9	-	+/-	++	++	++	++	+

Table 2. Growth of *msl5* mutants in the absence of *SUB2*

Strain	16°C	20°C	23°C	25°C	30°C	33°C	37°C
SUB2, MSL5	+++	+++	+++	+++	+++	+++	+++
Δsub2, MSL5	-	-	-	-	-	-	-
SUB2, msl5-2	+++	+++	+++	+++	-	-	-
Δsub2, msl5-2	-	-	-	-	-	-	-
SUB2, msl5-3	+++	+++	+++	+++	+/-	-	-
Δsub2, msl5-3	-	-	-	-	-	-	-
SUB2, msl5-5	-	+	+++	+++	+++	+++	+++
Δsub2, msl5-5	-	-	++	++	+	-	-
SUB2, msl5-9	+++	+++	+++	+++	+++	+/-	-
Δsub2, msl5-9	-	-	-	-	-	-	-

Table 3. Growth of *sub2* PS *prp* double mutants

Strain	16°C	20°C	23°C	25°C	30°C	33°C	37°C
SUB2, PRP	+++	+++	+++	+++	+++	+++	+++
sub2-1, PRP	+/-	+	++	++	++	+	+/-
sub2-5, PRP	-	+/-	++	++	++	++	++
SUB2, prp5-3	+++	+++	+++	+++	-	-	-
sub2-1, prp5-3	+/-	+/-	+/-	++	+/-	-	-
sub2-5, prp5-3	-	+/-	+/-	++	+/-	-	-
SUB2, prp9-1	+++	+++	+++	+++	+/-	-	-
sub2-1, prp9-1	+/-	+/-	+/-	++	+/-	-	-
sub2-5, prp9-1	-	+/-	+/-	++	+/-	-	-
SUB2, pr11-1	+++	+++	+++	+++	+/-	-	-
sub2-1, prp11-1	+/-	+/-	+/-	+/-	+/-	-	-
sub2-5, prp11-1	-	+/-	+/-	+/-	+/-	-	-
SUB2, prp21-1	+++	+++	+++	+++	-	-	-
sub2-1, prp21-1	+/-	+/-	+/-	+/-	-	-	-
sub2-5, prp21-1	-	+/-	+/-	+/-	-	-	-
*SUB2, PRP5**	+++	+++	+++	+++	+++	+++	+++
SUB2, prp5-1	+++	+++	+++	+++	+/-	-	-
sub2-1, prp5-1	+/-	+/-	+/-	++	+/-	-	-
sub2-5, prp5-1	-	+/-	+/-	++	+/-	-	-

CHAPTER 3

Biochemical and Genetic Analysis of the *SUB2-BRR1* Connection Implicates a Role for *SUB2* in snRNP Biogenesis

ABSTRACT

Originally isolated as a high copy suppressor of *brr1-1*, *SUB2* encodes a DEAD-box ATPase which function during the early stages of pre-mRNA splicing in yeast. These initial observations on the function of *SUB2* narrowed the potential mechanistic basis for the identification of *SUB2* as a high copy suppressor of *brr1-1*. Demonstration of a splicing function for *SUB2* raised the possibility that overexpression of *SUB2* suppresses the *brr1-1* growth defect through an indirect mechanism related to an effect on the overall efficiency of splicing in the *brr1-1* strain. We examined the impact of *GAL-SUB2* on the previously characterized splicing and snRNA biogenesis defects detected in the *brr1-1* mutant at the non-permissive temperature to test this hypothesis. In contrast to our expectations, overexpression of *SUB2* actually leads to an overall decrease in splicing efficiency, and confers only a weak, partial suppression of the temperature-dependent decrease in splicing efficiency detected in the *brr1-1* mutant. However, the decrease in snRNA levels in a *brr1-1* strain is clearly suppressed by *SUB2* overexpression. A subtle increase in the amount of 3' extended precursors of U4 and U5 is also detected in the *brr1-1* strain overexpressing *SUB2*. Moreover, *sub2-1* extracts phenocopy the decrease in snRNAs and snRNP complexes observed in splicing extracts derived from a Δ*brr1* strain. Directed genetic analyses suggest an antagonistic functional interaction between *SUB2* and *BRR1* that is influenced by the overall levels of each factor. Taken together, these preliminary experiments suggest that *SUB2* may play roles in snRNP biogenesis in combination with or in addition to its previously defined function in pre-mRNA splicing.

INTRODUCTION

At the outset of our studies with *SUB2*, the connection between *SUB2* function and snRNP biogenesis was mysterious. As its name conveys, *SUB2* (suppressor of *brr1-1*) was a novel factor isolated through its ability to suppress the cold-sensitive (cs-) growth defect of the snRNP biogenesis mutant, *brr1-1* (Noble 1995). *SUB2* was one of 4 genes capable of mediating overexpression-suppression of the cs- growth defect conferred by *brr1-1* at 16°C. In contrast, two of the other isolated high copy suppressors, *SMD1*, which encodes a core snRNP protein, and *LHP1*, the yeast homolog of the human La autoantigen, have clear functional links to a role in snRNA biogenesis. *SMD1* is a component of the common Sm core proteins that bind to, and are required for stabilization and maturation of spliceosomal snRNA precursors (Rymond et al. 1993; Roy et al. 1995; Seraphin 1995; Camasses et al. 1998). The La protein had been previously implicated in a number of diverse RNA processing events in higher eukaryotes (Maraia et al. 1994; Svitkin et al. 1994; Belsham et al. 1995), including association with 3' elongated cytoplasmic precursors of the U1 snRNA (Madore et al. 1984). Since the identification of these factors as high copy suppressors of *brr1-1*, independent studies have demonstrated a role for both *SMD1* and *LHP1* in snRNA stability and processing in yeast (Pannone and Wolin 2000; Xue et al. 2000). The mechanism by which the other high copy suppressor, *TBF4*, a transcription factor associated with the TFIIH component of RNA polymerase (Feaver et al. 1997), suppresses the *brr1-1* phenotype is less clear. However, a likely possibility based on the function of this gene in transcription was straightforward. Elevated levels of *TBF4* might suppress the snRNA biogenesis defect by altering the transcription of either the snRNAs

(U1, U2, U4, and U5 are all RNA polymerase II-dependent transcripts) or a factor involved in snRNA stability or biogenesis. Since *SUB2* encoded a novel gene, insight gained into possible mechanisms of suppression was derived solely from sequence analysis.

The presence of the seven conserved signature sequence motifs of the superfamily of DEAD-box proteins (Staley and Guthrie 1998; de la Cruz et al. 1999) in the *SUB2* gene raised the possibility that *SUB2* might play a role in an RNA rearrangement during pre-mRNA splicing or snRNP biogenesis. Indeed, genetic and biochemical analysis of several independently isolated conditional alleles of *SUB2* have demonstrated a function for *SUB2* in pre-mRNA splicing both before and after prespliceosome formation (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). Thus, a possible mechanism by which overexpression of *SUB2* is able to suppress the *brr1-1* cs- growth defect may be by increasing the efficiency of early transitions required during spliceosome assembly. If this is the case, overexpression of *SUB2* may suppress the cs- growth defect of *brr1-1* by functioning to increase the throughput of an RNA or RNA-protein rearrangement required for splicing that was made rate-limiting for growth in the cold in a *brr1-1* strain.

While this hypothesis is appealing given the data suggesting a role for *SUB2* in pre-mRNA splicing, it is also possible that the *SUB2* high copy suppression of *brr1-1* may be linked to an additional function of *SUB2* in snRNA biogenesis in the cell. A number of recent discoveries and advances since the original identification of *SUB2* are also consistent with such a possibility. Recent biochemical analyses of factors required for snRNP biogenesis in higher eukaryotes have suggested a function for a DEAD-box

ATPase in the snRNP biogenesis pathway. The DEAD-box protein GEMIN3 associates in a tight complex with SMN and SIP1, two factors demonstrated to function in both snRNA biogenesis and pre-mRNA splicing (Fischer et al. 1997; Liu et al. 1997; Pellizzoni et al. 1998; Charroux et al. 1999). Identification of clear sequence homologs of either SMN or SIP1 in S. cerevisiae has remained elusive. However, visual sequence comparison of *BRR1* and SIP1, combined with the previously characterized function in snRNA biogenesis (Noble and Guthrie 1996b), have identified *BRR1* as a candidate S. cerevisiae SIP1 factor (Fischer et al. 1997). Thus, despite the fact that GEMIN3 is distinct from UAP56, the human orthologue of *SUB2*, it remains an open possibility that in S. cerevisiae *SUB2* may function in both pre-mRNA splicing and snRNP biogenesis.

It is likely that there are sufficient quantities of *SUB2* protein to function both in splicing and snRNA biogenesis. Genome-wide expression data suggests that *SUB2* levels in the cell are 5-10 fold greater than the other spliceosomal DEAD-box ATPases (Velculescu et al. 1997). It may be that GAL-overexpression of *SUB2* suppresses *brr1-1* by increasing the amount of *SUB2* available to function in snRNA biogenesis.

These two different hypotheses on the mechanism of *SUB2* suppression of the *brr1-1* phenotype predict experimentally distinguishable effects of *SUB2* overexpression on the *brr1-1* splicing and snRNA biogenesis phenotypes. Thus, we investigated the impact of *SUB2* overexpression on the previously characterized defects of the *brr1* mutant strains to test if the effects of GAL-*SUB2* on the *brr1-1* phenotype were more consistent with a splicing-efficiency mediated mechanism of suppression, or an snRNA biogenesis-related mechanism of suppression. The results of this preliminary analysis favor a mechanism of GAL-*SUB2* suppression through a function in snRNP biogenesis.

RESULTS

SUB2 overexpression bypasses the requirement for BRR1 at 16°C

We began our investigation of the *SUB2-BRR1* interactions by further characterizing the *SUB2-BRR1* genetic interaction. Since the null allele of *BRR1* is viable, but cold sensitive, we were able to test if overexpression of *SUB2* bypasses the requirement for functional *BRR1*. We thus compared the growth of isogenic *BRR1*, *brr1-1*, and Δ*brr1* strains containing vector only, *GAL-BRR1*, or *GAL-SUB2* at 16°C on galactose media.

As previously reported (Noble 1995), overexpression of *SUB2* suppressed the growth defect of the *brr1-1* strain at 16°C (Figure 1). The *GAL-SUB2* plasmid also conferred growth to the Δ*brr1* strain at 16°C (Figure 1), demonstrating that overexpression of *SUB2* is sufficient to bypass the requirement for the *BRR1* gene product. Interestingly, this bypasss suppression phenotype was shared with all the other high copy suppressors of *brr1-1* except *SUB1*, which corresponds to the *LHP1* gene (M. Inada, personal communication, data not shown).

Although the detection of bypass suppression is often indicative of a less direct, non-specific interaction between two factors, the fact that this pattern of suppression is shared among the *brr1* high copy suppressors known or likely to be involved with spliceosomal snRNA biogenesis, is likely to be significant. Moreover, in independent experiments overexpression of *SUB2* confers a detectable growth defect to the wildtype *BRR1* strain (data not shown). This further suggests that the bypass suppression

conferred by *SUB2* is less likely to be due to a general, non-specific enhancement of growth. It may be that in this instance, bypass suppression of the *Δbrr1* growth defect by *GAL-SUB2* may indeed reflect a functional involvement for *SUB2* in a similar biological pathway.

GAL-SUB2 causes defects in splicing, but partially suppresses the brr1-1 splicing defect

To gain more insight into the mechanism by which overexpression of *SUB2* suppresses the cs- phenotype of *brr1-1*, we next examined if the cold sensitive splicing defect of the *brr1-1* mutant was suppressed by overexpression of *SUB2*. In *brr1-1* strains shifted to 16°C for 12 hours, the ratio of U3 pre-mRNA: U3 mRNA increases (Noble and Guthrie 1996a; Noble and Guthrie 1996b). To assess the impact of overexpression of *SUB2* on this *brr1-1* splicing phenotype, we performed primer extension analysis of U3 RNA isolated from cold-shifted *BRR1* and *brr1-1* strains carrying a plasmid vector, and *brr1-1* strains bearing either *GAL-BRR1* or GAL-*SUB2* plasmids. Results from analysis of a single sample from each of these strains before (0 hours timepoint) and after (12 hours timepoint) a shift in growth conditions from permissive temperature (30°C) to non-permissive temperature (16°C) are shown in Figure 2. These revealed an unexpected effect of *GAL-SUB2* on the splicing defect of the *brr1-1* mutant.

In the wildtype *BRR1* strain, growth at 16°C for 12 hours led to a mild increase in both the U3A and U3B pre-mRNA levels compared to the pre-mRNA levels detected before the shift (Figure 2, lanes 1 and 2). In contrast, growth of the *brr1-1* strain at 16°C leads to a significant increase in the U3 pre-mRNA levels (Figure 2, compare lanes 1 and 2 to lanes 3 and 4), as previously reported (Noble and Guthrie 1996b). Overexpression of

BRR1 fully suppresses the increase in U3 pre-mRNA levels detected in the *brr1-1* strain both before and after the shift to 16°C (Figure 2, ccompare lanes 3 and 4 to lanes 5 and 6). Interestingly in the *brr1-1* strain, the level of U3 pre-mRNAs appear elevated relative to those in wildtype strain even before the shift to 16°C (Figure 2, lane 3), despite the fact that equivalent amounts of total RNA were used in each primer extension reaction. This result raises the possibility of some leakiness in the *brr1-1* conditional phenotype. Consistent with this possibility, the growth of the *brr1-1* strain has been observed to be slower at permissive temperature on both plates and in liquid media (M. Inada and A. Kistler, data not shown).

Surprisingly, overexpression of *SUB2* appears to exacerbate, rather than suppress, the accumulation of U3 pre-mRNA detected in the *brr1-1* strain at permissive temperature (Figure 2, compare lane 3 and lane 7). After the 12 hour shift to 16°C, the U3 pre-mRNA levels in the *brr1-1* strains carrying the *GAL-SUB2* plasmid are similar or perhaps only slightly decreased compared to those detected in the *brr1-1* strain after the 16°C shift (Figure 2, compare lanes 4 and 8). Thus in contrast to the predictions for an indirect, increased splicing efficiency mechanism of *SUB2* overexpression suppression of *brr1-1*, it appears that at the permissive temperature, *GAL-SUB2* in a *brr1-1* strain leads to an overall decrease in the splicing efficiency relative to that observed in the *brr1-1* strain. However, in the *GAL-SUB2* strain, the relative increase in U3 pre-mRNA after growth at 16°C is indeed lower than in the *brr1-1* strain (Figure 2, compare lanes 7 and 8 to lanes 3 and 4). Thus, although *GAL-SUB2* overexpression in a *brr1-1* strain at the permissive temperature has a deleterious effect on overall splicing efficiency, it does

appear to weakly suppress the temperature dependent decrease in splicing efficiency conferred by *brr1-1*.

GAL-SUB2 confers growth defects and splicing defects to a wildtype strain at 30°C

Independent observations on the impact that *GAL-SUB2* expression has on the growth and splicing in an otherwise wildtype strain background provided further insight into this surprising effect of *SUB2* overexpression on the splicing phenotype of the *brr1-1* strain. For other purposes, we had created a wildtype strain in which the sole source of *SUB2* in the cell is the same centromeric plasmid bearing the GAL-cDNA *SUB2* isolated in the *brr1-1* high copy suppression screen. To investigate the effect of *SUB2* overexpression on growth and splicing activity, we compared the U3 pre-mRNA splicing in this strain to that of an isogenic strain that contains a centromeric plasmid bearing a genomic version of the *SUB2* gene (WT-*SUB2*). Both strains were grown at 30°C in rich media with galactose provided as a carbon source. The growth rate of these strains was measured, and their splicing activity was monitored by primer extension analysis of RNA samples collected from exponentially growing cultures.

We observed a subtle, but detectable difference in the growth rates of these strains. Under these conditions, the doubling time of a strain bearing the *GAL-SUB2* plasmid was approximately 1.2 fold slower than the strain with the WT-*SUB2* plasmid (data not shown), suggesting *SUB2* overexpression conferred some low level toxicity to a wildtype strain. Consistent with these observations, *SUB2* has been identified independently as one of 24 yeast SHE (Sensitive to High Expression) genes that cause overexpression-mediated growth arrest in wildtype strain background when expressed

from a multicopy (2 micron) GAL-driven plasmid at 30°C (Espinet et al. 1995). Our *GAL-SUB2* strains are not dead under these conditions because the plasmid employed in this study is only centromeric, and the strains were grown in rich media, rather than synthetic minimal media. It is likely that under such conditions there is less *SUB2* expression in galactose media from this construct than the 2 micron GAL construct utilized in the SHE screen.

Primer extension analysis of U3 RNA from two independent samples from these WT-*SUB2* and *GAL-SUB2* strains grown in galactose media is shown in Figure 3A (lanes 1 and 2, and lanes 3 and 4, respectively). Quantiation revealed no significant decrease in U3 mRNA levels (data not shown). However, there is a slight increase (approximately two fold) in the U3 pre-mRNA levels (Figure 3B) in the *GAL-SUB2* strain compared to the strain in which *SUB2* is driven by its endogenous promoter. Although subtle, the detection of a splicing defect in a wildtype strain overexpressing *SUB2* provides a potential explanation for the exacerbation of the splicing defect in the *brr1-1* strain bearing *GAL-SUB2* plasmid at the permissive temperature (Figure 2, compare lanes 3 and 7). It is possible that the exacerbation of the *brr1-1* splicing defect results from combining the leakiness of the *brr1-1* splicing defect with the decreased splicing efficiency conferred by overexpression of *SUB2* in wildtype strains (or near-wildtype strains as would be the case of the *brr1-1* strain at 30°C).

The brr1-1 snRNA biogenesis defect is partially suppressed by GAL-SUB2

To directly address the impact of overexpression of *SUB2* on the *brr1-1* snRNP biogenesis defect, we next examined if the *brr1-1* dependent decrease in steady state

levels of snRNAs was suppressed by *GAL-SUB2*. We isolated total RNA from isogenic *BRR1* and *brr1-1* strains carrying a plasmid vector, and *brr1-1* strains carrying either *GAL-BRR1* or *GAL-SUB2* plasmids both before and after growth at 16°C. The snRNA levels in each sample was assessed by Northern analysis (Figure 4).

In the first Northern, we examined the levels of all 5 snRNAs simultaneously (Figure 4, panel A). As expected, the wildtype *BRR1* strain exhibited little, if any, change in snRNA levels due to growth at 16°C (Figure 4, panel A, lanes 1-3). Consistent with previous observations (Noble and Guthrie 1996b), the *brr1-1* strain bearing only a vector plasmid appeared to have slightly less U1, U2, U4, and U5 snRNAs, (Figure 4, panel A, lanes 4-6). The U4 snRNA appears most sensitive to the shift to the non-permissive temperature in the *brr1-1* strain. The severity of the decrease in levels of this snRNA correlates with the amount of time grown at 16°C (Figure 4, panel A, compare lanes 4-6). The levels of the two different forms of the U5 snRNA (Figure 4, panel A, $U5_L$, U5 long; $U5_S$, U5 short) are also clearly altered after growth at 16°C in the *brr1-1* mutant. The $U5_S$ levels are lower than in wildtype, and decrease over the course of the shift. The $U5_L$ levels are also lower than in the wildtype strain, but surprisingly appear to accumulate slightly over the course of the 16°C shift. The U1 and U2 snRNA levels also appear to be decreased in the *brr1-1* mutant samples relative to the wildtype samples (Figure 4, panel A compare lanes 1-3 to lanes 4-6). However, the magnitude of the decrease in the levels of these snRNAs does not appear to correlate directly with the amount of time grown at 16°C, as seen with the U4 snRNA and the $U5_S$ snRNA. The U6 snRNA levels are not detectably affected in the *brr1-1* mutant after growth at 16°C. It is possible that differences in media or carbon source utilized in this

experiment and the previously published studies where U6 levels also decrease slightly (Noble and Guthrie 1996b) account for this difference. Regardless, the presence of the *GAL-BRR1* plasmid in the *brr1-1* strain restores the snRNAs to near-wildtype levels (Figure 4, panel A compare lanes 1-3 to lanes 7-9), demonstrating that these phenotypes result specifically from a defect in *BRR1* function.

When *SUB2* is overexpressed in the *brr1-1* strain (Figure 4, panel A, lanes 10-12), the levels of U4 snRNA remain constant, rather than decrease over the course of the shift to 16°C. Likewise, the decrease in both U5 snRNA species after the shift to 16°C appears to be less severe in the *GAL-SUB2* strain. Quantitation of U4 snRNA primer (Figure 5A) demonstrates that in a *brr1-1* strain shifted to 16°C, U4 levels decrease to approximately one-fourth of those in wildtype strain or a *brr1-1* strain carrying *the GAL-BRR1* plasmid. In contrast, the *brr1-1* strain carrying the *GAL-SUB2* plasmid contains lower levels of U4 snRNA than the *brr1-1* strain before the shift to 16°C, but appears to prevent further decrease in U4 snRNA levels upon growth at 16°C.

Quantitation of the U5 snRNA levels more clearly demonstrates *GAL-SUB2* suppression of the decrease in U5 snRNA levels conferred by the *brr1-1* mutant in the cold (Figure 5B). At time 0 in the *brr1-1* strain, U5 snRNA levels are slightly lower than all the other isogenic strains tested. As previously observed, these levels drop to approximately half of the amount of U5 in wildtype or *brr1-1* strains carrying *GAL-BRR1* after the 16°C shift. The presence of high copy *SUB2* appears to partially suppress this U5 snRNA depletion phenotype in the *brr1-1* strain. Although there is still a detectable decrease in the U5 snRNA levels in *GAL-SUB2* after growth at16°C, the overall U5

snRNA levels are still higher than the *brr1-1* strain, and only slightly lower than those present in wildtype or *brr1-1* carrying a *GAL-BRR1* plasmid.

In contrast to U4 and U5, the levels of U1 and U2 snRNAs are less clearly affected by overexpression of *SUB2* in the *brr1-1* strain background. More quantitative primer extension assays would be required to accurately determine if *SUB2* overexpression has any impact on the decrease of these snRNAs in a *brr1-1* strain.

GAL-SUB2 does not detectably suppress the U2 3'end processing defect of brr1-1

Previous analysis of the *brr1-1* function in snRNA biogenesis demonstrated a specific role for *BRR1* in the 3'end processing of the U2 snRNA. In transcriptional pulse-chase analysis experiments, a 3'end extended version of the U2 snRNA was detected in both wildtype and *brr1-1* strains. In the wildtype strain, this 3'end extended version of U2 is rapidly processed to the mature U2 snRNA. In contrast, in the *brr1-1* mutant, this 3'end extended version of U2 persists (Noble and Guthrie 1996b).

Since Northern and primer extension analysis suggested *GAL-SUB2* partially suppresses the U4 and U5 snRNA decreases observed in a *brr1-1* strain, we were curious to assess the impact of *GAL-SUB2* on the U2-3'end processing defect of the *brr1-1* mutant. Thus, further Northern analysis of the RNA samples from the wildtype, *brr1-1*, and *brr1-1* strains containing either *GAL-BRR1* or *GAL-SUB2* was performed using a U2-3'end probe. This probe was designed to hybridize specifically to the 20 nucleotides beyond the 3'end of the mature U2 snRNA. Because the U2 snRNA is so large, an oligo specific for this U2-3'end extended precursor is required to detect this U2-3'end extended precursor (M. Inada, personal communication). The membrane probed with all 5

snRNAs was stripped and re-probed with the U2-3'end oligo (Figure 4, panel B). As expected, the U2-3'end oligo hybridized to a high molecular weight band in the *brr1-1* strain (Figure 4, panel B, ←, lanes 4-6), but not the wildtype strain (Figure 4, panel B, ←, lanes 1-3), or the *brr1-1* strain carrying the *GAL-BRR1* plasmid (Figure 4, panel B, ←, lanes 7-9). Interestingly, the band which hybridizes to the U2-3'end oligo band is also present in the *brr1*-1 strain carrying the *GAL-SUB2* plasmid (Figure 4, panel B, ←, lanes 10-12). Thus, overexpression of *SUB2* does not significantly affect the U2-3'end processing defect of the *brr1-1* mutant.

Elongated U4 and U5 snRNAs accumulate in the brr1-1 strains overexpressing SUB2

A number of additional bands were detected in the Northern analysis of all 5 snRNAs (Figure 4, panel A, marked with *, **, and ***) in the *brr1-1* strains used in this study. From the initial Northern analysis with oligo probes directed against all five snRNAs, it was difficult to determine whether these extra bands were due to degradation of the snRNAs or accumulation of snRNA precursors. We were intrigued by the appearance of these bands and sought to determine their identity for three reasons. First, the additional bands appeared more prominent in the *brr1-1* strains and appeared to accumulate over the course of the shift, suggesting a link to the *brr1-1* snRNA biogenesis defect. Second, these additional unidentified bands were also detected in the *GAL-SUB2* strain, and appeared to accumulate to slightly higher levels than in the *brr1-1* strain over the course of the shift to 16°C (Figure 4, panel A, compare lanes to lanes 10-12). Finally, these unidentified bands were also detected in the other *brr1-1* high copy

suppressors tested (*SMD1* and *LHP1*), and were strongly enriched in the GAL-*LHP1* strains at all time points in the shift to 16°C (data not shown).

Since both *BRR1* and *LHP1* had been previously implicated in 3'end processing of RNAs (Madore et al. 1984; Noble and Guthrie 1996b), the appearance of these additional bands in our snRNA Northern potentially revealed additional defects in processing or stability of snRNA precursors in the *brr1-1* strains. Moreover, the accumulation of these additional bands in the *GAL-SUB2* strain suggested that overexpression of *SUB2* might suppress the *brr1-1* snRNA decreases via an influence on the generation, stability or processing of snRNA precursors.

We thus stripped the blot shown in panel A of Figure 4 and re-probed it sequentially with the U2-3'end, U4, and U5 oligo probes (Figure 4, panels B, C, and D, respectively). As discussed in the preceding section, the U2-3'end oligo probe hybridized to a series of high molecular weight bands on the blot in the *brr1-1* and *GAL-SUB2* strains only. The location of these U2-3' end hybridizing bands did not correlate to that of any of the additional mystery bands under study (Figure 4, panel B → compared to panel A * and **). In contrast, the longest and shortest additional bands (* and ***) hybridized to the U4 probe (Figure 4, panel C). These additional U4 hybridizing species were also detectable in the wildtype strain when only the U4 snRNA probe was used (Figure 4, panel C). In the *GAL-SUB2* strain, these U4-hybridizing bands appear to increase slightly over time, while in the other strains examined in this experiment the levels of these species appear to remain constant. The second longest additional band (**) hybridized to the U5 snRNA oligo probe (Figure 4, panel C). When probed with the U5 oligo alone, this U5-related species is not detectable in the wildtype strain or the *brr1-*

1 strain carrying the *GAL-BRR1* plasmid. Overexpression of *SUB2* also appears to lead to a slight increase in the amount of this U5-related species (Figure 4, panel C, compare ** in lanes 7-9 with lanes 10-12).

Since both of the additional higher molecular weight bands (* and **) detected in this study cross-hybridized to probes against lower molecular weight snRNAs, we concluded that the additional bands (* and **) correspond to extended precursors of the U4 and U5 snRNAs. Based on their similarity in migration pattern to previously detected U4 and U5 3' extended precursors ((Allmang et al. 1999; Xue et al. 2000); and data not shown) it is highly likely that these higher molecular weight species correspond to 3'extended U4 and U5 precursors. If this is indeed the case, detection of these bands in the *brr1-1* strain extends the detectable defect in 3'end processing due to loss of *BRR1* function to the U4 and U5 snRNAs (Noble and Guthrie 1996b). The detectable enrichment of the U4 and U5 snRNA precursors in the *brr1-1* strain carrying the GAL-*SUB2* plasmid, suggests that overexpression of *SUB2* may partially suppress the *brr1-1* snRNA biogenesis defect by influencing snRNA precursor generation, processing, or stabilization.

Primer extension analysis of the U4 and U5 snRNAs present in these samples provided a preliminary characterization of the higher molecular weight versions of these transcripts. In the primer extension analysis, the primers utilized would generate a higher molecular weight extension product only if the snRNAs were extended at the 5'end of the transcript. In neither U4 nor U5 primer extension reactions were we able to detect a higher molecular weight product consistent with the size expected for a 5'extended version of the respective snRNA (data not shown). Although more thorough primer

extension analyses with primers the 3'end of the mature versions of these snRNAs would be required to definitively confirm this preliminary data, the current results are indicate that the detected higher molecular weight versions of U4 and U5 are 3' extended snRNA precursors.

snRNP profiles are altered in both sub2 and brr1 mutant extracts

Partial suppression of the decrease in the snRNA levels in a *brr1-1* strain bolsters the hypothesis that *SUB2* might play a role in some aspect of snRNA biogenesis. We were thus interested in determining if any of the conditional *sub2* alleles characterized thus far had any additional effects on snRNA biogenesis. The availability of splicing extracts derived from *sub2-1*, *sub2-5*, and *Δbrr1* mutants grown at permissive temperature provided us with an excellent opportunity to examine both the snRNA levels and the profile of snRNPs in these extracts. These analyses also provided a chance to further assess the snRNP biogenesis phenotypes of *brr1* and *sub2* mutants using an assay in which potentially confounding secondary effects due to growth at the non-permissive temperature would be minimized.

The in vitro splicing, snRNA levels, and snRNP profiles of the Δ*brr1* extracts had been previously characterized (M. Inada,personal communication). The absence of *BRR1* has been observed to confer a mild (2-3 fold), yet reproducible defect in splicing of a pre-actin RNA substrate (M. Inada, personal communication). Primer extension analysis of snRNAs in these extracts incubated under splicing conditions at 16°C or permissive temperature (room temp) showed a similarly mild 2-3 fold decrease in snRNA levels compared to extracts from isogenic wildtype strains (M. Inada, personal communication

and Figure 6A). Native gel analysis to examine the snRNP profiles in these extracts revealed a striking decrease in the levels of all the snRNPs when incubated under splicing conditions, regardless of temperature (M. Inada and Figure 6B-F). Every detectable snRNP species was significantly reduced in the *Δbrr1* extract compared to the wildtype extract, regardless of incubation temperature.

The snRNA levels and snRNP profiles of the extracts derived from the *sub2* mutants had divergent phenotypes. The *sub2-1* extracts appeared to phenocopy the *Δbrr1* extracts. Regardless of preincubation treatment, the snRNA levels were only mildly reduced compared to the levels detected in an isogenic wildtype strain (Figure 6A). In contrast, native gel analysis revealed a significant decrease in all the snRNP species in the *sub2-1* extracts compared to the isogenic wildtype extracts (Figure 6B-F). In contrast, the snRNA levels and snRNP profile of the *sub2-5* extracts were indistinguishable from the isogenic wildtype extracts (Figure 6A-F). Thus, although the defect of the *sub2-5* mutant remains elusive, it appears that the *sub2-1* mutant harbors additional defects in snRNP integrity or stability, in addition to its previously characterized defects related to prespliceosome progression during pre-mRNA splicing (Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). In contrast to the previously characterized splicing defects observed in the *sub2-1* mutant extract, the decreased levels of snRNPs appear independent of temperature preincubation.

Loss of BRR1 both suppresses and exacerbates the growth defects of sub2 mutants

The observation of similar defects in snRNA levels and snRNP profiles in extracts derived from the *sub2-1* mutant and *Δbrr1* mutant suggest an impact on an additional

function of *SUB2* in some aspect of the snRNA transcription, processing, or stabilization. If this were the case, we might expect to detect an exacerbation of growth phenotypes or synthetic lethality in a *sub2-1 Δbrr1* double mutant. We thus generated a *Δsub2 Δbrr1* shuffle strain to investigate the growth phenotype of the *sub2-1 Δbrr1* double mutant by plasmid shuffle. As controls, we also examined the impact of *Δbrr1* on the growth of wildtype *SUB2* and *sub2-5* in the *Δsub2 Δbrr1* strain background. The results of this experiment are summarized in Table 1.

This *sub2-Δbrr1* double mutant analysis provided some unexpected results. Instead of exacerbating the *sub2-1* temperature sensitive growth defect, the *Δbrr1* mutant actually suppressed the *sub2-1* ts- phenotype. In contrast, the cold sensitive growth defects conferred by *sub2-1* and *sub2-5* appear to be exacerbated by *Δbrr1*. While the exacerbation of the cs- phenotypes in the *sub2 Δbrr1* double mutants conform to our expectations for the phenotype of two factors functionally involved in the same pathway, they are subtle. Thus, it remains possible that rather than a synthetic lethal interaction, the exacerbation of the *sub2-1* and *sub2-5* growth phenotypes by *Δbrr1* at low temperatures merely reflects an additive effect of combining two cold sensitive mutant alleles.

Overexpression of BRR1 exacerbates the growth defects conferred by sub2 mutants

Given that the defect in snRNA and snRNP profiles detected in *the sub2-1* mutant phenocopied those of the *Δbrr1* mutant, we were interested to determine if there was also any reciprocity of overexpression suppression between these two genes. We thus sought to determine if the ts- growth defects of the *sub2-1* allele could be suppressed by the

presence of a *GAL-BRR1* plasmid. To control for the specificity of any detectable *GAL-BRR1* impact we observed, we examined the growth phenotypes of isogenic wildtype *SUB2* and the *sub2-5* mutant in parallel with the *sub2-1* mutant. These strains were transformed with either the *GAL-BRR1* plasmid, or an isogenic vector plasmid. After selection and purification of transformants on glucose media, the growth phenotype on galactose media was compared side-by-side by streaking on plates. These results are summarized in Table 2.

The growth of the wildtype strain was unaffected by the presence of *GAL-BRR1* at any of the temperatures tested, suggesting that overexpression of *BRR1* has no deleterious effects on cell viability. Surprisingly, the growth defects associated with both the *sub2-1* and the *sub2-5* mutants were exacerbated, rather than suppressed, by the presence of the *GAL-BRR1* plasmid. These data point to the possibility of an antagonistic functional interaction between *SUB2* and *BRR1*, which may be influenced by expression levels. Biochemical follow-up on the impact of *GAL-BRR1* on the splicing phenotype, snRNA levels, and snRNP profile of both the *sub2-1* and *sub2-5* may provide invaluable insights into the basis of this exacerbation and the functional interplay between *SUB2* and *BRR1*.

Deletion of BRR1 can partially relieve the toxicity conferred by GAL-SUB2

Further genetic evidence for an antagonistic functional interaction between *SUB2* and *BRR1* which is influenced by the levels of each factor comes from additional studies of the impact of changes in *BRR1* levels on the overexpression toxicity conferred by the presence of a *GAL-SUB2* plasmid. The severity of the growth defect conferred *by GAL-*

SUB2 overexpression from a centromeric plasmid in an otherwise wildtype strain background is exacerbated at increased temperatures. In a W303 strain background, the presence of the centromeric *GAL-SUB2* plasmid is toxic at 37°C (Figure 5, top panels). Because we observed that *GAL-SUB2* is also mildly toxic to the growth of a wildtype strain at 16°C (Figure 1, left), yet capable of suppressing the growth defects of *brr1-1* and Δ*brr1* strains, we were curious if this trend of growth with *GAL-SUB2* also applied at higher temperatures. Thus we examined the impact of *brr1* mutants on the toxicity of *GAL-SUB2* we detected at temperatures above 30°C.

Intriguingly, we found that the Δ*brr1* strain appears to partially suppress the lethality conferred by high copy *SUB2* at elevated temperatures. In contrast to wildtype strains, Δ*brr1* strains containing the centromeric *GAL-SUB2* plasmid are capable of growth beyond the primary streak at both 30°C and 33°C (Figure 4, bottom panel). The growth of the *brr1-1* strain containing the *GAL-SUB2* plasmid exhibited a weaker, yet detectable partial suppression of the temperature sensitive growth defects conferred by *GAL-SUB2* (data not shown). The growth of the Δ*brr1* strains carrying a *GAL-SUB2* plasmid was also observed to be faster at the semi-permissive temperatures than the isogenic wildtype strains carrying a *GAL-SUB2* plasmid. In experiments where these strains were streaked side-by-side, at all temperatures where growth occurs, visible growth of the Δ*brr1* strain preceded that of the wildtype strain (data not shown).

In summary, growth defects due to loss of *BRR1* function can be partially suppressed by overexpression of *SUB2* in the cold (Figure 1), and growth defects due to overexpression of *SUB2* at high temperature can be partially suppressed by loss of *BRR1* (Figure 4). Taken together, these data are consistent with the possibility of antagonistic

roles for *SUB2* and *BRR1* in the cell, which can be influenced by an alteration in the balance of the levels of these factors. Proof that the balance of levels of these factors is important is found in experiments where both *BRR1* and *SUB2* are co-expressed in the same cell. Surprisingly, overexpression of *BRR1* also partially suppresses the ts- growth defects conferrend by *GAL-SUB2* (Figure 8).

DISCUSSION

Evidence for a role for SUB2 in snRNP biogenesis

This preliminary analysis of the impact of *SUB2* overexpression on the *brr1-1* splicing and snRNA biogenesis suggests that high copy *SUB2* suppresses the growth defect of *brr1-1* through influencing some aspect of snRNA biogenesis, stability, or processing. These conclusions are supported by the detection of defects in snRNP integrity in *sub2-1* splicing extracts. Like the *Δbrr1* extracts, the *sub2-1* mutant extract exhibits lower levels of all the snRNP complexes, regardless of temperature preincubation. Thus it appears that Sub2 may play a role in both pre-mRNA splicing and some aspect of snRNP formation or stability. Evidence from genetic analysis of interactions between *SUB2* and *BRR1* also substantiate the possibility of a role for *SUB2* in snRNP integrity. The complex interactions between *SUB2* and *BRR1* we detect display a pattern of functionally antagonistic interactions that can be modulated by the levels of each factor.

Parallels with snRNP biogenesis in higher eukaryotes?

The detection of a role for a DEAD-box ATPase in both splicing and snRNA biogenesis in yeast raises further parallels with factors recently identified in a large complex implicated in both splicing and snRNA biogenesis in higher eukaryotes (Fischer et al. 1997; Liu et al. 1997; Pellizzoni et al. 1998; Meister et al. 2000). Initially similarities between the mammalian snRNP biogenesis factor SIP1 and *BRR1* from yeast were drawn based on function, despite the lack of obvious sequence homology (Fischer et al. 1997). More recently, clear SMN and SIP1 homologs in Schizosaccharomyces pombe which bear little sequence homology to *BRR1* have been identified (Hannus et al. 2000; Owen et al. 2000). This lack of sequence homology has raised questions in light of a dearth of biochemical data as to whether *BRR1* is actually a functional homolog of SIP1 in S. cerevisiae (Hannus et al. 2000). Our initial genetic identification and characterization of *SUB2*, combined with the preliminary biochemical analyses presented here, strongly suggest that *SUB2* may be involved in both splicing and the *BRR1*-dependent snRNA biogenesis pathway *SUB2* (Noble 1995; Kistler and Guthrie 2001; Libri et al. 2001; Zhang and Green 2001). Moreover, our genetic analyses of the interplay of the levels of these two factors on cell viability also raises the possibility of a stoichiometric interaction between *SUB2* and *BRR1*.

In HeLa cells, the SMN-associated DEAD-box protein GEMIN3 interacts directly via protein-protein interactions with SMN, SIP1, and the Sm proteins SmB, SmD2, and SmD3 in a large complex (Charroux et al. 1999) implicated to function in both splicing and snRNP biogenesis (Fischer et al. 1997; Pellizzoni et al. 1998). While a direct role for GEMIN3 in splicing and snRNP biogenesis remains to be demonstrated, its association in

a complex with factors implicated in both of these processes is highly suggestive of a possible function in association with, or regulation of, this SMN splicing/snRNP biogenesis complex. Consistent with the possibility that Sub2 may exist in a complex, we have observed that efficient immunodepletion of two different epitope-tagged versions of Sub2 from splicing extracts requires the presence of high salt (data not shown). It is possible that the salt sensitivity of Sub2 immunoprecipitation reflects the existence of a Sub2-containing particle in yeast which functions similar to the SMN complex.

Further parallels between *SUB2* and *BRR1* interactions in yeast with those of the mammalian SMN complex can be gleaned from a recent report linking the nuclear SMN/SIP1 complex to RNA polymerase II. Analysis of the components of the SMN/SIP1 complex have revealed that RNA helicase A (RHA) is an SMN interacting protein (Pellizzoni et al. 2001). An RHA-dependent interaction between the C-terminal domain of RNA polymerase II (CTD) and SMN was also detected (Pellizzoni et al. 2001). Although the phosphorylation state of the CTD does not appear to influence this interaction, it is likely that these observations reflect some link or coupling between RNA polymerase II transcriptional activity and snRNP biogenesis or splicing.

As reviewed briefly in the introduction, links to transcription were also detected in our high copy suppression analysis of *brr1-1* (Noble 1995). *TBF4* was isolated as a strong high copy suppressor of *brr1-1*. It encodes a component of the TFIIH complex, which is thought to play an important role in transcription initiation and nucleotide excision repair (Feaver et al. 1997; Hampsey 1998; Feaver et al. 1999; Guzman and Lis 1999). While the nature or directness of the *BRR1-TBF4* interaction has yet to be fully

characterized, this genetic interaction may point to a parallel functional link between transcription elongation and snRNP biogenesis in yeast.

Overexpression of Sub2 has also been genetically linked to a function in transcription through suppression of a hyper-recombination defect. High copy *SUB2* was detected to suppress the temperature sensitive growth defect and hyper-recombination phenotype conferred by deletion of the *HPR1* gene. *HPR1* is required to maintain normal levels of recombination between directed repeats in the chromosome. It possesses some homology to *TOP1*, and has been implicated to play a role in transcription elongation. *HPR1* exhibits genetic interactions with components of the RNA polymerase II (pol II). These genetic links appears to reflect a physical connection to pol II, since *HPR1* protein has been found in affinity purified sub-complexes of pol II.

Thus, suppression of Δ*HPR1* by *SUB2* overexpression may reflect an additional role for Sub2 in transcription, or a general association of Sub2 with the transcription machinery. Consistent with this possibility, loss of function alleles of *sub2* display a hyper-recombination phenotype. Further biochemical analysis of the genetic interactions between these transcription components and *BRR1* and *SUB2* will likely clarify these possible links to transcription in yeast, and may further elucidate whether these genetic interactions with *HPR1* reflect further parallels with the mammalian SMN/SIP1 complex.

Insights into SUB2 BRR1 function from recent discoveries on snRNA biogenesis in yeast

Recent analyses of snRNA biogenesis in yeast since the initiation of the original *brr1-1* studies have identified a number of factors that influence spliceosomal snRNA biogenesis. While our understanding of snRNP biogenesis in yeast is far from complete,

the insights from these studies provide a framework for the development of testable models for the possible roles that *BRR1* and *SUB2* might play during snRNP biogenesis in yeast.

Processing of 3' elongated snRNA precursors: connections to Rnt1 and the exosome?

Analysis of the function of RNT1, the gene encoding the yeast RNAse III has demonstrated a role for this enzyme in the 3'elongated snRNA precursors of U1, U2, U4, and U5 spliceosomal snRNAs (Chanfreau et al. 1997; Abou Elela and Ares 1998; Allmang et al. 1999). The 3'-5' exonucleolytic processing of the 3'elongated Rnt1 cleavage products is likely to be carried out by the exosome, since genetic depletion of individual exosome components leads to the accumulation of these 3'elongated precursors (Allmang et al. 1999). In the absence of Rnt1 and the exosome, it appears that other processing pathways can perform the 3'end processing of snRNA precursors (Chanfreau et al. 1997; Abou Elela and Ares 1998; Allmang et al. 1999; Seipelt et al. 1999). However, the levels of precursor and mature forms of some of the snRNAs generated by these alternative pathways are significantly increased relative to those detected under normal conditions (Allmang et al. 1999).

The defects in 3'end processing of the snRNAs detected in *brr1-1* mutants ((Noble 1995) and data presented here) suggests that *BRR1* is involved in some aspect of this processing event during snRNP biogenesis. Identification of an increase in such snRNA precursors upon overexpression of *SUB2* in a *brr1-1* strain also point to a potential role for *SUB2* in this step as well. Since neither *BRR1* nor *SUB2* genes appear to encode proteins with nucleolytic activity, it may be that they play an indirect role in

3'end processing of snRNAs. For example wildtype *BRR1* may play some sort of regulatory role in Rnt1 or exosome activity. Overexpression of *SUB2* might be able to somehow modulate such a function of *BRR1*. Further biochemical and genetic analysis of the interactions between *SUB2*, *RNT1*, and *BRR1* may prove useful in dissecting the actions of these factors in this step of the snRNA biogenesis pathway in yeast.

Alternatively, it may be that Brr1p binds snRNA precursors as they are generated, and is required as a signal for further processing by the exosome to generate mature snRNAs. In the absence of Brr1 binding the precursor, snRNA precursor degradation may be more likely, thus leading to reduced levels of newly synthesized snRNAs. This appears to be the case in *smd1-1* strains with regard to U4 levels (Xue et al. 2000). The presence of additional copies of *LHP1* can suppress this defect by both an indirect effect, through stabilization of pre-snRNA levels, and a direct influence on the binding activity of Smd1p to the U4 snRNA precursors (Xue et al. 2000). Similarly, a mutant version of the Sm-like core protein, Lsm8 leads to decreases in U6 snRNA levels. Again, increasing the dosage of *LHP1* can suppress the decrease in mature U6 snRNA levels, perhaps through the stabilization of nascent U6 molecules (Pannone et al. 1998). If Brr1p normally performs a similar function, the activity of the *brr1-1* mutant may be suppressed similarly by *GAL-LHP1*.

Consistent with a possible function for Brr1p in binding to nascent snRNA precursors to influence mature snRNA levels, epitope-tagged Brr1p has been demonstrated to immunoprecipitate spliceosomal snRNAs (Noble and Guthrie 1996b). Interestingly, only a very small percentage of the total snRNAs are immunoprecipitated specifically with the epitope-tagged version of Brr1 ((Noble and Guthrie 1996b) and M.

Inada, personal communication). The sizes of the snRNAs that co-immunoprecipitate with Brr1 in these experiments appear to correspond to the mature snRNAs, rather than snRNA precursors. Although this appears contradictory to a model where Brr1 binds snRNA precursors, it is possible that the binding detected with the mature snRNAs is residual Brr1 remaining on the mature snRNAs after exosome processing has occurred. Detection of snRNA precursor binding to both Smd1p and Lhp1p in previous studies was enhanced in a distinct assay in which in vitro transcribed snRNA precursors or mature snRNA were utilized as substrates for co-immunoprecipitation studies in splicing extracts (Pannone et al. 1998; Xue et al. 2000). To better evaluate this possible function for *BRR1* in snRNP biogenesis, a similar assay may need to be employed due to the sparse and transient amounts of snRNA precursors present in whole cell extracts prepared from yeast.

How might overexpression of *SUB2* suppress a defect in this function? Overexpression of *SUB2* appears to lead to a small increase in the amount of the U4 and U5 precursors in a *brr1-1* strain background. In studies related to the decreased snRNA phenotype of *smd1-1*, overexpression of a combination of both U1 and U4 were sufficient to suppress the growth defect conferred by *smd1-1*, and suppress the synthetic lethality of the *smd1-1 Δlhp1* double mutant at the non-permissive temperatures (Xue et al. 2000). Preliminary experiments in our hands suggest that overexpression of U4 alone does not suppress the cold sensitive growth defect of the *brr1-1* or Δ*brr1* mutant (M. Inada, personal communication). Given the genetic links between both *BRR1* and *SUB2* to components of the RNA polymerase II complex, it is possible that overexpression of

SUB2 may function to increase the generation of a combination of snRNA precursors available for binding by *brr1-1*.

It may be that the binding of the snRNA precursors by Brr1p or Smd1p must occur co-transcriptionally to stabilize and mark precursors efficiently for further processing by downstream factors of the snRNA biogenesis pathway. It is possible that the bypass suppression of *GAL-SUB2* and GAL-*TBF4* may be due to possible roles these factors play in the generation of snRNA precursors. In the absence of *BRR1*, increasing the generation of snRNA precursors by *GAL-SUB2*, or GAL-*TBF4* may be effectively stabilized by the binding of other factors, such as SmD1, which may bind co-transcriptionally and may serve as a part of a redundant signal for exosome processing of Rnt1-cleaved snRNA precursors. Consistent with this model, GAL-*SMD1* on its own is also capable of bypassing the in vivo requirement for *BRR1*. The lack of bypass suppression by the *LHP1* strain raises the possibility that although Lhp1 can stabilize these snRNA precursors, it may act at a stage that may be downstream from the essential function of *BRR1*. Thus, if Brr1 protein is completely absent from the cell, an increase in the snRNA precursors at the stage after it Brr1 normally acts has no impact on the "throughput" of the essential Brr1-dependent snRNA biogenesis pathway.

This model for potential *SUB2-BRR1* functions during snRNA biogenesis could be easily tested by examining the impact of the simultaneous overexpression of a combination of snRNAs on the growth of the *brr1-1* and Δ*brr1* strains. If this model is indeed correct, there will likely be a combination of snRNAs that will phenocopy the *GAL-SUB2* suppression profile. Verification of a specific role for *GAL-SUB2* in transcriptional upregulation of snRNAs would require analysis of the alteration in

transcription profile of the snRNAs over the course of induction of *GAL-SUB2* overexpression. A direct correlation in *SUB2* levels and increased amounts of snRNA precursors would be strong evidence for a potential role for *SUB2* in a transcription-mediated suppression of defects in *BRR1* function. Distinguishing a role in transcription from a function in precursor stabilization could be accomplished by monitoring the fate of snRNA precursors after RNA polymerase II transcriptional shut-off in a *brr1-1* strain overexpressing *SUB2*.

FUTURE DIRECTIONS

Biochemical analysis of BRR1 and SUB2 function in snRNA biogenesis:

1. Confirmation and extension of the preliminary observations.

The preliminary biochemical analyses performed to analyze the impact *of GAL-SUB2* on the splicing, and snRNA phenotypes of *brr1-1* mutants merits repeating in these different strains. In particular, studies on the impact of *SUB2* overexpression in a wildtype strain at both permissive and non-permissive temperatures might shed light on how overexpression of *SUB2* influences splicing and snRNA biogenesis. Varying the gene dose of *BRR1* in these studies would further clarify the detected *SUB2-BRR1* genetic interactions, and allow for a direct test of the interpretation of the functional interaction of these two factors based on our preliminary genetic analysis. Small-scale bead-beat extracts (Rutz and Seraphin, personal communication) may provide a rapid and convenient method to prepare multiple samples from these strains for comparison of both in vivo and in vitro effects of the growth temperature on splicing, snRNA levels and snRNP profiles.

2. Biochemical analysis of BRR1 and SUB2 function in snRNA biogenesis:

Biochemical characterization of SUB2 and BRR1.

The interpretation of the results of our genetic matrix of interactions we detect between *BRR1* and *SUB2* is hampered by a dearth of detailed information on the physical interactions of these factors. When these studies were initiated, few reagents existed to address such questions. There are now multiple versions of epitope-tagged *SUB2* reagents available for biochemical analysis. Additionally, polyclonal antisera raised against purified, recombinant Sub2(HIS_6) protein has also been generated to facilitate analysis of native, untagged Sub2 protein in different strain backgrounds. Examination of the protein-protein interactions and RNA-protein interactions of Sub2 is now relatively straightforward and a should be a high priority.

Early experiments that suggested that Sub2 is not associated with snRNAs based on co-immunoprecipitation should be repeated, given the follow-up observations that a significant pool (approximately 50%) of Sub2 was inaccessible to immunoprecipitation in aqueous buffers containing less than 250mM monovalent cation. The apparent salt sensitivity of Sub2 immunoprecipitation may reflect the existence of two pools of Sub2 in extract: a large low-salt accessible pool, and an almost equivalent sized pool that is inaccessible in the absence of high salt. Although the accessible pool does not appear to be associated with snRNAs, its association with proteins has not been investigated, and merits study. Investigation of potential protein-protein and protein-RNA interactions in the pool of Sub2 inaccessible to immunoprecipitation under standard conditions should be pursued as a high priority, given the potential parallels between the functions of the

mammalian SMN/SIP1 complex and the apparent *SUB2-BRR1* functions in snRNP biogenesis. Additionally, independent studies suggest that the mammalian *SUB2* homolog, UAP56 may be present in a snRNP-enriched fraction of nuclear extracts (C. Newnham , C. Query, and M. Konarska, personal communication). Thus, it is possible that Sub2 does indeed associate with snRNAs, but in the context of a complex where the epitope tag is masked.

Simple first pass experiments to address this question are those described in the text of discussion covering the parallels between *SUB2* and *BRR1* interactions and the components of the mammalian SMN complex. In particular, Western blot analysis of native gels to compare the mobility of endogenous Sub2 in extracts compared to purified Sub2 would address if endogenous Sub2 is present in a complex in yeast extracts. If a complex were detected, the impact of salt, ATP, RNAses, and splicing substrates on the integrity of the complex could rapidly be addressed through a native gel assay. More sophisticated analytical techniques could also be applied to follow up any positive results obtained by native gel electrophoresis. Application of gel filtration chromatography or sucrose density gradient centrifugation could be employed to better characterize the size of any detected complexes, and perhaps ultimately purify and characterize the components of a Sub2 complex from yeast.

The physical interactions of *BRR1* also merit further study. The levels of Brr1p in yeast cells are far lower than Sub2. Genome wide expression data suggest a 10 fold difference in the amounts of these two factors (Velculescu et al. 1995). Thus, efforts to identify the snRNA interactions or protein-protein interactions of epitope tagged Brr1 have proven difficult (M. Inada, personal communication). Since GAL-*BRR1* does not

appear to have any deleterious effects on cell growth, it may be worthwhile to investigate the protein-protein interactions of Brr1p in cells overexpressing *BRR1* from the GAL promoter. Utilization of the TAP tag system (Rigaut et al. 1999) may also be a route to high yield purification of Brr1-containing complexes from yeast. Generation and purification of recombinant Brr1 protein may also prove to be a fruitful avenue for investigating the protein-protein interactions of Brr1. First, it is possible that a higher affinity polyclonal antibody against Brr1 could be obtained for use in further study. Second, a recombinant version of Brr1 may facilitate identification of protein interacting partners through affinity chromatography studies. Additionally, rBrr1p may also prove useful in directed analysis of possible snRNA interactions with in vitro transcribed precursor or mature snRNAs. Success on any of these fronts may help provide tools for analysis that would likely further our understanding of the biochemical and genetic functions of *BRR1*.

Genetic strategies for further insight into SUB2 and BRR1 function

Directed genetics with brr1 and sub2 alleles with candidate snRNA biogenesis factors

As described in the Discussion section, recent advances in the identification of different factors required for snRNA biogenesis in yeast may provide useful to further verify and pinpoint the functions of *BRR1* and *SUB2* in the snRNA biogenesis pathway. There is currently a short list of mutant versions of factors which would be predicted to exhibit genetic interactions with *SUB2* and *BRR1* if indeed they are acting in the same biological pathway. Investigation of genetic interactions with available alleles of *SMD1*, LSM8, deletion and overexpression versions of *LHP1*, alleles of RNT1, and components

of the exosome may provide further insight into the roles that *SUB2* and *BRR1* play in this pathway. Additionally, directed investigation of the impact of deletions of the C-terminal Domain (CTD) of RNA polymerase II on the snRNA biogenesis phenotypes associated with *brr1* and *sub2* mutants may shed further light on the potential connection between snRNA biogenesis and transcription.

Open-ended synthetic lethal screen with sub2-1

A number of attempts to identify *SUB2*-interacting factors through open-ended high copy suppression analysis of the *sub2-1* allele have been unsuccessful (J. Pan, personal communication). As an alternative strategy, synthetic lethal analysis may prove more successful in identification of factors interacting with *SUB2*, or pathways that *SUB2* may affect. This analysis may be particularly useful in elucidating the function of *SUB2* in snRNP biogenesis. With a series of straightforward, simple splicing and snRNA biogenesis assays available for secondary screens, it may be possible to sort any candidates obtained from such studies in a rapid manner. An added benefit of such a screen is that in addition to apparent defects in snRNA biogenesis, *sub2-1* has been demonstrated to possess defects in pre-mRNA splicing. Thus, additional factors required for the function of *SUB2* during pre-mRNA splicing are also likely be identified in such a screen. Such genetic analysis will likely further elucidate the function(s) of *SUB2* and the factors with which it collaborates during pre-mRNA splicing and snRNA biogenesis in yeast.

Open-ended synthetic lethal screen with brr1-1 or Δbrr1

Loss of *BRR1* function is deleterious to cell viability at low temperatures. To gain further insight into other factors which may possess functions that overlap with *BRR1*, a synthetic lethal screen may be an important direction to pursue, especially given the recent difficulties in biochemical attempts to analyze the protein-protein and protein-RNA interactions of *BRR1*. Identification of genes, which when mutated, cannot survive in the absence of *BRR1* may likely provide further insight into the functions that *BRR1* performs, or the factors with which *BRR1* interacts. Detection of synthetic interactions between *BRR1* and a number of factors involved in triple snRNP stability or integrity (SAD1, *PRP24*, and SNU66) through directed genetic analysis suggest that such a screen may turn up a number of factors involved in tri-snRNP formation or stability. Information gained from a more open-ended synthetic lethal analysis with *brr1-1* or Δ*brr1* may prove invaluable to our understanding of Brr1 function and snRNP biogenesis in yeast.

MATERIALS AND METHODS

Yeast strains and plasmids used in this study

yGS1	MATa ade2-100^0 ura3-52 lys2-801^a his3-Δ200 leu2-Δ1
yGS2	MATα ade2-100^0 ura3-52 lys2-801^a his3-Δ200 leu2-Δ1
ySN186	MATα *brr1-1* ade2-100^0 ura3-52 lys2-801^a his3-Δ200
ySN226	MATa Δ*brr1*::*LEU2* ura3 leu2 ade2 his3 lys2
yAK304	*MATa Δsub2::HIS3 ura3 leu2 trp1 his3*/pAK#291
yAK313	*MATa Δsub2::HIS3 ura3 leu2 trp1 his3*/pAK297

yAK317 *MATa Δsub2::HIS3 ura3 leu2 trp1 his3*/pAK320

yAK343 *MATa Δsub2::HIS3 ura3 leu2 trp1 his3*/pAK322

yAK424 diploid derived from ySN186 x yGS1

yAK427 diploid derived from ySN226 x yGS2

pRS315 (*LEU2 CEN* vector)

pRS316 (*URA3 CEN* vector)

pSN133 (*GAL-BRR1* in pRS316)

pSN120 (*GAL-SUB2* in pRS316)

pAK291 (*GAL-SUB2* in pRS315)

pAK297 (*SUB2* in pRS315)

pAK322 (*sub2-1* in pRS315)

pAK320 (*sub2-5* in pRS315)

pAK384 (*Δsub2::KANMX6* in pCR2.1 TOPO T/A vector)

Oligonucleotides used in this study

oCG368 (U3 EXON 2): C C A A G T T G G A T T C A G T G G C T C

oCG608 (U1-19G): CAGTAGGACTTCTTGATC

oCG609 (U2-L15): CAGATACTACACTTG

oCG350 (U2-3'END): A A A A T A A A A A A A T A A A A G A G C G A A C

oCG610 (U4-14B): AGGTATTCCAAAAATTCCCTAC

oCG611 (U5-7WTSMNR): AAGTTCCAAAAAATATGGCAAGC

oCG612 (U6-6D): AAAACGAAATAAATCTCTTTG

oCG90 (SSU1) GAATGGAAACGTCAGCAAACAC

oCG91 (SSU2) CTTAAAAAGTCTCTTCCCGTC

oCG92 (SSU4) ACCATGAGGAGACGGTCTGG

oCG325 (SSU5-120) ACACCCGGATGGTTCTGGTA

oCG95 (3'U6) AACGAAATAAATCTCTTTGTAAAAC

oAK93 (U14) ACGATGGGTTCGTAAGCGTACTCCTACCGTGG

SUB2-BRR1 genetic interactions

The impact of *GAL-SUB2* on the growth of wildtype, *brr1-1*, and *Δbrr1* strains was assessed by transforming yGS1, ySN186, and ySN226 with pRS316, *pSN133 (GAL-BRR1* in pRS316), and pSN120 (*GAL-SUB2* in pRS316) and streaking on synthetic galactose-uracil plates. These plates were incubated at various temperatures as indicated in Figures 1 and 7, and summarized in Table. To compare the growth of wildtype strains containing *GAL-SUB2* in the presence and absence of *GAL-BRR1*, strain yGS1 was transformed with four sets of plasmid pairs: pRS316 and pRS315; pSN133 and pRS315; pSN120 and pRS315; and pSN133 and pAK291 (*GAL1-SUB2* in pRS315). The impact of *BRR1* overexpression on the ts-growth defect of wildtype strains carrying *GAL-SUB2* was assessed by comparing the growth of these transformants on synthetic galactose plates lacking uracil and leucine, incubated at the temperatures shown in Figure 8. The impact of *GAL-BRR1* on the growth phenotypes of *sub2-1* and *sub2-5* mutants was analyzed by comparing the growth of yAK313, yAK343, and yAK317 transformed with pSN133 or pRS316 on synthetic galactose-uracil plates and incubated at the temperatures indicated in Table 2. To assess the growth impact of combining *sub2* and *brr1* mutants by plasmid shuffle (Boeke et al. 1987), strains ySN186 and ySN226 were crossed to

yGS1 and yGS2, to generate yAK 424 and yAK427, respectively. These diploids were transformed with the 2kb SpeI-Xho fragment of pAK348 (*Δsub2::KANMX* in pCR2.1 [Invitrogen]) to generate a chromosomal *Δsub2::KANMX* allele (Longtine et al. 1998). The *Δsub2::KANMX* allele was confirmed through whole cell PCR of the KAN^r transformants. The resulting strains were transformed with pAK269, and sporulated. Haploid *URA+* strains were screened for KAN^r, and the presence of *Δsub2::KANMX* in the chromosome was confirmed by whole cell PCR. *Δsub2::KAN brr1-1* strains were identified by their cs- growth, while *Δsub2::KAN Δbrr1::LEU2* strains were identified by growth on SD-LEU and cs- growth. The resulting haploid shuffle strains were transformed with pAK354 (*SUB2* in pRS313), pAK356 (*sub2-1* in pRS313), or pAK355 (*sub2-5* in pRS313). These transformants were streaked to 5FOA at 30°C media to lose pAK269. The growth of the resulting double mutant strains was assessed by streaking side-by-side with the parental *brr1-1* (ySN186), *Δbrr1* (ySN226), and the wildtype *BRR1 Δsub2::KAN* sister spores on YEPD media at the temperatures indicated in Table1.

Impact of GAL-SUB2 on in vivo splicing and snRNA phenotypes of brr1-1

Primer extension analysis of steady state levels of U3 RNA was performed to assess the impact of *GAL-SUB2* on the splicing defect conferred by *brr1-1*. Whole cell RNA was isolated from exponentially growing cells shifted from 30°C to 16°C for 0h, 6h, or 12h in synthetic galactose media lacking uracil. A ^{32}P-labeled oligonucleotide which anneals to exon 2 of U3 was used in an abbreviated primer extension protocol (Staley and Guthrie 1999) to detect both the U3 precursors and mRNA species. This same method was also applied to assess splicing in a set of RNA samples derived from

wildtype strains containing a *GAL-SUB2* plasmid or a *SUB2* plasmid driven by the endogenous *SUB2* promoter grown at 30°C. Oligonucleotide U5wtsmnr (oOG611) was used to detect U5 snRNA, which served as an internal control for RNA loading. The normalized amount of U3 pre-mRNA species in each of these samples was quantified by phosphorimager analysis. To assess the impact of *GAL-SUB2* on the decrease in snRNAs detected in *brr1-1* after shifting to 16°C, the whole cell RNA isolated in the temperature shift was also subjected to Northern hybridization analysis. A mixture of ^{32}P-labeled oligonucleotide probes (oCG608-oCG12) against all 5 spliceosomal snRNAs was initially used. To more carefully examine the impact on individual snRNAs, the blot was stripped and re-probed with ^{32}P-labeled oligonucleotide probes against the U2 3'end (oCG350), U4 (oCG610), and U5 (oCG611).

In vitro analysis of snRNA levels and snRNP profiles in splicing extracts

To assess the impact of *sub2* mutants and the *Δbrr1* mutant on snRNA levels and snRNP profiles, yAK313, yAK317, yAK343, and two independent cultures derived from yGS1 and ySN226 were grown at permissive temperature to an OD_{600nm} of 2 in YEPD media. Extracts derived from each of these cultures were prepared (Umen and Guthrie 1995). Extracts derived from the ts- *sub2-1* strain and an isogenic *SUB2* strain were preincubated in parallel for 10 minutes at 37°C or mock preincubated on ice for 10 minutes, then incubated with transcript in the presence of splicing buffer components (2mM ATP, 2.5mM MgCl2, 0.6mM Kphoshate, pH7, 3% PEG 8000). Extracts derived from the cs- *sub2-5*, *Δbrr1*, and isogenic wildtype control strains were incubated with

transcript in splicing buffer at 16°C or room temperature for 30 minutes. The levels of snRNA in these extracts were analyzed by primer extension analysis with a mixture of ^{32}P-labeled oligonucleotides against all 5 of the snRNAs (oCG90, oCG91, oCG92, oCG95, and oCG325) as previously described (Staley and Guthrie 1999). Samples were normalized by protein concentration present in each extract. The profile of snRNPs in these extracts was also analyzed through sequential Northern hybridization with ^{32}P-labeled oligonucleotides probes against each of the snRNAs (oCG608-oCG612) of samples separated by native gel electrophoresis (Cheng and Abelson 1987). Samples were again normalized by protein concentration present in each extract.

Figure 1. Impact of *GAL-SUB2* on the growth of isogenic wildtype, *brr1-1*, and *Δbrr1* strains at 16°C. Strains yGS1 (*BRR1*), ySN186 (*brr1-1*), and ySN226 (*Δbrr1::LEU2*) were transformed with the *GAL* vector only (left side) or *GAL-SUB2* (right side) and streaked to SGAL-URA plates at 16°C.

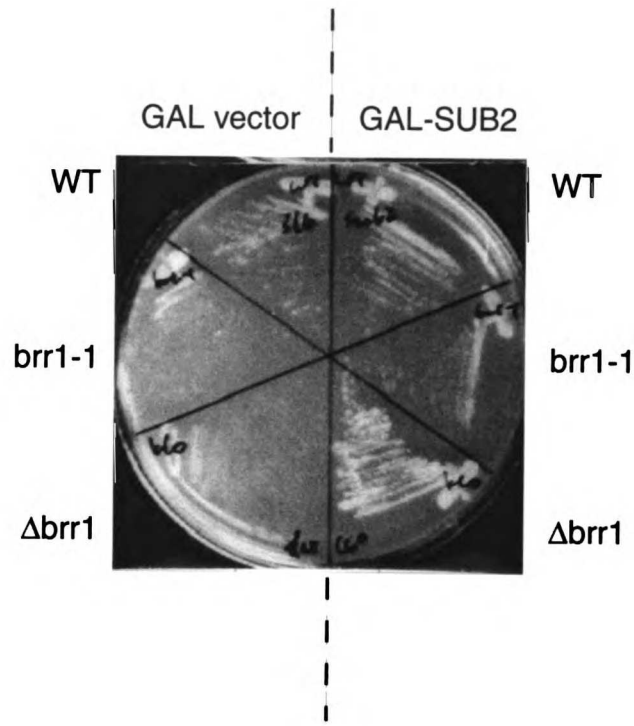

GAL vector
GAL-SUB2
WT
WT
brr1-1
brr1-1
Δbrr1
Δbrr1

Figure 2:

Impact of *GAL-SUB2* on the U3 pre-mRNA splicing defect in *brr1-1* strains. RNA was extracted from wildtype *BRR1*/pRS316 (lanes 1 and 2) or *brr1-1* strains carrying vector only (lanes 3 and 4), *GAL-BRR1* (lanes 5 and 6), or *GAL-SUB2* (lanes 7 and 8) at 0 hours (lanes 1, 3, 5, and 7) or 12 hours (lanes 2, 4, 6, 8) after a shift to 16°C. Primer extension of equal amounts of total cellular RNA was performed with a labeled oligonucleotide complementary to exon 2 of the U3 snoRNA. U3 pre-mRNA and mRNA primer extension products are indicated to the right of the gel.

GAL PLASMID	vector		vector	BRR1		SUB2	
GENOTYPE	BRR1		brr1-1	brr1-1		brr1-1	
TIME AT 16°C	0	12	0 12	0	12	0	12

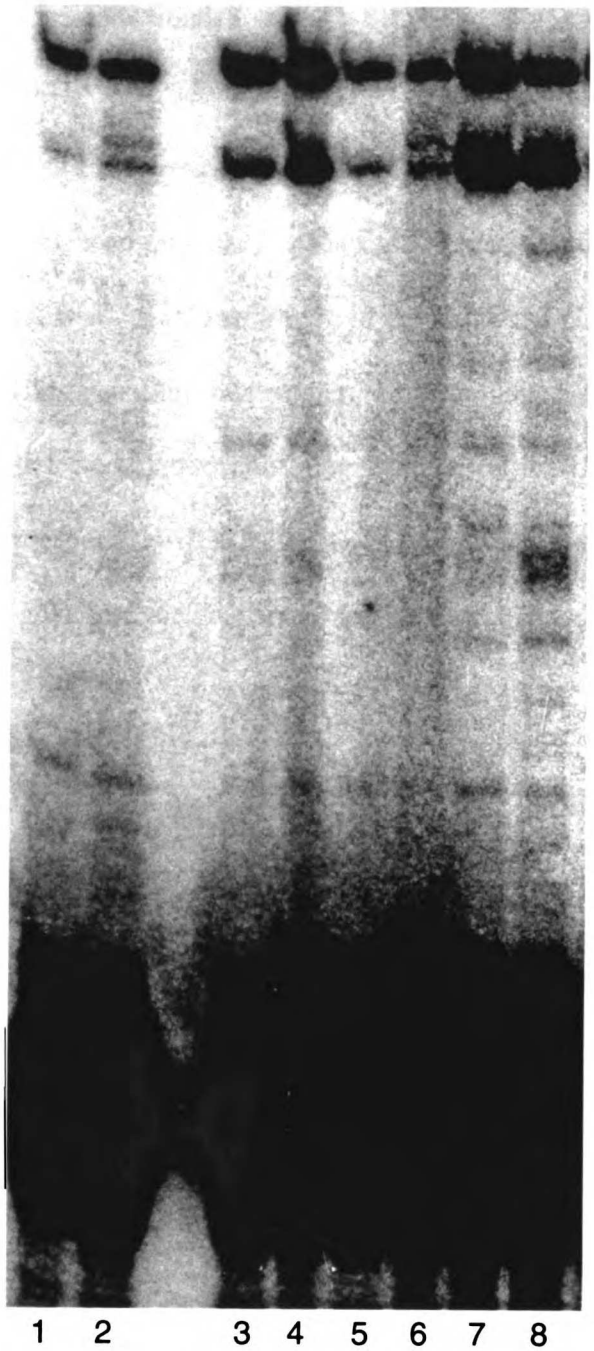
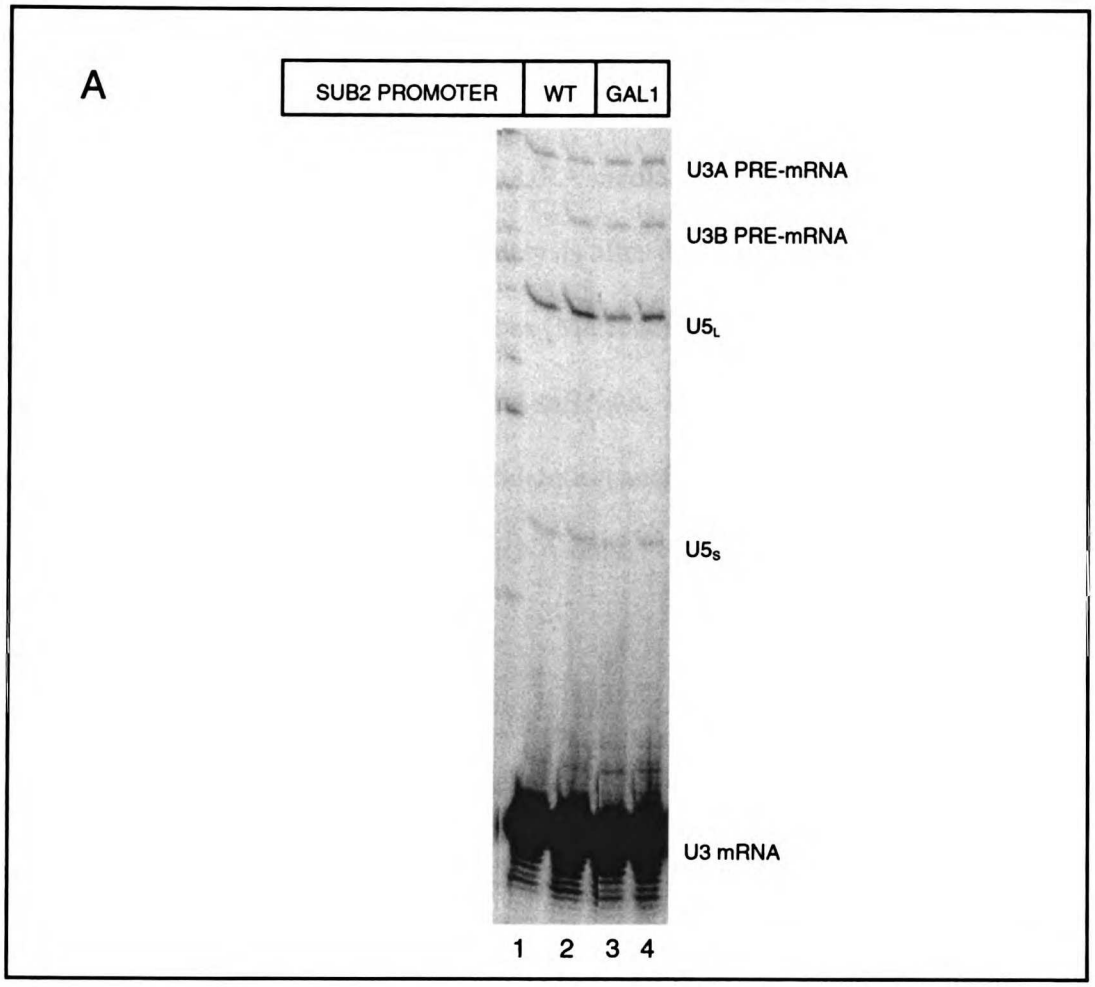


Figure 3: Impact of *GAL-SUB2* on splicing in a wildtype strain.

A. Primer extension analysis of the impact of *GAL-SUB2* on the U3 pre-mRNA splicing in a wildtype strain. RNA extracted from strains yAK313 (WT *SUB2* promoter, lanes 1 and 2) and yAK304 (*GAL SUB2* promoter, lanes 3 and 4) growing exponentially in YEP-galactose media was primer extended with a labeled oligonucleotide complementary to exon 2 of the U3 snoRNA. An additional labeled oligonucleotide complementary to the U5 snRNA was used as an internal loading control for RNA. U3 pre-mRNA and mRNA primer extension products are indicated to the right of the gel, and U5L and U5s snRNA products are also indicated. B. Quantitation of the impact of *GAL-SUB2* on U3 pre-mRNA levels in a wildtype strain. Average relative amount of U3 pre-mRNA (U3 pre-mRNA/U5L RNA) detected by phosphorimage analysis of the U3 pre-mRNA levels normalized to U5L present in yAK313 (WT *SUB2* promoter) and yAK304 (*GAL1-SUB2* promoter) grown in galactose media. Error bars indicate standard deviation, n=2.


A
SUB2 PROMOTER
WT
GAL1
U3A PRE-mRNA
U3B PRE-mRNA
U5L
U5S
U3 mRNA
1 2 3 4

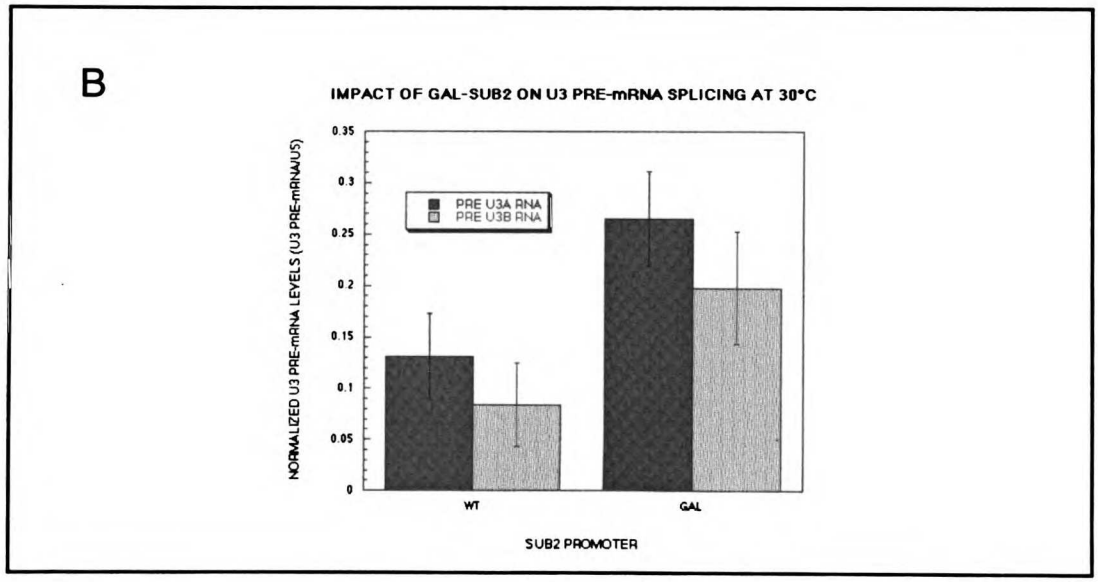

B
IMPACT OF GAL-SUB2 ON U3 PRE-mRNA SPLICING AT 30°C
NORMALIZED U3 PRE-mRNA LEVELS (U3 PRE-mRNA/U5)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PRE U3A RNA
PRE U3B RNA
WT
GAL
SUB2 PROMOTER

Figure 4. Impact of *GAL-SUB2* on snRNA levels in a *brr1-1* strain. RNA was extracted from *BRR1*/pRS316, and *brr1-1* containing either pRS316, *pGAL-BRR1*, or *pGAL-SUB2* after shifting to 16°C for 0h (lanes 1, 4, 7, and 10), 6h (lanes 2, 5,8, and 11), or 12h (lanes 3, 6, 9, 12) after growth at 30°C in SGAL-URA media. Equal amounts of total cellular RNA (5ug) were analyzed by Northern analysis after denaturing gel electrophoresis with the indicated labeled oligonucleotide probes (Materials and Methods). A. Northern hybridization analysis of all 5 spliceosomal snRNAs. B. Northern hybridization analysis of the U2 3'extension. C. Northern hybridization analysis of U4 snRNA only. D. Northern hybridization analysis of U5 snRNA only.

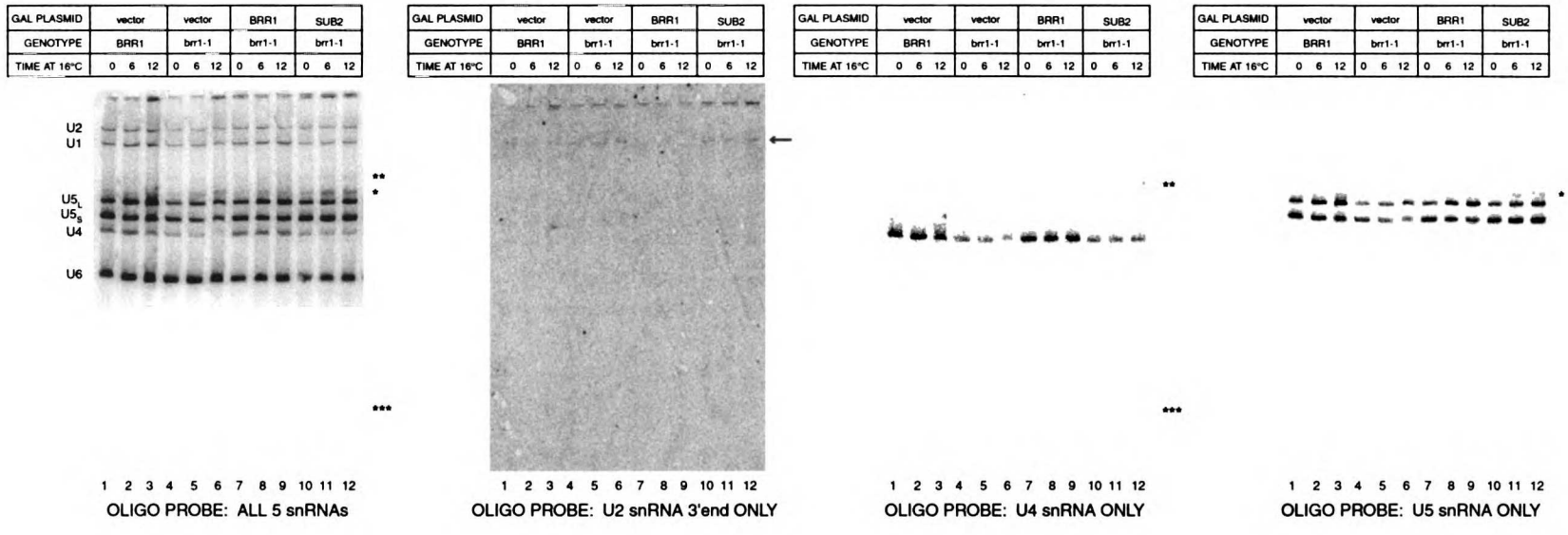
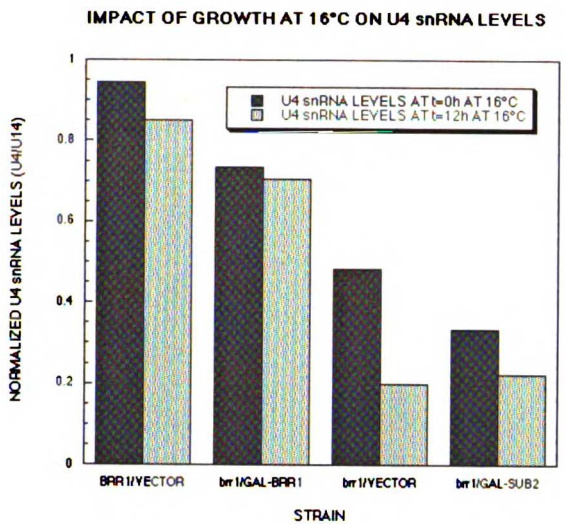

GAL PLASMID vector vector BRR1 SUB2
GENOTYPE BRR1 brr1-1 brr1-1 brr1-1
TIME AT 16°C 0 6 12 0 6 12 0 6 12 0 6 12
U2
U1
U5L
U5S
U4
U6
**
*
***
1 2 3 4 5 6 7 8 9 10 11 12
OLIGO PROBE: ALL 5 snRNAs
OLIGO PROBE: U2 snRNA 3'end ONLY
OLIGO PROBE: U4 snRNA ONLY
OLIGO PROBE: U5 snRNA ONLY

Figure 5. Quantitation of the impact of *GAL-SUB2* on U4 and U5. The amount of U4 or U5 snRNA relative to U14 snRNA detectable at t=0h and t=12h in theRNA samples shown in Figure 4 was quantitated by phosphorimage analysis of U4 and U5 primer extension reactions on these RNA samples. U14 was included as an internal control in these reactions. A. Quantitation of U4 snRNA levels. B. Quantitation of U5 snRNA levels.

A


IMPACT OF GROWTH AT 16°C ON U4 snRNA LEVELS
U4 snRNA LEVELS AT t=0h AT 16°C
U4 snRNA LEVELS AT t=12h AT 16°C
NORMALIZED U4 snRNA LEVELS (U4/U14)
0
0.2
0.4
0.6
0.8
1
BRR1/VECTOR
brr1/GAL-BRR1
brr1/VECTOR
brr1/GAL-SUB2
STRAIN

B

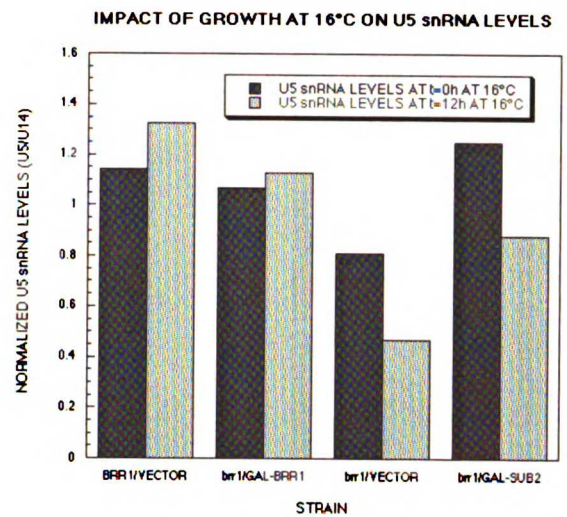
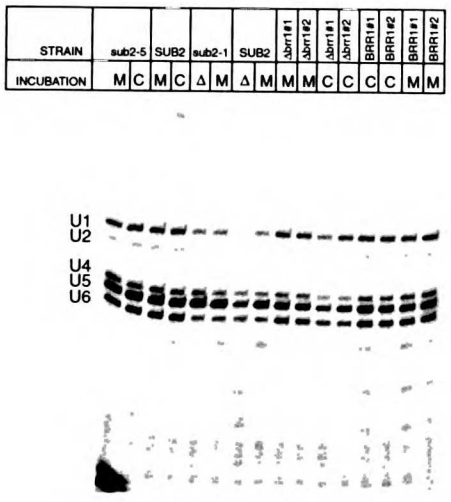
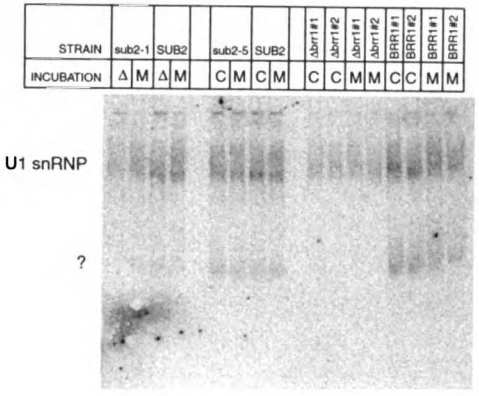

IMPACT OF GROWTH AT 16°C ON U5 snRNA LEVELS
U5 snRNA LEVELS AT t=0h AT 16°C
U5 snRNA LEVELS AT t=12h AT 16°C
NORMALIZED U5 snRNA LEVELS (U5/U14)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
BRR1/VECTOR
brr1/GAL-BRR1
brr1/VECTOR
brr1/GAL-SUB2
STRAIN

Figure 6. Impact *of sub2-1*, *sub2-5*, and *Δbrr1* on snRNAs and snRNPs. Splicing extracts derived from *sub2-1*, *sub2-5*, and *Δbrr1* strains and their isogenic wildtype parents grown at permissive temperature were examined by primer extension and Northern hybridization analyses after various preincubation treatments under splicing conditions (see materials and methods). For *sub2-1* and *SUB2* extracts: Δ, preincubation at 37°C for 10 minutes or M, mock heat incubation (treatment on ice for 10 minutes) followed by splicing at room temperature for 15 minutes. For *sub2-5*, *SUB2*, *Δbrr1*, and *BRR1* extracts; C, incubation under splicing conditions at 16°C for 30 minutes or M, incubation under splicing conditions at 23°C for 30 minutes. A. snRNA levels in *sub2-1*, *sub2-5*, and *Δbrr1* extracts. Primer extension analysis of the impact of *sub2-1*, *sub2-5*, and *Δbrr1* on the levels of all 5 of the spliceosomal snRNAs in splicing extracts derived from cells grown at the permissive temperature. B. U1 snRNP levels in *sub2-1*, *sub2-5*, and *Δbrr1* extracts. Northern hybridization of native gel analysis of the impact of *sub2-1*, *sub2-5*, and *Δbrr1* on the level and distribution of the U1 snRNP in splicing extracts derived from cells grown at the permissive temperature. C. U2 snRNP levels in *sub2-1*, *sub2-5*, and *Δbrr1* extracts. Northern hybridization of native gel analysis of the impact of *sub2-1*, *sub2-5*, and *Δbrr1* on the level and distribution of the U2 snRNP in splicing extracts derived from cells grown at the permissive temperature. D. U4 snRNP levels in *sub2-1*, *sub2-5*, and *Δbrr1* extracts. Northern hybridization of native gel analysis of the impact of *sub2-1*, *sub2-5*, and *Δbrr1* on the level and distribution of the U4 snRNP in splicing extracts derived from cells grown at the permissive temperature. E. U5 snRNP levels in *sub2-1*, *sub2-5*, and *Δbrr1* extracts. Northern hybridization of native gel analysis of the impact of *sub2-1, sub2-5*, and *Δbrr1* on the level and distribution of the

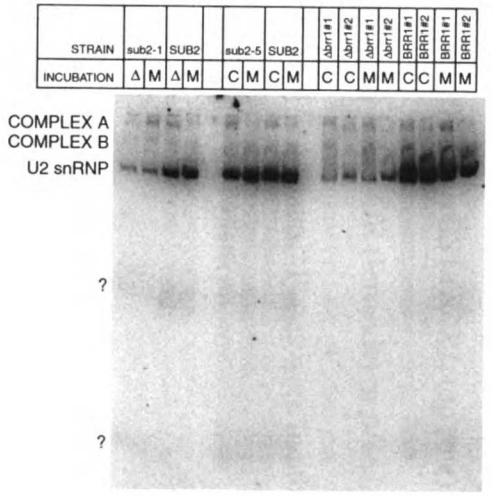
U5 snRNP in splicing extracts derived from cells grown at the permissive temperature. F. U6 snRNP levels in *sub2-1*, *sub2-5*, and *Δbrr1* extracts. Northern hybridization of native gel analysis of the impact of *sub2-1*, *sub2-5*, and *Δbrr1* on the level and distribution of the U6 snRNP in splicing extracts derived from cells grown at the permissive temperature.



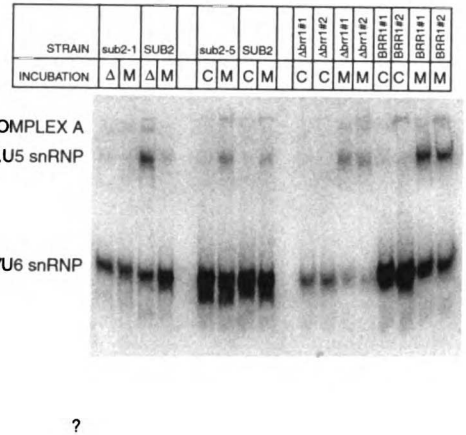
snRNA PRIMER EXTENSION



U1 snRNA PROBE



U2 snRNA PROBE



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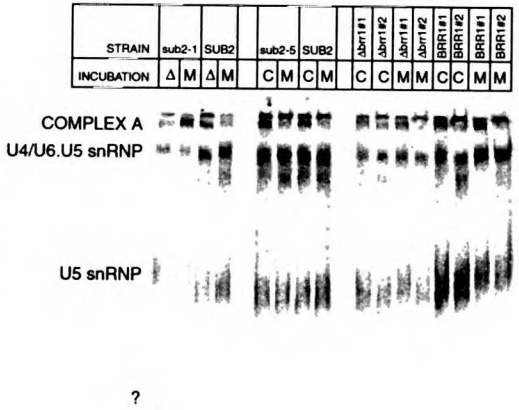
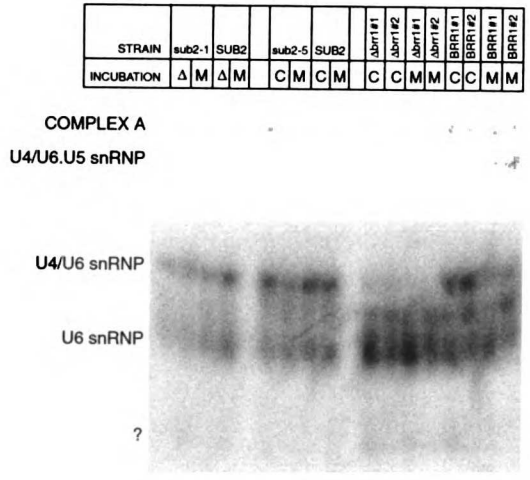
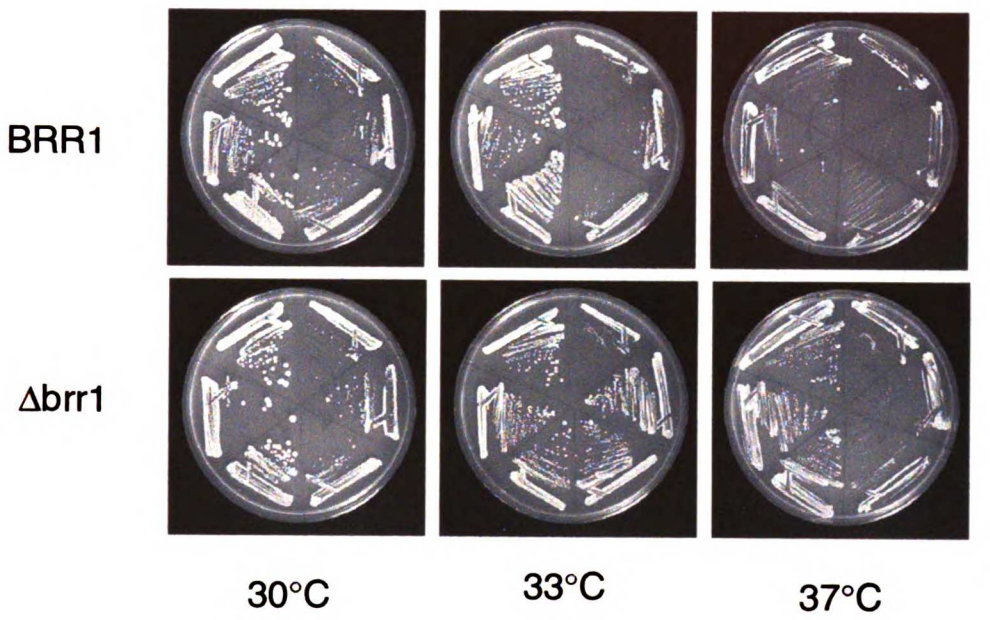


U5 snRNA PROBE



U6 snRNA PROBE

Figure 7: Impact of *Δbrr1* on the temperature sensitive growth defect conferred by *GAL-SUB2*. Strains yGS1 (*BRR1*, top row) or ySN226 (*Δbrr1*, bottom row) transformed with pRS316 VECTOR (left side) or *pGAL-SUB2* (right side), and streaked side-by-side on SGAL-URA plates. Plates were incubated at 30°C, 33°C, and 37°C, as indicated below each column of plate photos.


BRR1
Δbrr1
30°C
33°C
37°C

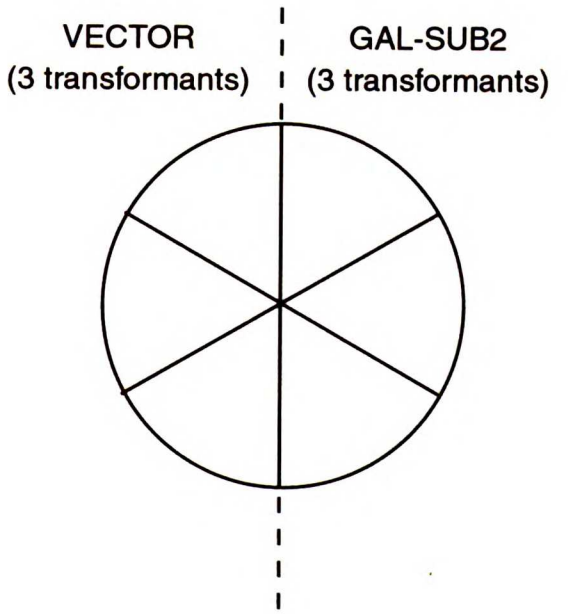
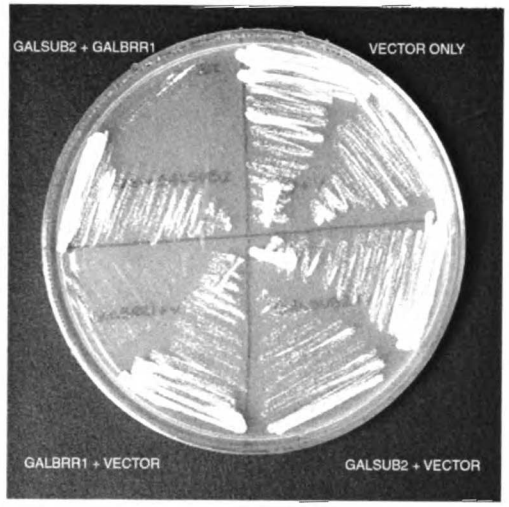

VECTOR
(3 transformants)
GAL-SUB2
(3 transformants)

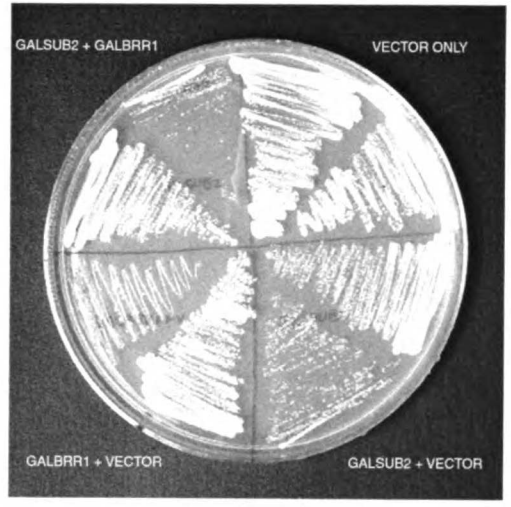
Figure 8. Impact of *GAL-BRR1* on the temperature sensitive growth defect conferred by *GAL-SUB2*. Strain yGS1 was transformed with four plasmid combinations: pRS315+pRS316 (VECTOR ONLY, upper right quadrant), *pGAL-SUB2*+pRS315 (*GAL-SUB2*+VECTOR, lower right quadrant), *pGAL-BRR1*+pRS315 (*GAL-BRR1*+VECTOR, lower left quadrant), and *pGAL-SUB2+pGAL-BRR1* (*GAL-SUB2+GAL-BRR1*) and streaked onto SGAL-URA-LEU plates. These plates were incubated at the temperatures above (for 16°C, 20°C, and 25°C) and below (for 30°C, 33°C, and 37°C) each plate.

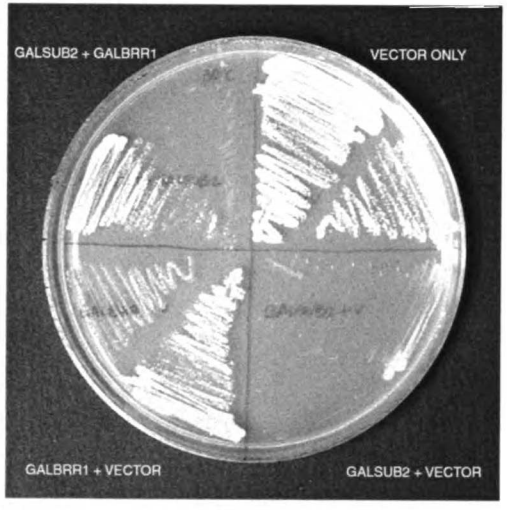
16°C

20°C

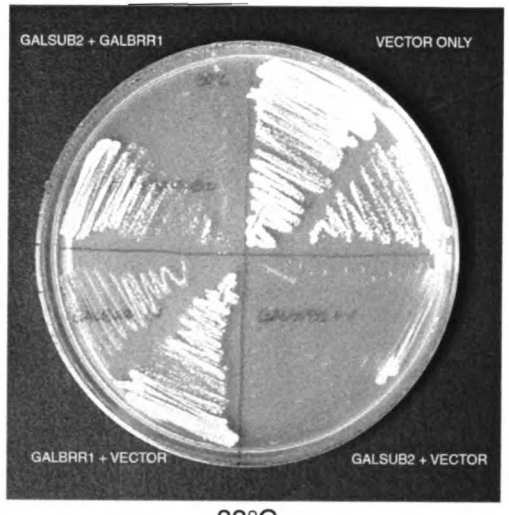


25°C

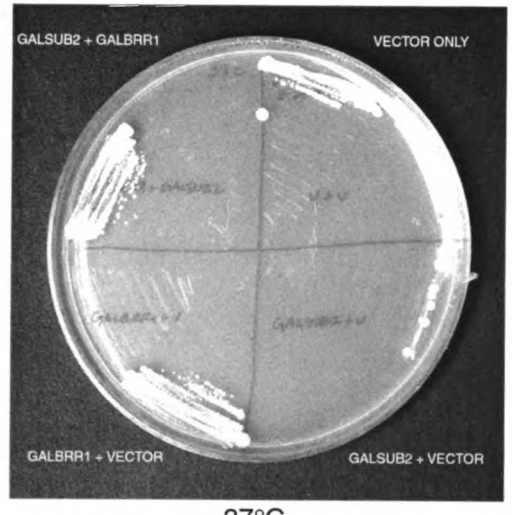




30°C



33°C



37°C

Table 1. Growth of *sub2-brr1* double mutants

Genotype	16°C	20°C	23°C	25°C	30°C	37°C
SUB2 BRR1	+++	+++	+++	+++	+++	+++
SUB2 Δbrr1	+	++	++	+++	+++	+++
sub2-1 Δbrr1	+/-	++	+/-	+++	++	+
sub2-1 BRR1	+/-	++	+	+++	++	+/-
sub2-5 Δbrr1	-	+/-	+/-	+++	+++	+++
sub2-5 BRR1	-	+	+	+++	+++	+++

Table 2. Growth of *sub2* mutants with *GAL-BRR1*

Genotype	16°C	25°C	37°C
SUB2 BRR1	+++	+++	+++
SUB2 GAL-BRR1	+++	+++	+++
sub2-1 GAL-BRR1	++	+	-
sub2-1 BRR1	++	++	+/-
sub2-5 GAL-BRR1	+	++	+++
sub2-5 BRR1	++	+++	+++

Genetic and biochemical studies of Sub2 function in have provided insight into the role that this factor plays in the early recognition events that occur at the BPS of a pre-mRNA substrate before splicing catalysis in yeast. In the first part of this chapter I will review our current picture of BPS recognition during pre-mRNA splicing in light of the work presented in chapters 1 and 2, and recent advances that have occurred since I began this work. I will next point out emerging patterns of spliceosomal ATPase activity that have been reinforced by these and other recent studies of spliceosomal ATPases. I will highlight how recent studies of DEAD-box ATPases have advanced our understanding of the possible functions and potential substrates of spliceosomal DEAD-box RNA factors. Finally, I will discuss the exciting possibilities that further analysis of Sub2 function is likely to hold.

Branchpoint recognition events before splicing catalysis

I. Possible function for Sub2 in formation of a productive PS complex

Accurate recognition and activation of the branchpoint sequence appears to be of critical importance to pre-mRNA splicing. In the first step of splicing, the branchpoint adenosine attacks the 5'ss, resulting in the formation of free upstream exon and a lariat intermediate. Work in mammalian extracts suggests that the identity of the attacking residue in the first step of splicing is important for the U2-branchpoint sequence interaction (Reed and Maniatis 1988) (Wu and Manley 1989; Zhuang and Weiner 1989), which is required to activate the branchpoint residue for attack in the first step of splicing (Wu and Manley 1989; Zhuang and Weiner 1989). The efficiency of both the first and the second step of splicing were observed to decrease in the presence of nucleotides other

than A at the branchpoint position (Query et al. 1995; Query et al. 1996). Detection of an early interaction with a protein with an apparent molecular mass of 14kDa was also highly sensitive to changes in the identity of the branchpoint residue (Query et al. 1996). Moreover, recent studies of BBP and U2AF65, factors which interact at the branchpoint sequence in an ATP-independent manner, also suggest that the branchpoint adenosine and the neighboring nucleotides present at the BPS exert a strong impact on ATP-independent protein interactions at this splice consensus signal (Berglund et al. 1997). Thus it appears that even in mammals, where the consensus signal for the BPS is highly degenerate, the identity of the branchpoint residue is still of great importance for numerous stages of the pre-mRNA splicing reaction.

Recent genomic analysis of the yeast genome confirms previous observations that the BPS is highly conserved in this organism (Spingola et al. 1999). Of the 228 identified intron-containing genes in yeast, 209 contain a full match to the BPS consensus sequence (UACUAAC). Of the genes containing BPS divergence from consensus, 18 contain single point mutations, and only 1 contains two point mutations in the BPS consensus. As in mammals, work in yeast has also demonstrated that mutation of the branchpoint adenosine to a different nucleotide strongly depresses splicing activity (Parker et al. 1987; Rymond and Rosbash 1992). Deleterious effects in splicing upon mutation of other nucleotides of branchpoint consensus sequence have also been observed (Parker et al. 1987). Compensatory mutation of the U2 snRNA branchpoint sequence interacting region does not completely relieve the effects conferred by mutations in the BPS (Parker et al. 1987). It is likely that other factors, which either bind the BPS directly, such as BBP (Berglund et al. 1997), or inspect the branchpoint sequence/conformation later in

splicing, such as Prp16 (Couto et al. 1987; Burgess and Guthrie 1993a), contribute to these residual defects detected even in the presence of the U2 compensatory mutations. In mammals the branchpoint sequence is much less conserved (c/uUa/gAC) (Reed and Maniatis 1988). Instead, there is a stronger requirement for a polypyrimidine tract (Py tract) of nucleotides between the branchsite and the 3'ss (Ruskin et al. 1988; Zamore et al. 1992). Intron-containing genes in yeast do contain Py tract elements, and these elements can influence the splicing of transcripts in yeast (Patterson and Guthrie 1991; Umen and Guthrie 1995); however such elements are not as highly conserved as in mammals. Recent analysis of the yeast and mammalian factors that bind these elements suggests that the difference in degree of conservation of each element reflects the relative contribution of each factor to the total binding affinity of the ATP-independent complex which initially forms on the branchpoint region of the transcript (Berglund et al. 1997; Berglund et al. 1998a). In mammals, U2AF65 binds to the Py tract and stabilizes the binding of mBBP at the degenerate BPS (Berglund et al. 1998a). In yeast, the sequence-specific binding of BBP at the BPS contributes to the binding of Mud2 at the less conserved Py tract region(Berglund et al. 1997). In both cases, the binding of each factor has been proposed to contribute to the stability of the complex of both factors bound at the BPS in the absence of ATP (Berglund et al. 1998a).

The ATP-independent binding of BBP to the BPS is likely to be mutually exclusive with the basepairing interaction of the U2 snRNA with the BPS. BBP binds the BPS in a sequence specific fashion, which is resistant to competition by a complementary 2'OMe RNA oligonucleotide (Berglund et al. 1998a). In yeast, BBP and its binding partner Mud2 have only been detected in the early ATP-independent CC2 complex (Rutz

and Seraphin 1999). Once U2 snRNP is stably bound to the pre-mRNA substrate, these factors are no longer detectable in splicing complexes containing transcript (Rutz and Seraphin 1999). Although both BBP and the U2 snRNP have recently been detected in the ATP-independent early (E) spliceosome complex in mammalian extracts, the presence of the U2 snRNP in this complex does not appear to require an intact BPS (Das et al. 2000). Thus, it is likely that even though these factors may bind simultaneously to the transcript, they are still in competition for access to binding to the BPS.

The work presented in chapters 1 and 2 of this thesis, combined with independent studies performed in both yeast and mammalian extracts (Fleckner et al. 1997; Libri et al. 2001; Zhang and Green 2001), suggest that Sub2 plays an important role in facilitating the exchange of BBP and Mud2 at the BPS, for the the subsequent U2 snRNA basepairing interaction at the BPS. A model based on this work thesis that reflects this possible function for Sub2 is presented in Figure 1. Genetic interactions between *sub2* mutants and mutant versions of both BBP and MUD2 suggest a function for Sub2 in collaboration with both of these factors in an early, ATP-independent step of spliceosome assembly (CC2). Biochemical studies of the spliceosome activity and assembly phenotype of genetically depleted extracts, and extracts derived from the ts- allele, *sub2-100* support this hypothesis (Zhang and Green 2001). Both of these extracts are defective for splicing in vitro, and exhibit defects in formation of the CC2 complex.

Our observation that loss of function alleles of both MUD2 and BBP could suppress the ts- growth defect of the *sub2-1* allele suggests that Sub2 may promote ATP-dependent removal of these factors during splicing. The fact that the same alleles of MUD2 and BBP can also bypass the requirement for Sub2 function in vivo further

supports the hypothesis that the major function of Sub2 is to promote the removal of Mud2 and BBP from the BPS during splicing. Based on biochemical analysis of the spliceosome assembly defect of *sub2-1*, it appears that failure to carry out such a function leads to a defect in splicing in vitro and the formation of a PS-like complex which accumulates, rather than efficiently converting to a spliceosome. These results were somewhat surprising; we had expected PS formation to be blocked. It remains possible that this defect reflects a function for Sub2 later in splicing, perhaps at the stage of tri-snRNP addition. This possibility has not been ruled out in the experiments I've carried out during my thesis work, and merits further investigation.

However, detection of genetic interactions between the *sub2* alleles and mutant alleles of factors required for PS formation support the possibility that Sub2 is indeed having some impact on PS formation (Ruby et al. 1993). I have observed that *sub2* mutants exacerbate the growth defects of mutant alleles of the U2 snRNP components *PRP9*, *PRP11*, and *PRP21*, all of which are defective for PS formation. Additionally, I have also detected an exacerbation of growth of *prp5* alleles defective for PS formation. Surprisingly, I observed a distinct bypass suppression of the *SUB2* null allele by deletion of the non-essential splicing factor *CUS2*. This bypass suppression occurs only in the cold, where the bypass suppression exerted by the MUD2 null allele or the *msl5-5* allele of BBP is no longer detected. This result was striking in terms of its connection to PS formation and Sub2 function. In a wildtype cell, the presence of Cus2 appears to impose the ATP requirement for PS formation; deletion of *CUS2* allows for formation of a functional PS complex in the absence of ATP (Perriman and Ares 2000). In summary, combining mutant alleles of *SUB2* and mutants which lead to defects in PS formation is

synthetically lethal; in contrast, complete loss of function of *SUB2* can be conditionally suppressed by other mutants which promote PS formation. I have interpreted these data as supportive of a function for Sub2 in the formation of a functional PS complex.

I would also like to point out that the genetic interactions between the *sub2* alleles and the PS splicing factors raise the possibility of a balance of affinities between the mutually exclusive ATP-independent and ATP-dependent binding interactions at the BPS. It seems plausible that during PS formation, Sub2 and Prp5 utilize ATP to modulate RNA-protein interactions at the BPS, and the structure of the U2 snRNP, respectively, to influence this balance of binding interactions at the BPS. Such activities result in efficient formation or progression of PS complexes to mature spliceosome complexes during splicing.

Additional results from in vitro studies of splicing in the absence of Sub2 are consistent with this hypothesis. In the absence of Mud2, or when BBP binding to the BPS is likely reduced (as may also be the case in the *msl5-5* cs- mutant), Sub2 is dispensable. Thus, we can envision two alternative splicing pathways, a *Δsub2-bbp-* pathway (Figure 2) and a Sub2-Mud2 independent splicing pathway (Figure 3A) through which splicing may be occurring in these double mutant strains. Although Sub2 appears to be dispensable in both of these strains, its absence does impart a slow growth phenotype at the permissive temperature, and lethality at both high and low temperatures.

If Sub2 and Prp5 utilize ATP to influence a balance of competing binding interactions at the BPS, then it is likely that the growth defects in these strains may reflect the disruption of that balance in the absence of Sub2. Biochemical analysis of extracts derived from the *Δsub2 Δmud2* strain allowed the predictions of this hypothesis to be

directly tested. Intermediate/permissive temperatures would allow for sufficient balance of interactions with the BPS to support splicing and thus viability, in the absence of Sub2 (Figure 3A). This also appears to be the case. In *the Δsub2 Δmud2* extracts, splicing and spliceosome assembly both occur in a manner similar to isogenic wildtype extracts, but are kinetically slowed in the absence of Sub2.

Low temperatures may stabilize the BBP-BPS interaction normally disrupted by Sub2, and thus tip the balance of interactions at the BPS towards the ATP-independent interactions (Figure 3B). Indeed we observed very little splicing activity in these extracts at 16°C, and a defect in spliceosome assembly before PS formation. In contrast, high temperatures would likely further destabilize BBP-BPS, perhaps resulting in a BPS that is more accessible for U2 snRNP association. This would in turn shift the balance of the interactions towards the premature addition of U2 to the BPS (Figure 3C). Our analysis of splicing at 33°C supports this prediction: PS complexes form efficiently, but accumulate dramatically relative to PS complexes observed in isogenic wildtype extracts. Despite this assembly defect, the splicing activity of this extract is not significantly defective relative to that of extract derived from an isogenic wildtype strain. This could reflect a requirement for a higher temperature to fully inactivate splicing. Alternatively, the lack of detectable defect in splicing activity in the *Δsub2 Δmud2* extract at 33°C may be a manifestation of decreased accuracy of BPS recognition by the U2 snRNP. A decrease in the fidelity of BPS recognition combined with the formation of less productive PS complexes may explain the ts- phenotype of this strain, in the absence of an obvious splicing activity defect.

The hypothesized influence of Sub2 on the balance of interactions at the BPS raises further predictions for future biochemical experiments with *the Δsub2 Δcus2* strain. It is likely that the *Δsub2 Δcus2* strain is viable only at lower temperatures because the loss of *CUS2* leads to a conformation of the U2 snRNP that is somehow more competent to add to the BPS (Perriman and Ares, 1999, Figure 3A). This may result in the formation of a productive PS in the absence of Sub2 only at lower temperatures where BBP and Mud2 binding might be most stable. It may be that in the *Δsub2 Δcus2* strain at low temperatures, the balance of the BBP and U2 snRNP BPS interactions is similar to that at the higher "permissive" temperatures for the *Δsub2 Δmud2* and *Δsub2 msl5-5* strains. At temperatures greater than 20°C, the BBP and Mud2 interactions may be sufficiently destabilized in the absence of both *SUB2* and *CUS2* to tilt the balance of interactions towards premature addition of U2 to the BPS (Figure 3B).

Branchpoint recognition events before splicing catalysis

I. Links between Sub2 function and nuclear retention of pre-mRNA and splicing fidelity?

The strong genetic interactions reported in chapter 2 between alleles of BBP defective for pre-mRNA retention in the nucleus (Rutz and Seraphin 2000), and *the sub2-5* cs- allele provide further food for thought with regard to potential mechanisms of fidelity regulation at the very early, ATP-independent stages of BPS recognition during splicing. As suggested in chapter 2, it may be that the early, ATP-independent interaction between Sub2 with BBP and Mud2 may imply a role in targeting the pre-mRNA transcript to the splicing pathway for further processing, rather than export from the nucleus. In this model (Figure 5), factors which associate with nascent pre-mRNA

transcripts in an early ATP-independent manner are likely to be the major determinants of early targeting events distinguishing transcripts to be retained in the nucleus for splicing from transcripts ready for export to the cytoplasm.

Previous studies examining the factors required for the nuclear retention of pre-mRNA has provided evidence for this possible mechanism for targeting transcripts to the splicing pathway. Mutations in the 5'ss or branchpoint sequence lead to dramatic increases in pre-mRNA leakage from the nucleus (Legrain and Rosbash 1989). The amount of detectable pre-mRNA leakage can be seen to further increase in the absence of the non-essential UPF1 gene (Long et al. 1995), which is required for cytoplasmic degradation of transcripts containing premature stop codons (He et al. 1993). Mutations in U1 which decrease its ability to basepair with the 5'ss also lead to an increase in pre-mRNA transcripts in the cytoplasm (Legrain and Rosbash 1989). Three splicing mutants, *prp2-1*, *prp9-1*, and *prp6-1* were found to exhibit increased levels of pre-mRNA leakage at the non-permissive temperature (Legrain and Rosbash 1989). While it is unclear exactly why mutant alleles of these factors seemed most defective for pre-mRNA retention, one pattern did emerge: all of these splicing factors act at an early stage of splicing, at or before the first chemical step of splicing. Not surprisingly, further independent studies identified a similar defect for pre-mRNA retention in strains deleted for MUD2 (Rain and Legrain 1997).

We envision a mechanism of pre-mRNA retention in which a nascent transcript is initially maintained in the nucleus due to the formation of the CC2 complex (Figure 5). Thus, the presence of BBP, Mud2, and U1 snRNP on a transcript may be an initial signal for retention. Our genetic data suggests that Sub2 may interact with such a complex.

Thus it is possible that Sub2 serves as an early "receptor" for the pre-mRNA splicing pathway. It may be that Sub2 binding to a pre-mRNA bound by BBP and Mud2 both stabilizes and targets the CC2 complex for further interactions with other splicing factors (Figure 5). Given the genetic interactions detected between Sub2 and both the ATP-independent splicing factors and PS *prps*, it is possible that Sub2 effectively serves as a splicing pathway "receptor" by virtue of its ability to interact with downstream, ATP-dependent components of the splicing pathway.

Once Sub2 has associated with the CC2 complex, it may be that the stability of the Sub2-BBP-Mud2-BPS complex also serves as a possible regulation point in monitoring the fidelity of early BPS recognition events before further commitment to the splicing pathway. If this complex falls apart before further interactions with downstream components of the splicing pathway, the transcript may be degraded in the nucleus, or exported to the cytoplasm and degraded by the nonsense mediated decay pathway. In either scenario, this model posits an additional factor that is monitoring the association of Sub2 with BBP and Mud2 at the BPS. This factor could actually be the U2 snRNP, or a component associated with the U2 snRNP, such as Cus2 or Prp5. In such a scenario, the U2 snRNP or a conformational change in the U2 snRNP could further serve as a trigger for Sub2 to promote the removal of BBP and Mud2 from the BPS in a timely manner. A defect in such a coordinated mechanism of PS formation might also explain the accumulation of the PS-like complex that is detected in the *sub2-1* mutant extract and the *Δsub2 Δmud2* extract.

Expanded notions of DEAD-box ATPase function and potential substrates

At the beginning of my graduate studies, numerous advances in the understanding of a series of RNA interactions and rearrangements required for the formation of a functional, catalytically active spliceosome had been defined (Madhani and Guthrie 1994). Clearly, a major goal for the field since then has been to understand how these RNA rearrangements are regulated. The spliceosomal DEAD-box ATPases have long been suspected to be the catalysts for the rearrangements required during spliceosome assembly. Indeed, genetic, structural, and functional studies have tied the functions of these DEAD-box factors to each of the major rearrangements and recognition events throughout the course of splicing (Staley and Guthrie 1998). However, the mechanism by which these factors functioned to catalyze these rearrangements remains unclear. Homology among these spliceosomal DEAD box factors with a class of proteins known to function as RNA and DNA helicases (de la Cruz et al. 1999) points to the compelling possibility that the spliceosomal DEAD box factors utilize the energy derived from ATP hydrolysis to unwind the RNA-RNA duplexes required during spliceosome formation and splicing catalysis.

However, despite the fact that many of the spliceosomal DEAD-box proteins have been demonstrated to exhibit ssRNA stimulated ATPase activity (Schwer and Guthrie 1991; Kim et al. 1992; O'Day et al. 1996; Schwer and Gross 1998; Wagner et al. 1998), detection of canonical helicase activity as that observed for the prototypical RNA helicase, eIF4a, has only recently been described for a subset of the spliceosomal DEAD-box factors. Independent analyses of Prp22 have demonstrated its ability to unwind a synthetic RNA-RNA duplex (Schwer and Gross 1998; Wagner et al. 1998). Similar

studies with synthetic RNA duplex substrates also demonstrated a helicase activity for Prp16 (Wang et al. 1998). Studies with the purified, mammalian version of the tri-snRNP associated DEAD box factor, Brr2/Snu246 have also demonstrated unwinding activity with a synthetic RNA duplex as well (Laggerbauer et al. 1998). Independent work in yeast also showed that Brr2/Snu46 was responsible for the release of free U4 and free U6 snRNA from a tri-snRNP particle in the presence of ATP (Raghunathan and Guthrie 1998a). Thus, although the nature of the physiological substrates that these factors might act on had not been fully elucidated, the detection of RNA unwinding activity in the spliceosomal DEAD-box protein family members was an important "proof-of-principle" result.

Our understanding of the relevance of these detected in vitro unwinding activities remains incomplete in the absence of identification of the physiological substrates of these spliceosomal ATPases. A number of candidate RNA duplexes exist in the spliceosome (Staley and Guthrie 1998). However, with the exception of the U4/U6 RNA duplex, the bulk of the RNA duplexes to be exchanged or disrupted during spliceosome assembly are short in length. This has raised further questions as to whether the spliceosomal ATPases actually function as RNA helicases during pre-mRNA splicing. Thus, to avoid misattribution of the function of these spliceosomal ATPases, their recently discovered in vitro capabilities have been termed RNA unwinding activity, rather than RNA helicase activity (Lorsch and Herschlag 1998; Staley and Guthrie 1998).

Indeed, it has been suggested that the in vitro RNA unwindase activity exhibited by RNA associated DEAD box factors may actually reflect an unwindase capability that is a consequence, rather than a reflection of the actual biological function of these DEAD

box proteins (Lorsch and Herschlag 1998; Staley and Guthrie 1998). Recent work on the gp41 and Dda DNA helicases (Morris and Raney 1999) have demonstrated that force production by these DNA helicases translocating on a ssDNA substrate increased the dissociation rate of streptavidin bound on the end of a biotinylated oligo as much as 500 fold over the rate of spontaneous dissocation. These observations raise the possibility that members of the superfamily of helicases may utilize the energy of ATP hydrolysis or the force of translocation along a nucleic acid to disrupt nucleic acid-protein interactions. Consistent with this possibility, another DNA helicase homolog, Mot1, has been demonstrated to regulate transcription by displacing TBP bound at the TATA box DNA element in an ATP-dependent manner (Auble et al. 1994; Auble et al. 1997).

Recent work with the vaccinia virus NPH-II DExH-box protein provides further evidence for such activity in the RNA unwindase homologs (Jankowsky et al. 2001). NPH-II has been demonstrated to exhibit highly processive unwinding activity with duplex RNA substrates in vitro. Analysis of this unwinding activity in the presence of an RNA-protein interaction within the RNA duplex substrate was performed to assess the whether NPH-II was also capable of dislodging a protein bound to the duplex RNA substrate. NPH-II was capable of unwinding the RNA duplex regardless of whether the protein was present or not. However, the dissociation rate of the protein was increased by several orders of magnitude in the presence of NPH-II and ATP. Although this scenario is yet another non-physiological context for assessing the function of RNA unwindases, it raises the possibility that the RNA unwindase activity of the RNA specific members of the superfamily of DEAD box proteins may indicate a biological "RNPase" function for these factors (Jankowsky et al. 2001).

This possibility is especially relevant for consideration of functions for the spliceosomal DEAD-box factors. These helicase homologs function in the context of a large ribonucleoprotein complex. Thus, it seems highly likely that these factors may perform some sort of "RNPase" function during splicing. The spliceosomal DEAD-box factors may harness the energy of ATP hydrolysis towards RNPase functions that involve disruption of tertiary RNA interactions, short RNA-RNA basepairing interactions, or RNA-protein interactions that exist in the spliceosome to effect the larger scale alterations in RNA interactions that are required during spliceosome formation and catalysis. Our work on Sub2 (Kistler and Guthrie 2001) in combination with work on Prp28 (Chen et al. 2001) provide in vivo support for the RNPase mode of function for spliceosomal DEAD-box factors. In both of these studies, the requirement for these DEAD box factors is bypassed by mutation or loss of function alleles in proteins that stabilize interactions at splice site consensus sequences. Detection of bypass suppression of these essential spliceosomal ATPases suggests that the critical function of both of these spliceosomal DEAD-box factors is to destabilize RNA-protein interactions which are mutually exclusive with subsequent interactions required for spliceosome assembly to proceed.

Exciting possibilities from future analysis of Sub2

Definitive proof that spliceosomal DEAD box factors function in a manner mechanistically similar to helicases or RNPases or another, unanticipated manner, awaits identification of the specific substrates for each of these DEAD box ATPases. Thus, the results from the genetic and preliminary biochemical analysis of Sub2 and Prp28 are

particularly exciting, in that they provide specific candidate ribonucleoprotein complex substrates to examine. Our genetic analyses with Sub2 provide a clear candidate ribonucleoprotein complex formed very early in splicing (CC2) on a standard RNA substrate (pre-mRNA containing an intact BPS) to directly test for RNPase functions associated with Sub2. This substrate is particularly appealing since the number of components present on a pre-mRNA substrate at such an early stage of splicing is likely to be lower than that present in the complexes modulated by later acting spliceosomal ATPases. Thus, studies with native CC2 complexes isolated from splicing extract could provide a means to directly address if Sub2 is present in this complex, or if its presence and ATP leads to the dissociation of this complex in vitro.

Alternatively, a further reduced system, similar to that described with Mot1 and TBP could be employed for such analysis (Auble et al. 1994; Auble et al. 1997). An RNA oligo containing the BPS and a Py tract, and purified recombinant mBBP and U2AF65 have already been shown to interact stably in vitro by gel shift assays (Berglund et al. 1998a). An additional RNA oligo mimic of the U2 snRNA BPS-interacting region can compete with BBP and U2AF65 for binding to the BPS-Py tract oligo (Berglund et al. 1998a). This does not require ATP (Berglund et al. 1998a). It may be that additional stabilizing interactions from other factors present in a normal pre-mRNA substrate (eg, the U1 snRNP) are required to impose an ATP-dependence on this exchange. However, analysis of the influence of Sub2 and ATP on these interactions among purified componenets may also provide an even simpler system to assess Sub2 function. In either case, more detailed analysis of Sub2 function with a specific substrate provides the

exciting and unique opportunity to directly analyze the possible mechanisms by which a spliceosomal DEAD-box factor may function during pre-mRNA splicing in yeast.

Emerging patterns of spliceosomal ATPase activity

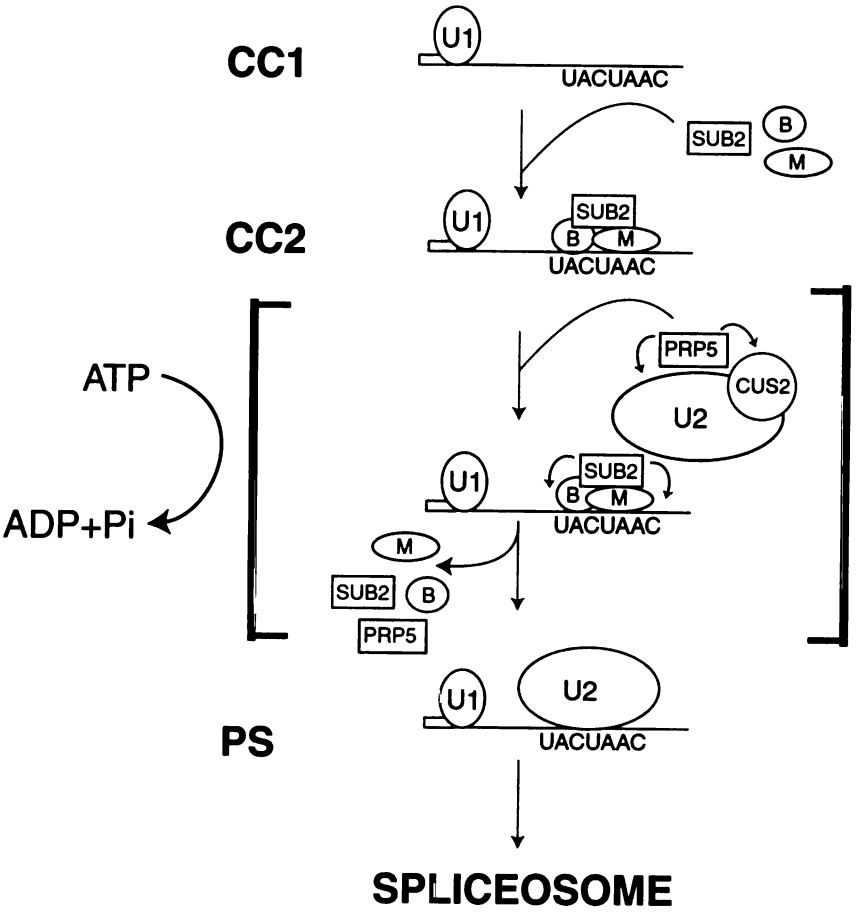
Our model for the role of Sub2 in the early stages of BPS recognition during pre-mRNA provides another example of possible emerging patterns in the activities that spliceosomal ATPases appear to share. First, as described above, Sub2 may first interact with the spliceosome, and perhaps effectively perform a function in an ATP-independent manner. In a subsequent function, likely to involve ATP hydrolysis, Sub2 may promote the removal of BBP and Mud2 at the BPS. Although direct biochemical data supporting these functions remains to be obtained, these two hypothesized functions bear similarity to functions detected in more biochemically characterized spliceosomal ATPases.

Prp2, Prp16, and Prp22 have all been observed to associate with the spliceosome in the absence of ATP (Schwer and Guthrie 1991; Kim and Lin 1993; Teigelkamp et al. 1994; Schwer and Gross 1998). In the presence of ATP, these factors each have been identified to catalyze some sort of conformational rearrangement in the spliceosome (Schwer and Guthrie 1992; Teigelkamp et al. 1994; Kim and Lin 1996; Schwer and Gross 1998; Wagner et al. 1998). The catalysis of these conformational changes in the spliceosome is usually accompanied by the dissociation of these spliceosomal ATPases from the spliceosome (Schwer and Guthrie 1992; Kim and Lin 1993; Schwer and Gross 1998; Wagner et al. 1998) (Teigelkamp et al. 1994). As biochemical studies with Sub2 progress, it will be of great interest to determine if these patterns in ATP-independent physical association with the spliceosome and execution of ATP-dependent functions will

bear out. Much is likely to be gained through biochemical add-back experiments with *Δsub2* spliceosomes formed in the cold or at high temperature, where spliceosome assembly and splicing activity is most drastically affected in the absence of Sub2. The ability of purified Sub2 to bind and alter such complexes in the presence and absence of ATP will likely provide further insight into Sub2 function.

It also seems likely, based on the recent analyses of Sub2 and other spliceosomal ATPases, that a pattern of a division of labor among the spliceosomal ATPases is emerging. The rearrangements that have now been assigned to the known spliceosomal ATPases can be assigned to 3 main classes: 1) RNA-RNA or RNA-protein interactions at the splice sites of a pre-mRNA substrate, 2) snRNA remodeling in the context of a snRNP, and 3) RNA or ribonucleoprotein remodeling events required for catalysis or large scale remodeling of the spliceosome (Staley and Guthrie 1998). Our work with Sub2 (Kistler and Guthrie 2001) and the recent work with Prp28 (Chen et al. 2001) suggest that these two factors function on a class 1 rearrangement. Studies on the function of Brr2 (Laggerbauer et al. 1996; Xu et al. 1996; Raghunathan and Guthrie 1998a; Kim and Rossi 1999) and Prp5 (O'Day et al. 1996; Wiest et al. 1996) suggest a role for these factors in class 2 rearrangements. The functions of Prp2, Prp16, Prp22, and Prp43 are consistent with a class 3 rearrangement (Schwer and Guthrie 1992; Kim and Lin 1996; Arenas and Abelson 1997; Schwer and Gross 1998; Wagner et al. 1998).

Within this division of labor, there also appears to be an emerging pattern of coupling or coordination between the steps catalyzed by different spliceosomal ATPases. This is suggested by the recent detection of multiple ATPases participating in distinct steps of spliceosome assembly events. For example, Prp28 is required to catalyze the


CC1
U1
UACUAAC
SUB2
B
M
CC2
U1
SUB2
B
M
UACUAAC
ATP
ADP+Pi
PRP5
CUS2
U2
U1
SUB2
B
M
UACUAAC
M
SUB2
B
PRP5
PS
U1
U2
UACUAAC
SPLICEOSOME

Δsub2-msl5-5 SPLICING PATHWAY AT RT

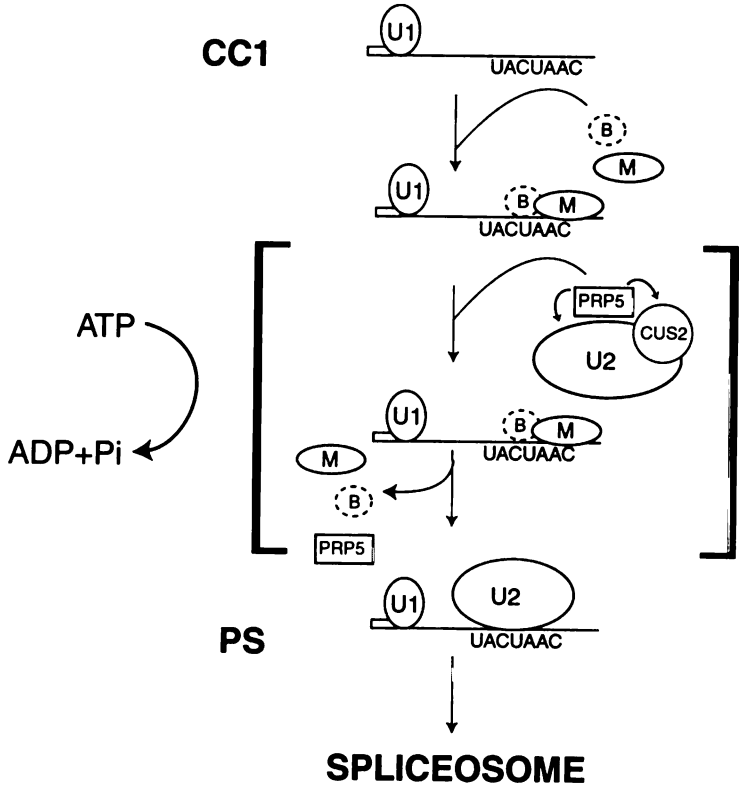


Figure 3: Model for the Sub2-Mud2 independent splicing pathway activity at permissive and non-permissive temperatures. A, room temperature; B, low temperature (16°C); C, high temperature (33°C).

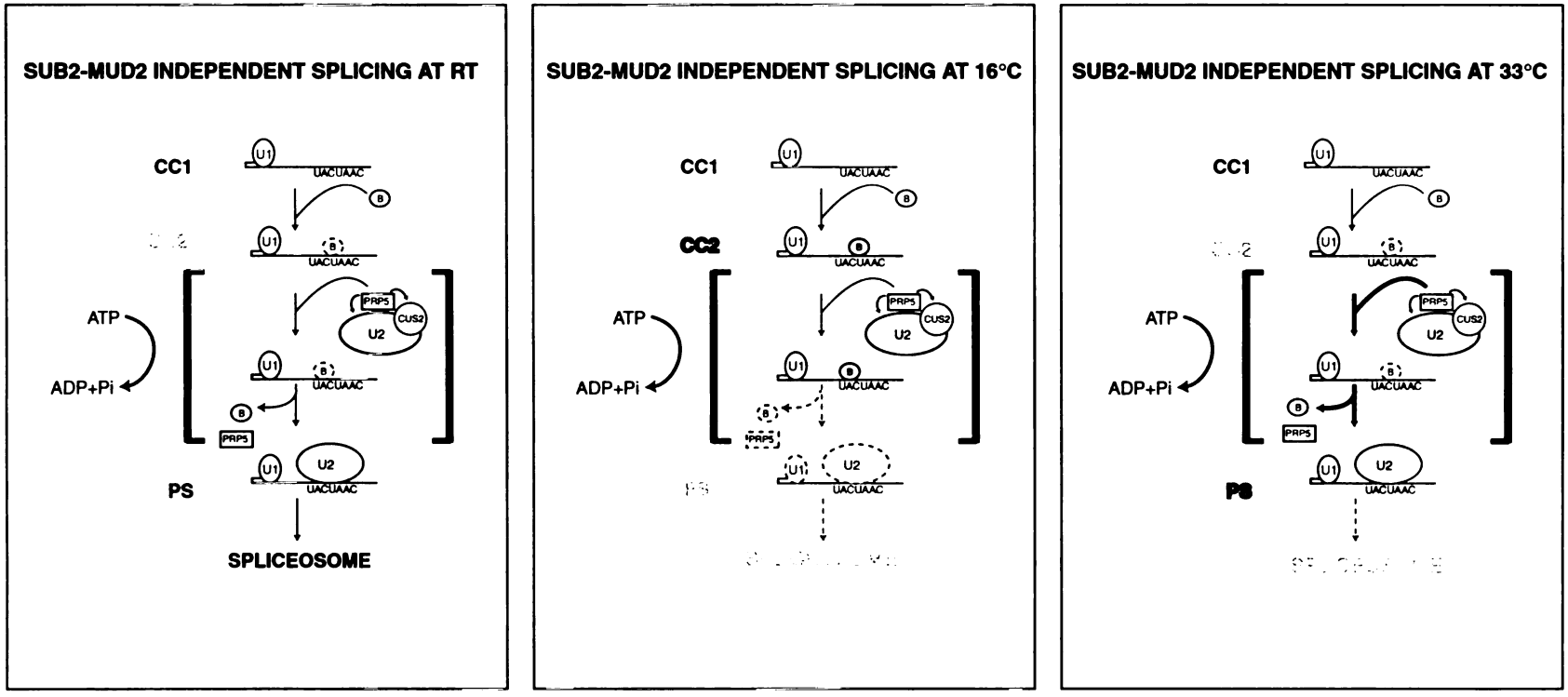
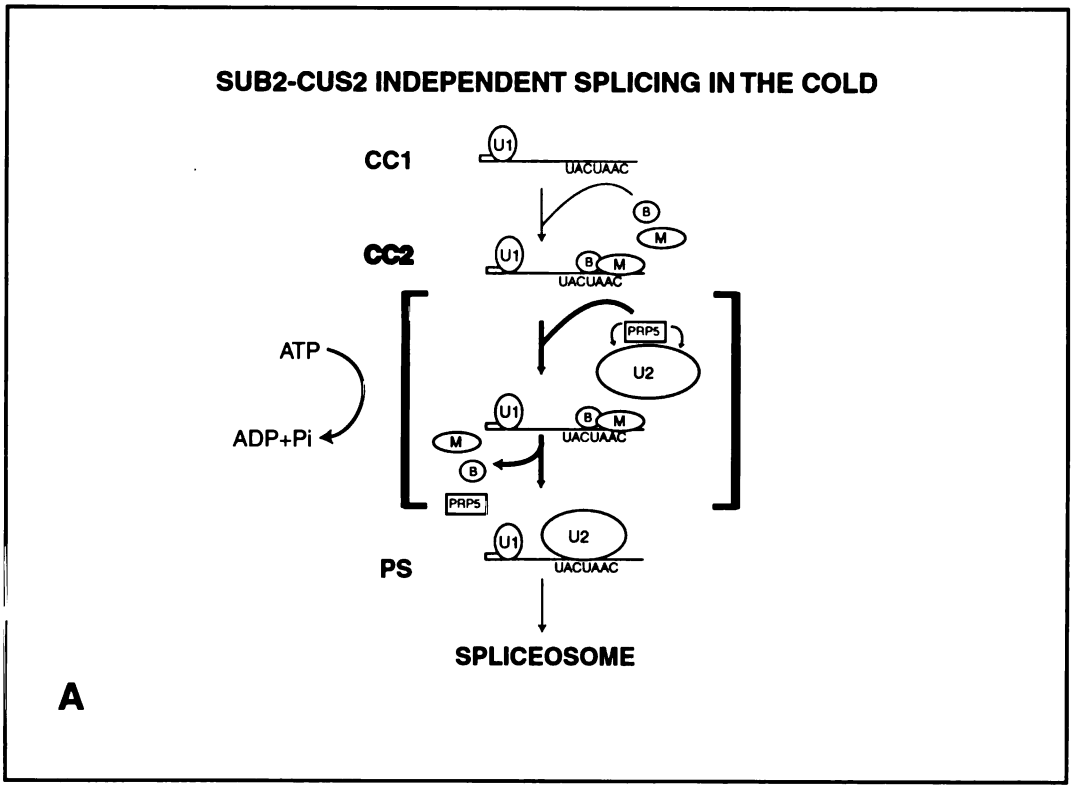

SUB2-MUD2 INDEPENDENT SPLICING AT RT
CC1
CC2
ATP
ADP+Pi
PRP5
CUS2
U2
U1
B
UACUAAC
PS
SPLICEOSOME
SUB2-MUD2 INDEPENDENT SPLICING AT 16°C
SUB2-MUD2 INDEPENDENT SPLICING AT 33°C

Figure 4: Model for the Sub2-Cus2 independent splicing pathway at different temperatures. A, cold temperatures (≤20°C); B non-permissive temperatures (≥20°C).


SUB2-CUS2 INDEPENDENT SPLICING IN THE COLD
CC1
U1
UACUAAC
B
M
CC2
U1
B
M
UACUAAC
ATP
ADP+Pi
PRP5
U2
U1
B
M
UACUAAC
M
B
PRP5
U1
U2
UACUAAC
PS
SPLICEOSOME
A

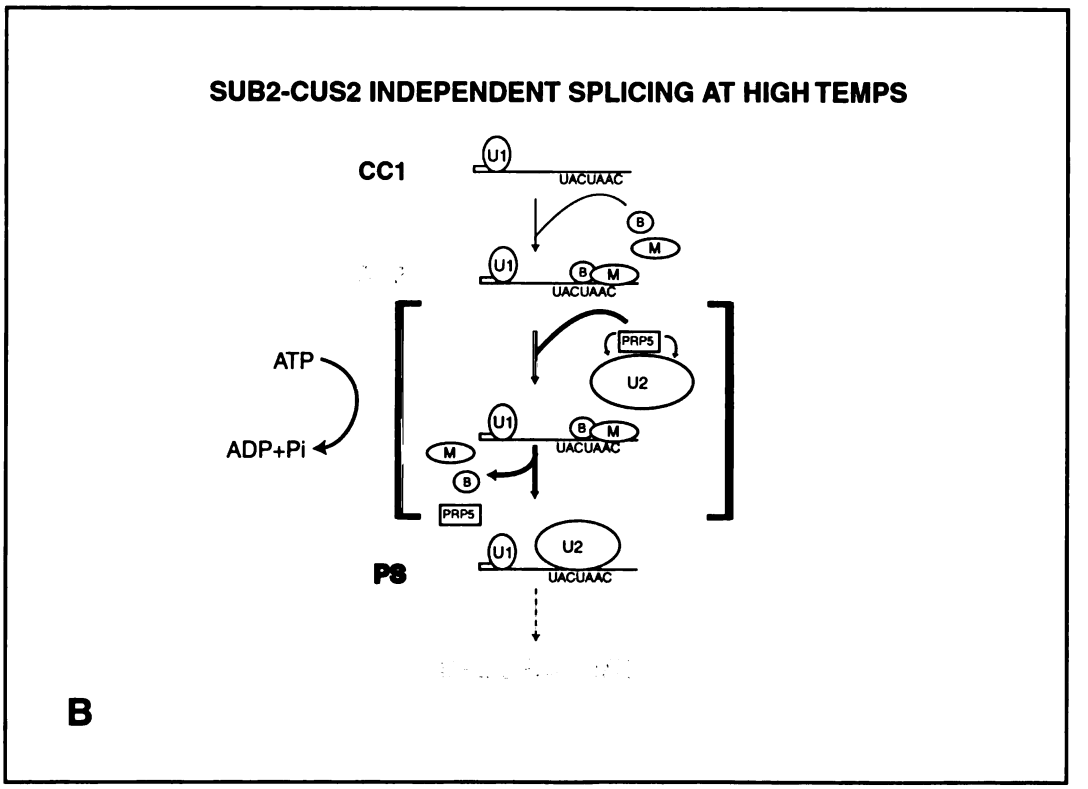
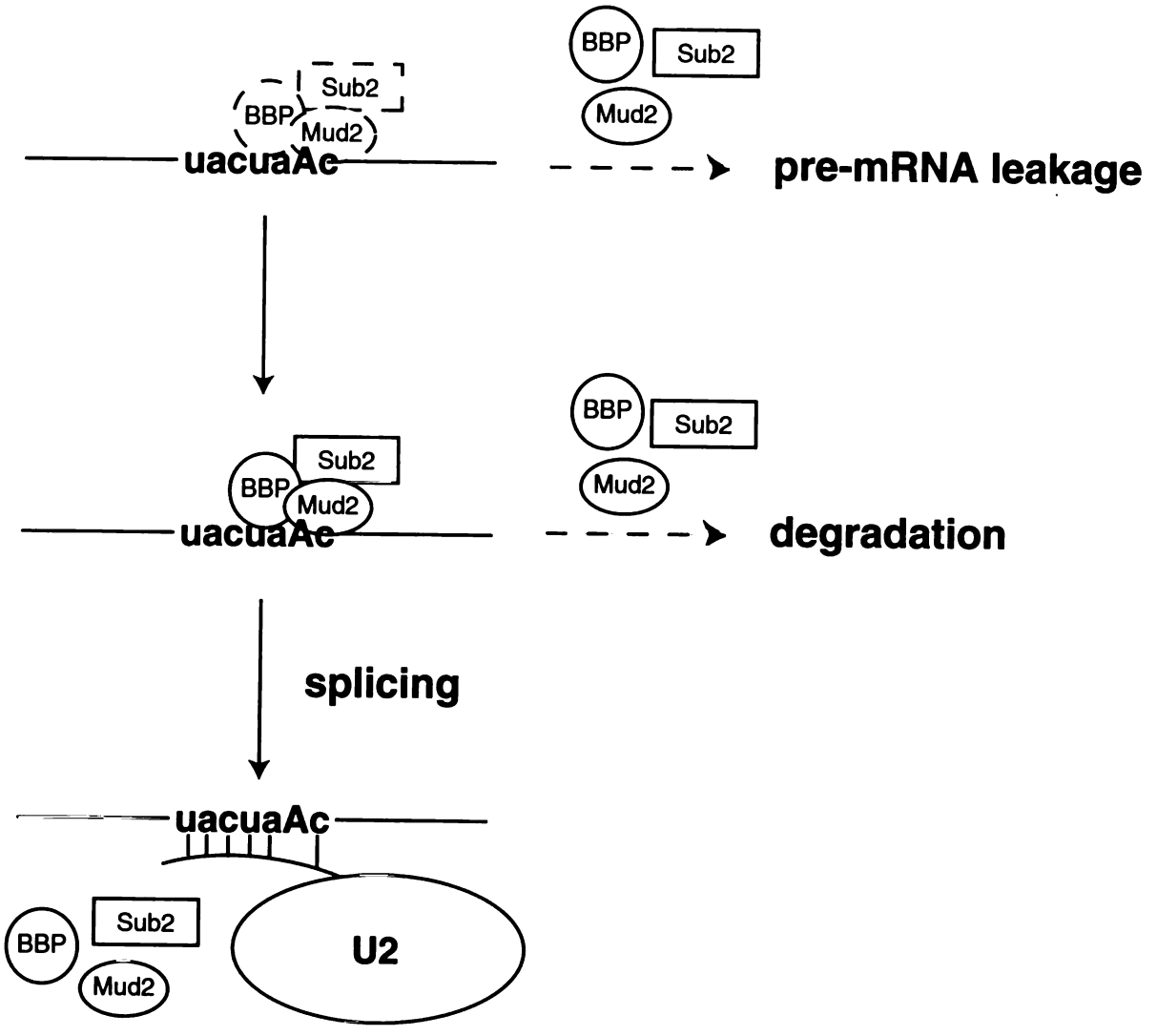

SUB2-CUS2 INDEPENDENT SPLICING AT HIGH TEMPS
CC1
U1
UACUAAC
B
M
U1
B
M
UACUAAC
ATP
ADP+Pi
PRP5
U2
U1
B
M
UACUAAC
M
B
PRP5
U1
U2
UACUAAC
PS
B

Figure 5: Model linking the early ATP-independent function of Sub2 with possible roles in pre-mRNA retention and potential discard pathway.


BBP
Sub2
Mud2
uacuaAc
pre-mRNA leakage
degradation
splicing
U2

Appendix 1

Some Last Words On Evidence of Possible Additional Functions For Sub2

Is splicing the whole story?

A number of links between Sub2 function and disparate RNA processing pathways in the cell have been reported. These links between Sub2 function and snRNP biogenesis, RNA transcription, and RNA transport are summarized below. I close by proposing a model which incorporates the recent data implicating Sub2 function to RNA processing events ranging from transcription to RNA export.

I. Genetic interactions with BRR1: a link to a snRNP biogenesis function?

The data presented in chapter 3 summarize preliminary results from our efforts to revisit and understand the original connection between Sub2 and the snRNP biogenesis factor, Brr1. In these experiments, we observed a series of complex genetic interactions that suggest that these factors may functionally interact antagonistically, in a manner that is dependent on the levels of the two proteins. Biochemical analysis of the impact of high copy *SUB2* on the phenotypes associated with the *brr1-1* mutant indicate that overexpression of *SUB2* may suppress *brr1-1* through an impact on some aspect of snRNP biogenesis or stability. Consistent with this possibility, the alterations in the snRNP profile of *sub2-1* extracts phenocopies that detected in *Δbrr1* extracts. In each case, regardless of temperature preincubation conditions, both extracts exhibit lower levels of all the snRNPs relative to similarly treated isogenic wildtype extracts. Whether this phenotype is a cause or an effect of the *sub2-1* mutant remains to be tested. Moreover, the impact of *GAL-SUB2* overexpression in a wildtype strain on the biogenesis or stability of snRNPs also remains to be investigated. A recent link between overexpression of *SUB2* and RNA transport defects (see below) raises the possibility that

the link between *SUB2* and *BRR1* may reflect a role for RNA transport in the snRNP biogenesis pathway. Such studies merit further attention, as it has remained an unresolved question if snRNA biogenesis in yeast follows a similar transport dependent pathway as that which has been described in mammals.

II. Genetic interactions with HPR1: a link to a function in transcription?

Sub2 has also been genetically linked to a function in transcription and repression of hyper-recombination. In an independent study, high copy *SUB2* was detected to suppress the temperature sensitive growth defect and hyperrecombination phenotype conferred by deletion of the *HPR1* gene (H. Klein, personal communication). *HPR1* is required to maintain normal levels of recombination between directed repeats in the chromosome (Aguilera and Klein 1988; Aguilera and Klein 1989). It possesses some homology to *TOP1*, and has been implicated to play a role in transcription elongation (Aguilera and Klein 1990; Chavez and Aguilera 1997). *HPR1* exhibits genetic interactions with components of the RNA polymerase II (pol II) (Fan et al. 1996). Additionally, *HPR1* protein has been found in pol II subcomplexes purified through affinity chromatography with antibodies raised against the C-terminal domain of the polymerase (Chang et al. 1999).

Suppression of *Δhpr1* by *SUB2* overexpression may reflect an additional role for Sub2 in transcription, or a general association of Sub2 with the transcription machinery. Consistent with this possibility, loss of function alleles of *sub2* display a hyper-recombination phenotype (H. Klein, personal communication). Additionally, recent data suggests that BBP is associated with the CTD of RNA polymerase II (M. Carmo-

Fonseca, personal communication), thus raising the possibility that all the early-acting BPS factors bind a nascent transcript co-transcriptionally. Finally, the Drosophila homolog of Sub2 exhibits phenotypes consistent with members of the polycomb group of transcription regulatory factors (Eberl et al. 1997). If Sub2 is indeed associated with RNA polymerase to perform its normal splicing function, or some additional function linked to transcription, it could be that overexpression of Sub2 may lead to a gain of function substitution for the normal function of the *HPR1* gene.

However, an alternative possibility has been raised through preliminarly analysis of genome wide transcription in the *Δhpr1* strain. In these studies, transcription of the endogenous *SUB2* gene is decreased in this strain context (J. Jaehning, personal communication). Thus, it is possible that the hyper-recombination and transcription elongation phenotypes are a consequence of the downstream loss of *SUB2* gene expression and function—either in a direct role in transcription which inhibits hyper-recombination, or through some less direct, downstream impact on a hyper-recombination monitoring system in the cell. In this scenario, the high copy suppression of the *Δhpr1* phenotypes does not reflect a functional interaction, but an epistatic interaction between these factors.

These two distinct hypotheses can be easily distinguished with tools currently available. Analysis of affinity purified fractions of RNA polymerase complexes or holoenzyme and mediator fractions of transcription factors with Sub2 polyclonal antibodies should address whether Sub2 associates with RNA polymerase complexes. Reciprocal analysis of affinity purified fractions of Sub2 with antibodies against pol II or factors known to associate with pol II should provide further biochemical tests of an

association of Sub2 and the transcription complex. Performing such experiments in both wildtype and *Δhpr* strains overexpressing *SUB2* would further address the possible gain of transcription function in the absence of *HPR1* hypothesis.

III. Genetic interactions with Yra1: a link to a function in RNA transport?

Even more recent evidence raises the possibility that Sub2 may play a role in RNA transport. A recent genetic screen identified a ts- *sub2* mutant that is synthetically lethal with nonessential deletions of the RNA export factor *YRA1* (K. Strasser and E.C. Hurt, personal communication). This ts- allele of *sub2* accumulates poly A+ RNA at the non-permissive temperature in a manner consistent with a direct role in RNA transport. Both overexpression and underexpression of *SUB2* also appeared to cause the accumulation of poly A+ RNA in the nucleus. These genetic interactions and cytological phenotypes may reflect a bona fide role for Sub2 in RNA export from the nucleus. Protein-protein interactions performed in these studies in both yeast and E. coli extracts suggest the inferred interactions between Yra1 and Sub2 result from a direct physical interaction.

Similar implications for such a function for the mammalian homolog of Sub2, UAP56, and Aly, the mammalian version of Yra1 have also recently been reported (M-J Luo, Z. Zhou, K. Magni, C. Christoforides, J. Rappsilber, M. Mann, and R. Reed, personal communication). Aly has previously been implicated in facilitating the export of spliced transcripts from the nucleus (Zhou et al. 2000). It has been proposed to serve as part of a "mark" for the completion of splicing (Le Hir et al. 2000; Zhou et al. 2000). Analysis of the proteins which biochemically co-purifying with the Aly-mRNP complex

revealed that UAP56 was a component of this complex (M-J Luo, Z. Zhou, K. Magni, C. Christoforides, J. Rappsilber, M. Mann, and R. Reed, personal communication).

Kinetic analysis of proteins associated with transcripts incubated in splicing extract over time suggest that UAP56 associates with transcript in a U2AF65-dependent fashion, and remains bound to the transcript over the course of the splicing reaction (M-J Luo, Z. Zhou, K. Magni, C. Christoforides, J. Rappsilber, M. Mann, and R. Reed, personal communication). As splicing proceeds, U2AF65 is released, as previously observed. This release is likely catalyzed by UAP56. However, concomitant with UAP56 release, Aly association with the transcript is observed. Both Aly and UAP56 remain bound throughout the course of the splicing reaction. Consistent with these observations, UAP56 and Aly protein-protein interactions can be detected in both HeLa extracts and E. coli extracts. Additionally, microinjection of molar excess quantities of UAP56 into Xenopus nuclei, where Aly has been demonstrated to be limiting for the export of spliced mRNA, inhibits mRNA export. These data suggest that the excess UAP56 may be titrating and inactivating the limiting Aly. Consistent with this hypothesis, co-injection of excess Aly with the UAP56 rescues the mRNA export defect. Thus it appears that in both yeast and mammals, in addition to a role in splicing, Sub2/UAP56 may also interact with a key factor required for RNA transport from the nucleus.

IV. Broader implications of functions for Sub2 in diverse RNA processing pathways

Taken together, these new studies in yeast and mammals raise the possibility that Sub2 may not dissociate from a transcript destined for the splicing pathway. Instead,

they suggest that Sub2 may serve as a marker for entering the splicing pathway, and remains as a marker for exiting the pathway, in order for spliced mRNAs to be exported from the nucleus. Although such a function for Sub2 may be farfetched, there are certainly sufficient quantities of protein present in the cell to perform such a function. Moreover, the genetic and biochemical data from both yeast and mammals make persuasive cases for Sub2 functions at both ends of this RNA processing pathway. Taken together, it is possible to imagine a pathway where Sub2 functions in such a manner.

It is possible that the data indicating a function for Sub2 in transcription reflects a co-transcriptional association of Sub2 with a nascent pre-mRNA molecule. The stable association of Sub2 would target this pre-mRNA transcript to the splicing pathway. As suggested earlier, destabilization of the interaction between Sub2 and a nascent pre-mRNA molecule would likely lead to leakage of that transcript from the nucleus, resulting in cytoplasmic decay. Alternatively, destabilization of this interaction could also result in nuclear degradation of the transcript. Stably associated Sub2 would target the transcript to the splicing pathway. The proposed ATP-dependent function wherein Sub2 removes BBP and Mud2 from the BPS, would promote further spliceosome assembly on the pre-mRNA transcript. To function at a later stage, after the completion of pre-mRNA splicing, Sub2 would have to remain associated with the pre-mRNA transcript. It is unclear how this occurs. A "handing-off" of the interaction between Sub2 and BBP and Mud2 for a Sub2-Yra1 interaction could facilitate the retention of Sub2 in a complex with a transcript committed to splicing. Kinetic analysis of the association of Aly with a pre-mRNA transcript in mammalian extracts suggest such an exchange could occur in this manner (see above).

In order to serve as a signal for completion of splicing, Sub2 and Yra1 would ultimately have to remain bound to the transcript throughout the course of splicing, likely via an interaction with the exon junctions generated by splicing of the pre-mRNA substrate. Since Yra1 contains RNA recognition motifs, it is likely that it mediates the association of Sub2 with the transcript throughout the later stages of splicing. Moreover, Aly, the mammalian homolog of Yra1 has been detected to bind near exon junctions generated during splicing in mammalian extracts (Le Hir et al. 2000). Preliminary immunofluorescence analysis suggests that Sub2 is a nuclear protein (M. Inada personal communication). Thus, at some point, the interaction between Yra1 and Sub2 is likely exchanged for Mex67, an RNA transport factor (Katahira et al. 1999; Hurt et al. 2000; Strasser et al. 2000)which has been shown to bind Yra1 to facilitate transport of the spliced RNA molecule from the nucleus (Strasser and Hurt 2000). Consistent with this possibility, analysis of the protein-protein interactions of Yra1 and Sub2 and Mex67 indicates that Sub2 and Mex67 bind the same region of Yra1 (K. Strasser and EC Hurt, personal communication). Preliminary studies with recombinant proteins demonstrate that these binding interactions are competitive. Thus the Yra1-Mex67 and Yra1-Sub2 interactions are likely to be mutually exclusive. This may indicate that RNA transport of a spliced pre-mRNA transcript is achieved by a final "hand-off" or displacement of Sub2 from the spliced mRNA particle in exchange for interactions with Mex67. Release of Sub2 from such a particle may provide a mechanism by which Sub2 may re-enter this pathway, perhaps through a re-association with RNA polymerase II subunits.

Appendix 2

Analysis Of The cis Elements Required For Exon Inclusion During Mata1 Splicing

SUMMARY

At the time these studies were initiated, MATa1 was one of 4 genes in yeast known to contain more than one intron. I focused on analyzing the cis elements for exon inclusion during MATa1 splicing. I chose the MATa1 transcript because both the exon-included and exon-skipped mRNAs from encode transcripts without an in-frame nonsense codon. Thus the potentially complicating issue of differential stability of the different messages (skipped vs included) due to nonsense mediated decay was likely to be minimized with this transcript, in contrast to the other multi-intronic substrates available in yeast. Additionally, the distance between exons 1 and 3 was within the normal distribution of intron sizes known for yeast transcripts. Through these studies, I hoped to gain insight into how efficient exon inclusion was in yeast, and whether yeast utilized an intron-defined or exon-defined system for pairing multiple exons of a single transcript during splicing. My ultimate goal was to utilize the knowledge gained in the study of the cis elements required for exon inclusion to carry out a genetic screen for splicing factors required for efficient exon inclusion during splicing in yeast. Based on the results obtained from the analysis of the cis elements required for efficient exon inclusion during MATa1 splicing, I hypothesize that genetic screens for increased exon skipping may prove useful in efforts to identify factors involved in the coupling of transcription and splicing.

RESULTS

1. Architectural features of the MATa1 intron and basal splicing activity

The MATa1 transcript contains two introns (Figure 1). In this transcript, both exon inclusion splicing and exon skip-splicing pathways can generate in-frame transcripts. The distance between the exon skip-splicing splice sites (5'ss of intron 1 and 3'ss of intron 2) is not unusually large (346 nucleotides) relative to the distribution of intron sizes in yeast genes containing introns (REF SPINGOLA). However, the length of the branchpoint sequence-3'ss distance of this intron is short (10 nucleotides) compared to a typical intron of this length (18-40 nucleotides). In contrast to the size of the individual introns of MATa1, which are at the very low end of the distribution of intron lengths (54 nucleotides and 52 nucleotides for intron 1 and intron 2, respectively). Additionally, the second intron of MATa1 is located 424 nucleotides beyond the 5'end of the transcript. Thus it seems that architecturally, neither splicing pathway is signficantly optimized in this transcript.

2. Analysis of exon inclusion splicing efficiency

However, exon skipping during MATa1 splicing appears to be rare (Figure 2). Despite the fact that the exon included mRNA and exon skipped mRNA accumulate to similar steady state levels in vivo (Figure 2 "long" primer extension gel), exon skipped mRNA generated from the MATa1 pre-mRNA is barely detectable by primer extension analysis. A low level of exon skipping, approximately 0.1% of the total mRNA generated from the MATa1 pre-mRNA transcript, can be detected using an oligonucleotide optimized to anneal specifically to the junction of exon1 and exon 3

(Figure 2 "skipped" primer extension gel). Primer extension analysis using an oligonucleotide in exon 3 and ddGTP also allows for detection of an exon-skipped version of the MATa1 mRNA (data not shown). Thus although a significant amount of exon skipped mRNA can be detected to be generated from the MATa1 pre-mRNA, it appears that the exon inclusion splicing pathway dominates the splicing pattern of this transcript.

3. Intron definition vs exon definition in MATa1 splicing

In higher eukaryotes, the pairing of splice sites of multi-intronic transcripts have been observed to occur via two distinct sorts of mechanisms. In an intron-defined system, the boundaries of introns and exons are thought to be defined through stabilization of early-acting splicing factors across the introns (Figure 3A). In contrast, in an exon-defined system, interactions of early acting splicing factors at the splice sites occurs across the exons (Figure 3B). Mutation of the downstream 5'ss leads to distinct splicing phenotypes in each of these systems, and thus provides a way to assess the type of definition operating in a given multi-intronic transcript. Because the spliceosome complexes are formed and stabilized independently on each intron in the intron-defined system, mutation of the downstream 5'splice site leads to a defect in the splicing of the second intron. Thus, such transcripts retain intron 2 upon mutation of the downstream 5'ss (Figure 3A). In contrast, in exon-defined transcripts, mutation of the downstream 5'ss destabilizes both the splicing complexes at that splice site and the complexes present at the 3'ss of the upstream intron. The stabilizing effects of the cap binding complex and the polyadenylation machinery at the 5' end and 3'end of the transcript, respectively lead

to activation of splicing at the first 5'ss and the last 3'ss. Thus, mutation of the downstream 5'ss in exon-defined transcripts leads to exon skipping.

To address which mechanism operates in yeast to maintain the high accuracy of exon inclusion during MATa1 splicing in yeast, we mutated the 5'ss of intron 2 in MATa1 and examined the impact on the profile of MATa1 mRNAs (Figure 4). As expected, mutation of the downstream 5'ss abolished exon inclusion splicing. Instead, a significant amount of singly spliced MATa1 RNA was detected. The identity of this mRNA as intron 2 retained was confirmed by independent primer extension studies using an intron 2 oligo primer (data not shown). There was no detectable increase in exon-skipped mRNA in this mutant, regardless of which oligo primer or primer extension method was applied (Figure 4 and data not shown). Taken together, these data indicate that MATa1 splicing is intron-defined.

A. Evidence for co-transcriptional splicing?

Intron definition and co-transcriptional splicing mechanisms are hard to distinguish in vivo through mutation of individual splice sites. Both models predict that introns will behave independently. However, the splicing profile of MATa1 provides a possible opportunity to test the possibility that exon inclusion during its splicing may occur co-transcriptionally. The steady state profile of splicing products and intermediates suggests that the splicing of intron 2 of MATa1 may be less efficient than intron 1. A singly spliced intermediate can be detected (Figures 3, 4, and 5). In parallel primer extension studies we have found that this intermediate corresponds to intron 1 spliced transcript.

This splicing profile may be due to the sequences or architecture of the second intron. Alternatively, this splicing profile may be a result of co-transcriptional splicing, and decreased efficiency of second intron splicing due to its lack of proximity to the 5' end of the pre-mRNA transcript. If the splicing profile is simply a result of the sequence or architecture of the second intron, then replacing this intron with intron 1 will likely alter the splicing profile such that all the transcript will be spliced to mRNA. However, if the splicing of the intron is influenced by its location in the transcript relative to the 5'end of the molecule, then the splicing profile will remain unaltered in a MATa1 pre-mRNA in which both introns contain identical sequences. We thus substituted intron 2 with intron 1 sequences and then compared the splicing profile of this intron 1 (i1x2) reiterated MATa1 to the profile of wildtype MATa1 (Figure5). Substitution of intron 2 sequences with intron 1 sequences had no detectable impact on the profile of splicing products and intermediates generated from the MATa1 pre-mRNA. Independent primer extension analysis with exon 2 and exon 2/3 junction primers confirmed these results (data not shown). Thus, it appears that the sequence of intron 2 is not responsible for the difference in the profile of the splicing products of the MATa1 pre-mRNA. In fact, in a matrix of swaps and reiterations of both the introns and introns plus flanking sequences, we found no case where the profile of the MATa1 splicing products were altered (Table 1 and data not shown). Moreover, preliminary in vitro splicing experiments suggest that intron 1 and intron 2 splice with similar efficiencies in vitro when present as single intron splicing substrates (data not shown). Thus, it is possible that these splicing profiles reflect co-transcriptional splicing of MATa1 pre-mRNA in vivo.

MATERIALS AND METHODS

Oligonucleotides used in this study

oAK22 CGCGGATCCGCGAAGGACAACATATGGATGAT

oAK23

CGGGATCCCGTTTAGATCTCATACGTTTATTTATGAAATTATAAAGATTATTA

GTATTGAGA

oAK35 GAATTTATTTAGATCTCATACGTTTATTT

oAK36 CGCGGATCCGCGGCAAGTAGATAAGGGTATAG

oAK49 TTTTCCTTTTGCGGCCGCTTTTTTCCTTAAGAGCAAGACGATGGGG

oAK65 GTAAGAGTTTGGCAATGTAATATGAG

oAK69 CGTTTATTTATGAAATTATAAAGATTATT

oAK129

CTACAAATTGTTAGTATAGGATATATTTAAGTTTGATTCTCATATTACATACA

TTATAAAGATTATTAGTATTGAGAT

oAK0130 ACTAACAATTTGTAGTTTATAATGGAAAGTAATTTGACT

oAK0131 CTTCTTTTTCCTTGGAATTAAGGCTTTGCTTTCTTC

oAK0140 CAGGTTTTTAGAAGAAAGCAAAGC

oAK0141

CTGAAGTATTGTTAGTAAATTGATTCAAAGGCTATCCTTGAAATGAAAACAT

ACCCAAACTCTTACTTGAAGTGG

oAK0142 TTACTAACAATACTTCAGTTCATAAATAAACGTATGAGATCTAAAT

oAK0143 AACATTCTAGGTACTGAGATTGAT

oAK93 (U14) ACGATGGGTTCGTAAGCGTACTCCTACCGTGG

Plasmids and strains used in this study

pBSKS(+)	
pRS316	
pTCAΔR1	EcoR1-BamH1 fragment of the GPD promoter in pRS314 polylinker
pAK71	pRS316 containing the GPD promoter
pMS11	MATa1 gene in pGEM3
pAK117	MATa1 in pAK71
pAK137	MATa1 exon 2 skipped cDNA in pAK71
pAK91	MATa1 in pBSKS(+)
pAK119	MATa1 5'ss#2 mutant (CAAUGU) in pBSKS(+)
pAK127	MATa1 5'ss#2 mutant (CAAUGU) in pAK71
pAK233	MATa1 i2x2 mutant in pAK71
pAK248	MATa1 i1x2 mutant in pAK71
pAK252	MATa1 i2/i1 mutant in pAK71
pAK256	MATa1 (i1+C)x2 mutant in pAK71
yCL51	MATa ade2 cup1::ura3 leu2 trp1 ura3-52 lys2his3

pTCAΔR1 was provided by the Yamamoto lab. pAK71 was generated by subcloning the polylinker of pTCADR1 polylinker into pRS316. A BamH1-NotI cleaved PCR product was generated with oAK22 and oAK49 using pMS11 as template, then

subcloned into the BamH1-NotI sites of pAK71 to generate pAK117. An exon-skipped cDNA version of MATa1 was generated by PCR with oAK22 and oAK23, cleaved with BamH1 and BglII and subcloned into pAK117 cleaved with the same enzymes to generate pAK137. A BamH1 fragment of MATa1 was generated by PCR using oAK22 and oAK36. This fragment was subcloned into pBSKS(+), to generate pAK91, which was used as a source of ssDNA template for site directed mutagenesis (REF). Mutation of the second 5'ss of MATa1 was carried out by site directed mutagenesis using oAK65. PAK127 was generated by subcloning the BamH1-BglII fragment of pAK119 into pAK117. MATa1 constructs with swapped intron sequences were generated through restriction digest of the StyI-BglII fragment from MATa1 mutants generated by sequential PCR reactions using oligos oAK22, oAK129-oAK131, oAK140-oAK143, and oAK49. The wildtype and mutant MATa1 plasmids in pAK71 were transformed into yCL51 (Lesser and Guthrie 1993) to assess their impact on exon inclusion and the profile of MATa1 RNA in vivo.

In vivo analysis of MATa1 splicing

Total cellular RNA was isolated from logarithmically growing cells and analyzed by an abbreviated primer extension protocol (Lesser and Guthrie 1993). A ^{32}P-labeled oligonucleotide primer which anneal specifically to exon 2 of MATa1 (oAK35) was used in the "long" primer extension analyses to analyze the distribution of all the MATa1 RNA species. This same primer could be used to specifically detect exon skipped mRNA when ddGTP was substituted for dGTP in the primer extension reactions (not shown). The oligonucleotide primer oAK69 was used to specifically detect exon-skipped MATa1

RNA in the "short" primer extension analyses. In all these experiments, a primer which anneals to the U14 snoRNA (oAK93) was used as an internal RNA loading control.

Figure 1: Architecture of MATa1 gene. Boxes denote exon sequences, lines introns. Lengths of introns used for exon inclusion or exon skipping are indicated above the arrows spanning these possible introns.

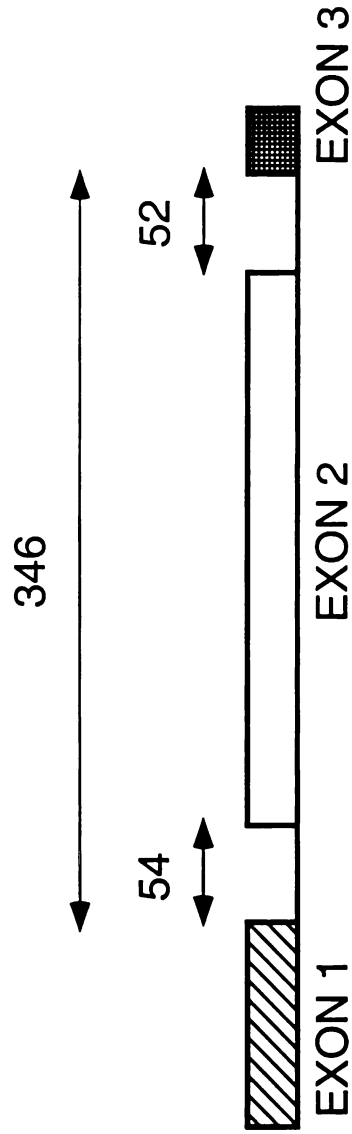

346
54
52
EXON 1
EXON 2
EXON 3

Figure 2: Primer extension analysis of MATa1 RNA. The steady state levels of MATa1 RNA generated from plasmids bearing wildtype MATa1 or an exon-skipped MATa1 cDNA were compared. MATa1 splicing intermediates and products are diagrammed at left and right of the two gels. The left panel (LONG) utilizes an oligonucleotide primer in exon 3, the right panel are the same samples primer extended with an oligonucleotide that hybridizes specifically to the exon 1/exon 3 junction of an exon skipped version of MATa1 (SKIPPED).

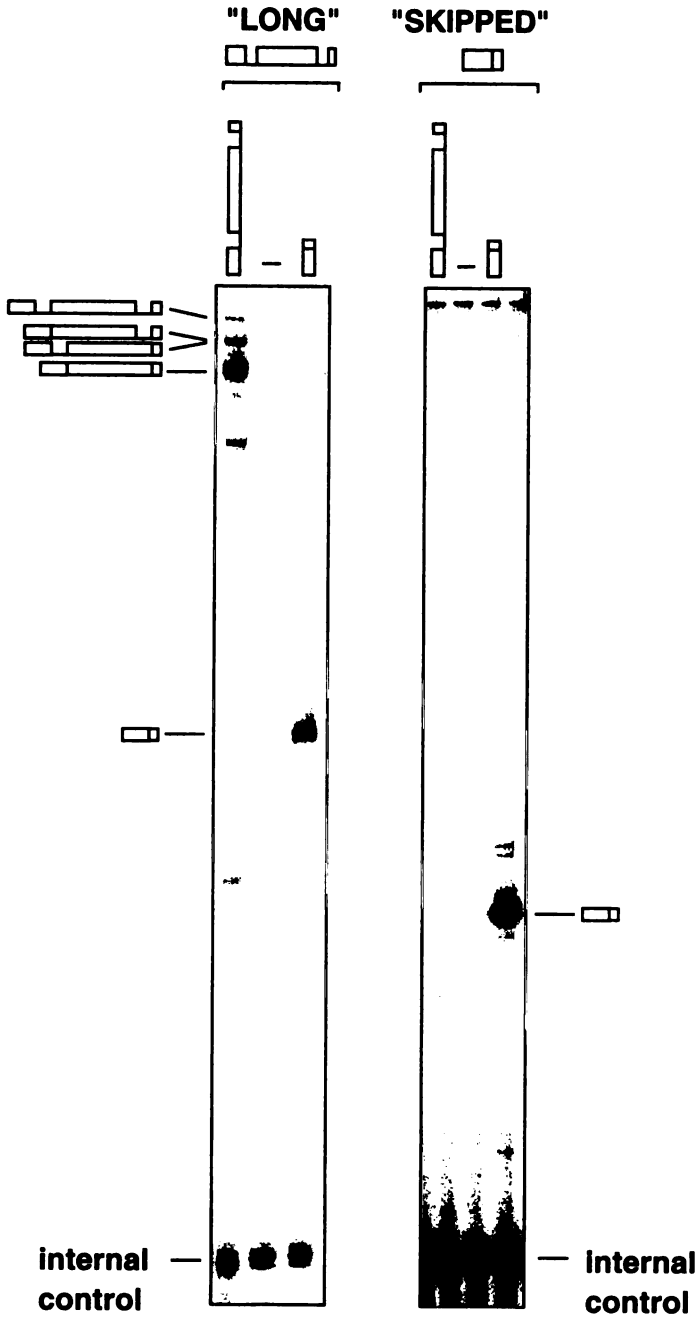
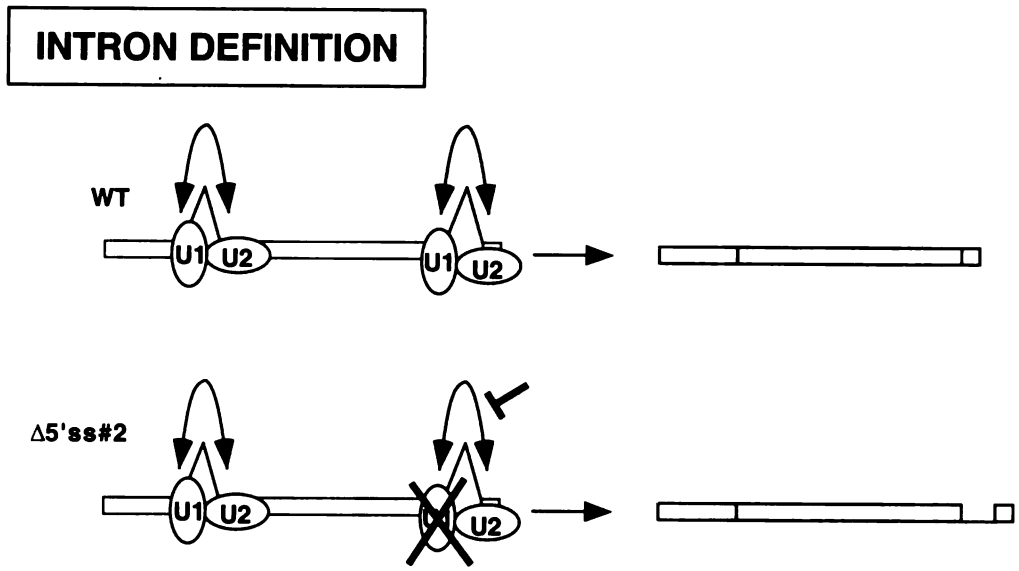

"LONG"
"SKIPPED"
internal control
internal control

Figure 3: Models for exon inclusion during MATa1 splicing. A, Intron Definition. B, Exon Definition. Shown are the interactions between early splicing factors thought to define the boundaries between exon and intron sequences of multi-intronic transcripts. The impact of deletion of the second 5'ss on the splicing of the transcript in each context is diagrammed below the schematic illustrating each method.


INTRON DEFINITION
WT
U1
U2
U1
U2
Δ5'ss#2
U1
U2
U1
U2

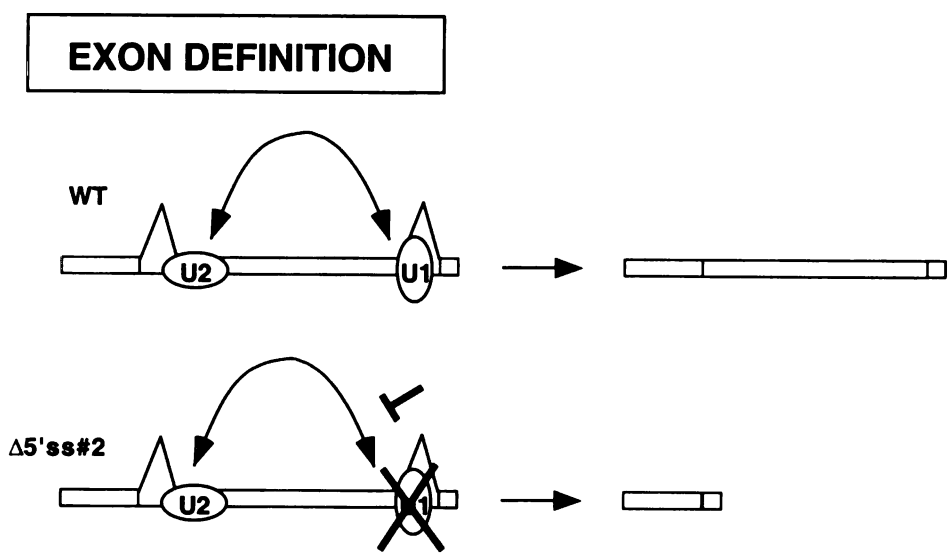

EXON DEFINITION
WT
U2
U1
Δ5'ss#2
U2
U1

Figure 4: Impact of mutating the second 5'ss of MATa1 on exon inclusion. Primer extension analysis of MATa1 transcripts containing a mutation in the second 5'ss. The profile of MATa1 RNA generated from strains transformed with a plasmid bearing MATa1 exon skipped cDNA, wildtype MATa1, or MATa1 with a defective 5'ss in the second intron was assessed by primer extension analysis with an oligo that hybridizes to exon 3.

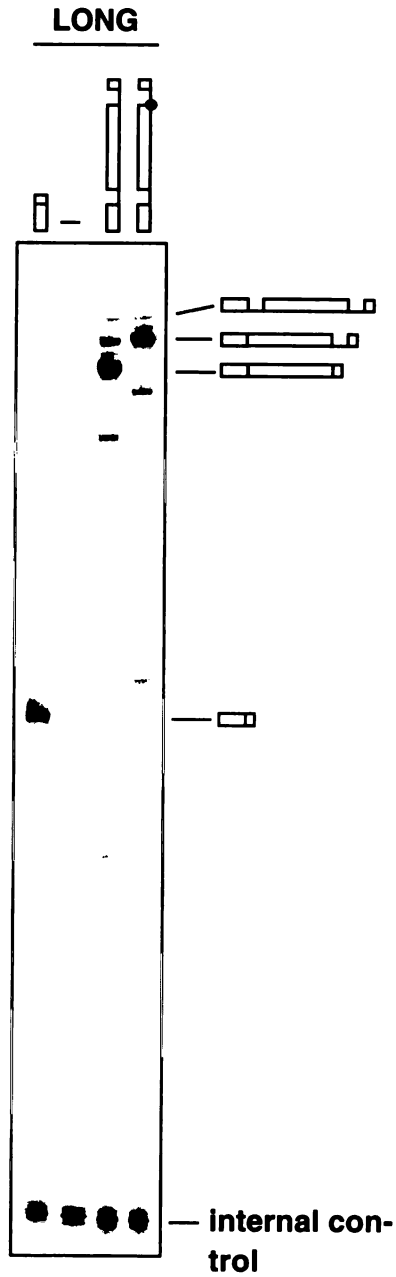

LONG
internal con-
trol

Figure 5: Impact of substituting intron 1 sequence for intron 2 sequence on the steady state profile of MATa1 RNA. Primer extension analysis of MATa1 transcripts in which intron 1 sequences replace intron 2 sequences. The profile of MATa1 RNA generated from 4 independent transformants containing either wiltype MATa1 plasmids, or plasmids bearing MATa1 in which the intron 2 sequences were replaced with intron 1 sequences were assessed by by primer extension analysis with an oligo that hybridizes to exon 3.

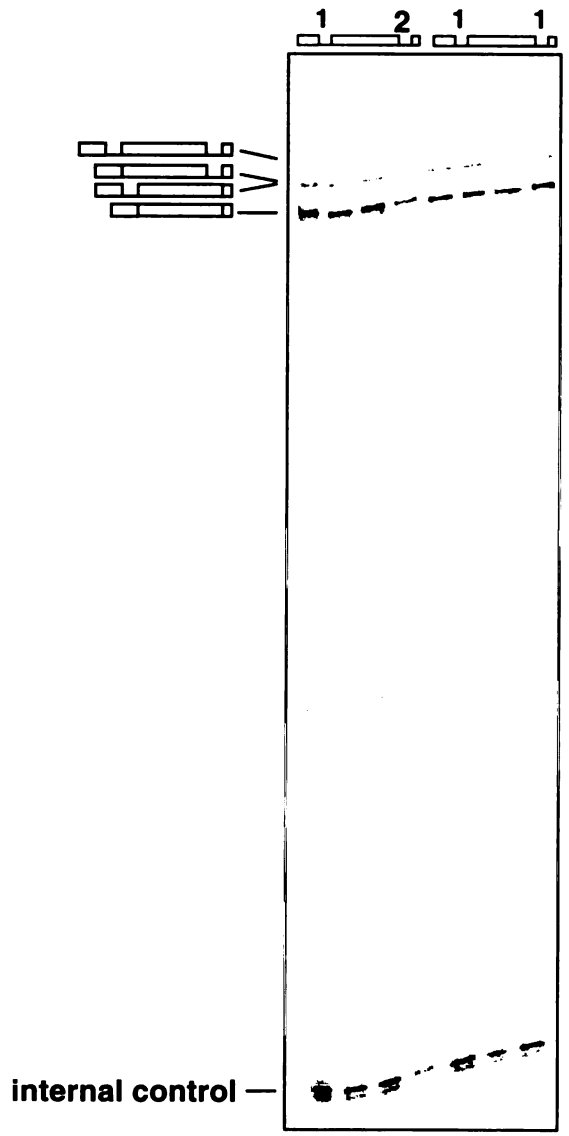
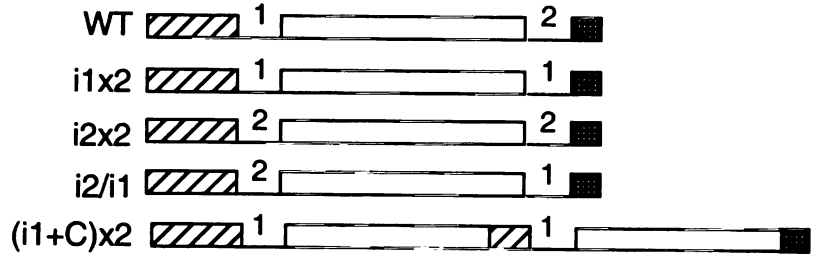

1
2
1
1
V
I
internal control

Figure 6: Summary of the impact of altering the identity of the MATa1 introns on thesteady state profile of MATa1 RNA. WT, wildtype MATa1; i1x2, MATa1 in which intron 1 sequences replace intron 2 sequences; i2x2 MATa1 in which intron 2 sequences replace intron 1 sequences; i2/i1, MATa1 in which intron 1 sequences are swapped with intron 2 sequences; (i1+C)x2, MATa1 in which intron 1 sequences plus flanking exon context replace intron 2 sequences.

MATa1 CONSTRUCT	ALTERED SPLICING PROFILE?
	
WT	NO
i1x2	NO
i2x2	NO
i2/i1	NO
(i1+C)x2	NO

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