DISCUSSION

In the first part of this study, we investigated the presence of BBP and Mud2p in splicing complexes and the requirement for BBP in the formation of the spliceosome. We showed that BBP is required for the formation of CC2. In addition, we demonstrated that BBP is not detectable in CC1 and is present in CC2. Our data indicate that the transition from CC1 to CC2 involves the addition of Mud2p and BBP to the former complex. Furthermore, we provide evidence that BBP and Mud2p join CC1 as a binary complex. This is supported by the absence of detectable intermediates between CC1 and CC2 in wild type extracts.
Importantly, we found that both BBP and Mud2p are not detectable in pre-spliceosomes. Moreover, BBP depletions or a *MUD2* disruption had no significant effect on (pre)-spliceosome formation or pre-mRNA splicing *in vitro*.
In the second part of this study, we generated conditional mutants of *MSL5*, the gene coding for BBP/ScSF1, to further analyze its function *in vivo*. Using a PCR mutagenesis strategy we could isolate 11 ts mutants and one cs mutant. The mutants showed differences in the severity of their temperature-sensitivity and were found not to result from common amino acid changes. These are the first conditional mutants reported for *msl5*, which so far has escaped all large-scale screens for splicing mutants.
The *in vitro* analysis of the obtained mutants confirmed the results obtained with BBP depleted extracts, namely a block in CC2 formation, but no inhibition of mature spliceosome formation or splicing. *In vivo*, we detected decreased splicing efficiencies for introns with non-consensus splice sites, but no significant effect on the splicing of a wild type intron. Interestingly all mutants showed a strong increase in leakage of pre-mRNA to the cytoplasm.

1. Transient interaction of BBP/ScSF1 and Mud2 with the splicing machinery affects the kinetics of spliceosome assembly

Although both Mud2p and BBP seem to be associated in a complex and to act in the same step of splicesome assembly, they differ in their requirement for yeast viability. Therefore the results obtained for both proteins, despite being similar, will be interpreted separately.

1.1. Role of Mud2p in spliceosome assembly and splicing

Mud2p, is the closest homologue of U2AF^{65} in yeast. It is not essential for viability and has been shown to be part of CC2 and to be required for the formation of CC2 (Abovich *et al*., 1994). These results, and our finding that pre-spliceosome formation and splicing were not affected in extracts from a *MUD2* disrupted strain (Figure 22), indicate that accumulation of CC2 as detected in native gels is not absolutely required for pre-spliceosome formation and pre-mRNA splicing. In addition, we could show that Mud2p is not detectable in pre-spliceosomes. In the mammalian system U2AF^{65} has been detected in complex E (the commitment complex) and A (the pre-spliceosome) (Bennett *et al*., 1992b; Champion-Arnaud and Reed, 1994). It is likely that the presence of U2AF^{65} in complex A reflects a slight difference between the yeast and metazoan splicing processes. In yeast, *MUD2* is not essential for viability while its *Drosophila* homologue, dU2AF^{50}, is an essential gene (Kanaar *et al*., 1993). In fission yeast, *Schizosaccharomyces pombe*, the homologue of U2AF^{65}, U2AF^{59}, is also required for viability (Potashkin *et al*., 1993) indicating the early divergence of U2AF dependent and independent splicing processes.
In addition, the 3' splice site region of introns in most higher eukaryotes shows strong conservation of a polypyrimidine stretch downstream of the branchpoint region. In yeast, this polypyrimidine tract is often missing while the branchpoint region is highly conserved with only a few introns showing one or two nucleotides difference from the consensus (Spingola *et al*., 1999; Lopez and Séraphin, 2000). Mud2p is clearly lacking the RS domain present in U2AF^{65} which has been proposed to help base pairing of U2 snRNA with the branchpoint (Valcárcel *et al*., 1996). This suggests that Mud2p is less strongly interacting with the other components of the splicing machinery and the pre-mRNA substrate than its metazoan counterpart.

1.2. Role of BBP/ScSF1 in spliceosome assembly and splicing

For BBP the results obtained create a paradox: BBP has been reported to be essential for viability and to be involved in splicing (Abovich and Rosbash, 1997), yet, the depletion of this protein or its conditional inactivation in mutants did not block (pre)-spliceosome assembly or splicing *in vitro*. We are presenting and evaluating below three alternative possibilities to explain this apparently conflicting data.

a) Is BBP essential because of its role in pre-mRNA splicing?

BBP might function in processes other than splicing that could account for its essentiality in yeast. While human SF1 has been proposed to participate in transcriptional repression

(Zhang and Childs, 1998) and macrophage inherent functions (Wrehlke *et al*., 1999), the purification of SF1 as an activity required for pre-spliceosome formation and splicing (Krämer, 1992) and its interaction with U2AF^{65} (Abovich and Rosbash, 1997; Berglund *et al*., 1998a; Rain *et al*., 1998) suggest that BBP/SF1 is a bona fide splicing factor. This is supported by the observation that the interaction between BBP/ScSF1 and the closest homologue of U2AF^{65}, Mud2, is conserved in yeast (Abovich and Rosbash, 1997; Fromont-Racine *et al*., 1997; Rain *et al*., 1998; this study). Yeast and mammalian BBP/SF1 have also been shown to bind specifically to the branchpoint region (Berglund *et al*., 1997; Berglund *et al*., 1998b) confirming a role in splicing. The yeast BBP/ScSF1 in addition interacts with the U1 snRNP protein Prp40 suggesting again that it is a splicing factor (Abovich and Rosbash, 1997). Last, but not least, the experiments shown above clearly demonstrate that BBP is a component of CC2, a precursor of the spliceosome (Séraphin and Rosbash, 1989). While this possibility was suggested by previous studies (Abovich and Rosbash, 1997), it was still formally possible that BBP was not present in splicing complexes and interacted only transiently with the pre-mRNA substrate. This study and work by others (Abovich and Rosbash, 1997) also indicate that BBP is required for CC2 formation.

Our *in vivo* studies on *msl5* mutants showed a role for BBP/ScSF1 in splicing (see 2.3.). A possible essential function of BBP/ScSF1 could be in pre-mRNA retention in the nucleus (see 2.4.). Further studies will be required to demonstrate whether BBP has, in addition, a role in other cellular processes.

b) Is BBP a general splicing factor?

BBP could be a splicing factor needed only for the splicing of specific introns in yeast with at least one of the corresponding genes being essential for viability. However, CC2 formation has been shown to occur with a variety of substrates (Séraphin and Rosbash, 1991; Vilardell and Warner, 1994; Wells *et al*., 1996), implicating a non-discriminatory role of BBP in splicing. Analysis of the binding specificity of BBP (Berglund *et al*., 1997) also indicates that it will interact with all or nearly all yeast introns (Spingola *et al*., 1999; Lopez and Séraphin, 2000). Our *in vivo* analysis, revealed a stronger sensitivity of introns with non-consensus splice sites to mutations in BBP/ScSF1 (see 2.3.). However, the mutations used in this analysis are in absolutely conserved positions, meaning that pre-mRNA with these divergent splice sites are not existent in yeast (Burge *et al*., 1998a). *In vivo* splicing of a non-consensus branchpoint containing intron (*YRA1*) in a BBP depleted strain showed no decrease in efficiency, but mRNA levels remained constant while pre-mRNA levels were diminished (see 2. 5.). A comprehensive analysis of the splicing of all yeast introns in a

BBP depleted or mutated strain would have to be performed to rule out a specific role of BBP in the splicing of certain introns.

c) Recyling model for BBP/Mud2p

A trace amount of BBP present in our depleted extracts could be sufficient for (pre)-spliceosome formation and splicing, even though our depletions were nearly complete (99-99.9%). It is noteworthy that the GAL-ProtA-BBP strain did not stop growing after several days of incubation in glucose media, even though cells divided with increased doubling time (Figure 11). This suggests that a low level of BBP is sufficient for splicing *in vivo*, albeit at reduced rate. Consistent with an essential role of BBP in splicing, the mammalian homologue of BBP, SF1, is required for pre-spliceosome formation and splicing (Krämer, 1992; Rain *et al*., 1998). However, these studies in the mammalian system also suggested that trace levels of SF1 were sufficient for activity. Considering the difference in binding affinities of human and yeast BBP/SF1 for the conserved branchpoint sequence (30 µM compared to 0.5 µM respectively, Berglund *et al*., 1997) one would expect a lower threshold level for an effect of the depletion in yeast. This is consistent with our results.

Our data indicate that BBP is released from splicing complexes during the formation of pre-spliceosome. Two other studies identified proteins in HeLa cells that crosslinked to the pre-mRNA in close proximity or at the branchpoint in complex E, but crosslinks were no longer detectable in complex A (Chiara *et al*., 1996; MacMillan *et al*., 1994). Both proteins could be identical to SF1 (Berglund *et al*., 1997). These crosslinking studies would therefore be in agreement with our results obtained for BBP.

Taken together with the lack of inhibition of pre-spliceosome formation upon BBP depletion, this suggests a model, where BBP is released during or immediately after the CC2 to pre-spliceosome transition and is efficiently recycled to promote maturation of CC1 into CC2 (Figure 43). In this case only catalytic amounts of BBP would be sufficient to assure normal spliceosome formation and splicing. However, when no spliceosomes are formed (e.g. in the absence of ATP) the release of BBP from CC2 cannot occur. Since BBP is necessary for the formation of CC2, this complex can not accumulate any higher than the level of BBP in the extract. In our experiments, these levels are below the detection limit. The accumulation of CC1 during spliceosome formation in a BBP depleted extract (Figure 24A, lanes 11-15) is consistent with this model. Indeed, when BBP is limiting, the transition from CC1 to CC2 would be slowed down by the need to recycle BBP, leading to accumulation of CC1. This suggests that, under these conditions, the transition of CC2 to pre-spliceosome is faster than the CC1 to CC2 transition. In contrast, in a complete extract, BBP is not limiting and the transitions from CC1 to CC2 and from CC2 to pre-spliceosome

are fast thereby preventing the accumulation of intermediate complexes. *In vivo* this could lead to a change in kinetics of the splicing reaction which could explain the slow growth phenotype observed in a BBP depleted strain.

An absolute requirement for BBP in pre-spliceosome formation is also suggested by the observation that pre-mRNAs containing point mutations of the branch residue were not able to form CC2 and pre-spliceosomes, but instead accumulated CC1 in the presence or absence of ATP (Pascolo and Séraphin, 1997). One could have anticipated that these substrates should form pre-spliceosomes because the U2 snRNA-pre-mRNA recognition does not involve the branch residue (Ares, 1986; Parker *et al*., 1987; Pascolo and Séraphin, 1997). The observation that splicing complex assembly on a branchpoint mutant is blocked at the CC1 step, suggests that the branch residue is recognized by an essential factor other than the U2 snRNA. As BBP has been reported to require the branch residue for binding (Berglund *et al*., 1997) it is a likely candidate for this factor.

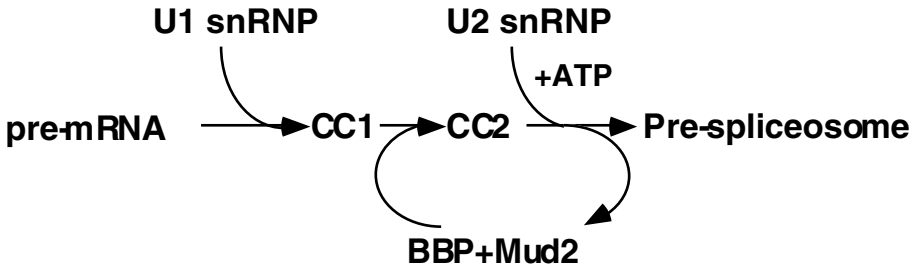

Figure 43. Model for the recycling of BBP and Mud2p during pre-spliceosome formation.

The 5' splice site of the pre-mRNA is recognized by U1 snRNP leading to the formation of CC1. The BBP/Mud2p complex joins this complex to form CC2. During the formation of pre-spliceosome (the first ATP dependent step) the BBP/Mud2p complex is released and can promote a new round of CC2 formation.

2. A dual role for BBP/ScSF1 in nuclear pre-mRNA retention and splicing

2.1. Functionally relevant mutations in *msl5* are located in evolutionary conserved domains

The definition of "minimal" mutants that still show a growth defect proved the usefulness of our PCR mutagenesis strategy. Only a high mutagenesis rate could create the pattern of mutations required for the strong phenotype observed in some of the mutants. This could indicate that several domains of the protein act synergistically, each of them contributing only partially to the growth phenotype observed.

Clearly the KH domain, which is required for RNA binding (Arning *et al*., 1996; Berglund *et al*., 1998b; Rain *et al*., 1998), is essential for SF1 function. This is reflected by a relevant point mutation in this region in both mutants analyzed. The mutation (P155→S) in *msl5-2* is located immediately after a predicted β-strand which is present at the same position in five structures of KH domains (Figure 30; Musco *et al*., 1996; Musco *et al*., 1997; Lewis *et al*., 1999; Lewis *et al*., 2000). The proline in this position is one of the most conserved residues in the KH domain (Lewis *et al*., 1999) and is absolutely conserved in all members of the STAR family (Signal Transduction and Activation of RNA, Vernet and Artzt, 1997). Two additional relevant mutations are located in the N-terminus of *msl5-2*. This part of SF1 (aa 41-141 in yeast) has been shown to interact with Mud2p/U2AF65 (Rain *et al*., 1998) and could therefore be required for the cooperative binding to the branchpoint (Berglund *et al*., 1998a). However, since a complete knock-out of *MUD2* shows no growth phenotype, this alone can not account for the strong ts phenotype observed in *msl5-2*. Either the combination with the mutation in the KH domain increases the importance of the interaction with Mud2p or additional interactions are affected by the mutations in this region. One obvious candidate would be the proposed bridging interaction connecting U1 snRNP at the 5' splice site via Prp40p with the branchpoint region (Abovich and Rosbash, 1997). Alternatively, the mutations could distort structures necessary for the proper folding of the protein. However, previous data suggest that the N-terminal region of BBP/ScSF1 contains a functional domain required for other functions than RNA binding. Indeed, a truncated BBP/ScSF1 protein (aa 145-330) shows strong affinity and high specificity for a branchpoint sequence RNA (Berglund *et al*., 1997; Berglund *et al*., 1998b), while a construct lacking the N-terminus (aa 146-476) is not sufficient to complement a disruption of SF1 in yeast (Rain *et al*., 1998).

The cs mutant contains a mutation (V195→D) in a region that is less conserved among KH domains and also not absolutely conserved between members of the STAR family. However, only conservative substitutions seem to occur in this position in the STAR family (V, I or M). It lies in a flexible loop that could account for the sequence specificity of the KH domain (Figure 30 and Lewis *et al*., 2000). The second mutation (E258→V) lies inside the conserved STAR-QUA2 domain, besides the KH domain another hallmark of the large family of STAR proteins. In a study with both human and yeast BBP/SF1 this region was shown to help RNA binding and specificity when present together with the KH domain (Berglund *et al*., 1998b), but the exact function of this domain has not yet been determined.

2.2. *In vitro* analysis of *msl5* mutants shows minor defects in (pre)-spliceosome assembly and splicing

Extracts from the *msl5* mutants showed very similar *in vitro* phenotypes compared to the extracts depleted for BBP/ScSF1. They were all defective in CC2 formation, but showed (with the exception of the cs mutant) no effect on (pre)-spliceosome formation and splicing *in vitro*. The cs mutant extracts were blocked in (pre)-spliceosome assembly at the non-permissive temperature. However, splicing activity was still detectable suggesting that functional spliceosomes still formed. This could indicate a more assembly-related phenotype of the cs mutant, which still allows for splicing but does not allow (pre)-spliceosomes to accumulate to detectable levels in native gels. The fact that BBP/ScSF1 was not found stably associated with pre-spliceosomes (see above) suggests that the mutant form of BBP/ScSF1 affects the assembly of pre-spliceosomes at a very early step. Taken together these results confirm our depletion studies and suggest that the requirement of BBP/ScSF1 in splicing is not universal for all substrates or that only very small amounts of the functional protein are required for proper splicing.

2.3. *In vivo* analysis of *msl5* mutants reveals splicing defect of non-consensus introns

We further investigated splicing of the different mutants *in vivo* using a sensitive reporter assay based on the RP51A intron and mutants thereof. This analysis revealed that splicing of the wild type intron was only very modestly affected by the mutations in *msl5*, while introns with mutated 5' splice site and/or branchpoint region showed a strong decrease in splicing efficiency. We conclude that BBP/ScSF1 may be required only for the splicing of introns with weak splice sites or otherwise impaired splicing efficiency. This observation was not specific for the RP51A intron mutants as it was also observed for a weakly spliced synthetic intron (see above, Legrain and Rosbash, 1989). Because weak introns are not spliced *in vitro*, we could not test whether this phenotype could be reproduced *in vitro*.

2.4. Pre-mRNA leakage to the cytoplasm

A few splicing mutants have been reported to affect pre-mRNA retention in the nucleus. In contrast to *msl5*, these mutants (e.g. prp6, prp9) also showed very pronounced splicing defects detectable *in vitro* and *in vivo* (Legrain and Rosbash, 1989; Abovich *et al*., 1990). Analysis of the *msl5* mutants for their ability to retain unspliced pre-mRNA in the nucleus showed that all 12 mutants allowed leakage of unspliced pre-mRNA to the cytoplasm. Furthermore, the strength of this effect correlated well with the severity of the growth

defect of the specific mutant. It is important to note that all 12 mutants qualitatively showed the same *in vivo* and *in vitro* phenotype regarding splicing and pre-mRNA retention (with the exception of the cs mutant that differed in its block of (pre)-spliceosome formation). Sequencing of several mutants (Figure 28) and the mapping of relevant mutations in two mutants (Figure 29) demonstrated that this was not due to identical point mutations present in these mutants. The phenotype for *msl5* reported here is therefore not allele specific, but appears to reflect the true function of the gene. Overall these data suggest that the increased leakage of pre-mRNA to the cytoplasm accounts, at least partially, for the essential phenotype of *MSL5*.

The parallel analysis of splicing and pre-mRNA leakage in an isogenic strain lacking the *MUD2* gene showed that the effect of this non-essential gene is much less pronounced than for the *msl5* mutants. This suggests that the difference in essentiality between *MSL5* and *MUD2* could be related to the quantitatively different effects on nuclear pre-mRNA retention and splicing.

2.5. Synthetic lethality of *msl5* in combination with a disruption of the NMD pathway

The importance of BBP/ScSF1 in preventing pre-mRNA leakage is further strengthened by our finding of a synthetic lethal phenotype between three *msl5* mutants and a disruption of the NMD pathway. The close correlation between the severity of the pre-mRNA retention defect of the different *msl5* mutants with the strength of the synthetic lethal phenotype underlined the importance of pre-mRNA retention for viability. This suggests that the leakage of pre-mRNA in the *msl5* mutants causes a lethal phenotype if the cellular control mechanism for these aberrant messages is disrupted.

It is difficult to imagine that an elaborate control mechanism like NMD would have evolved only to prevent the accumulation of aberrant messages from very rare genomic mutation events. Given the importance of splicing in yeast, where about every third message contains an intron, many of which are located in essential genes (Lopez and Séraphin, 1999), it seems likely that the NMD pathway has evolved to prevent the accumulation of pre-mRNA in the cytoplasm. This idea was first suggested by experiments showing accumulation of the inefficiently spliced CYH2 pre-mRNA in *upf1* null mutants (He *et al*., 1993) and was further confirmed by an analysis of the spatial distribution of pre-mRNAs and their degradation by the NMD pathway in yeast (Long *et al*., 1995). Additional support for this hypothesis comes from our analysis of *in vivo* splicing of the *YRA1* intron in a BBP depleted strain. We observed a strong decrease in the levels of pre-mRNA, while the mRNA

levels were not significantly changed (Figure 23). This phenotype could be explained with the increased leakage of pre-mRNA to the cytoplasm in a BBP depleted strain. The cytoplasmic pre-mRNA would be subjected to the NMD pathway and therefore an overall decrease in the pre-mRNA levels would be detected.

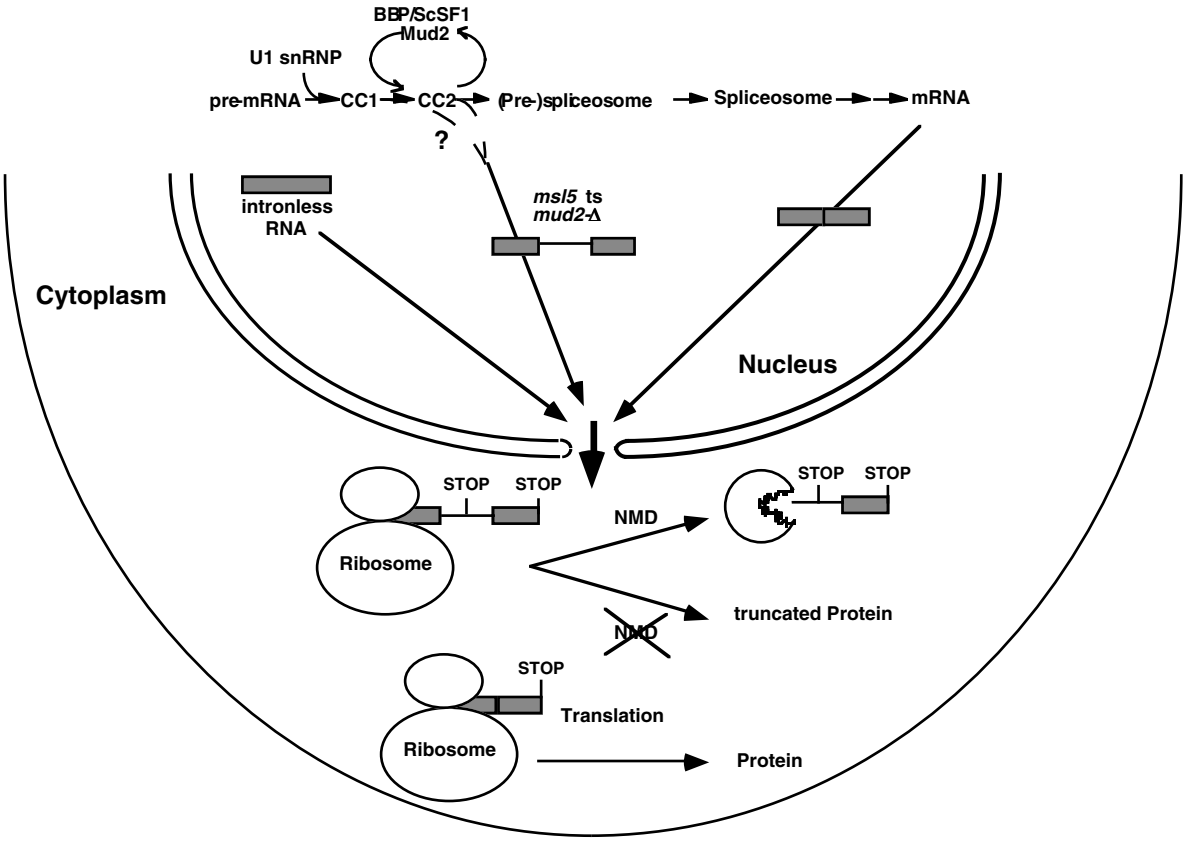

Figure 44. Model for a role of BBP/SF1 in nuclear pre-mRNA retention and splicing

Two major classes of mRNA are exported from the nucleus to the cytoplasm: intronless mRNAs and spliced mRNAs. Pre-mRNAs or partially spliced mRNA species are retained in the nucleus until the splicing process is completed. If *msl5*, the gene coding for BBP/ScSF1, is mutated pre-mRNAs can escape to the cytoplasm before being spliced. Because BBP/ScSF1 is involved in CC2 formation and plays a kinetic role for the assembly of pre-spliceosomes this is likely to occur before or after the formation of CC2. Pre-mRNAs that appear in the cytoplasm are subject to nonsense-mediated decay (NMD) if they contain in frame stop codons in the intron. If the NMD pathway is disrupted cytosolic pre-mRNAs can accumulate and be translated into truncated and potentially toxic proteins. Although under wild type conditions the NMD pathway is not essential, this pathway becomes limiting for yeast growth when *MSL5* is mutated.

3. Outlook

In this study we functionally characterized the BBP/ScSF1 protein in yeast. This analysis helped to better understand the early steps of spliceosome assembly, but also generated new insights in the mechanism of nuclear pre-mRNA retention and its connection with the nonsense-mediated decay pathway.

Using biochemical methods we investigated the interaction of BBP/ScSF1 with splicing complexes. This revealed that BBP/ScSF1 together with Mud2p is present in CC2, but not detectable in CC1 and the pre-spliceosome. Depletion of BBP/ScSF1 to more than 99 % showed no significant effect on mature spliceosome formation and *in vitro* splicing, although formation of CC2 was blocked. This led us to propose a recycling mechanism that would allow catalytic amounts of BBP/ScSF1 to promote spliceosome assembly.

We generated and analyzed a battery of conditional mutants for BBP/ScSF1. We could show *in vivo* that BBP/ScSF1 is a splicing factor that affects splicing of introns with mutated splice sites, but has only a minor effect on the splicing of introns with consensus splice sites. This could indicate a more specialized function for SF1 in splicing. To analyze defects in splicing in *msl5* mutant strains, DNA arrays displaying all yeast introns could be a valuable tool. In higher eukaryotes, it will be interesting to learn if the different isoforms of SF1 are specific for different introns and if they are used in a tissue or development specific manner.

Although a model has been proposed for the bridging of introns by interaction of BBP/ScSF1 with the U1 snRNP (Abovich and Rosbash, 1997), conclusive evidence for this is lacking. So far no specific interaction of BBP/ScSF1 with the U1 snRNP has been shown to be required for spliceosome formation and interaction studies using recombinant proteins or the yeast two-hybrid system differed in their results (Prp40p versus Prp39p as interaction partners; Abovich and Rosbash, 1997; Fromont-Racine *et al*., 1997). For mammalian cells the data is even more difficult to interpret, since two proteins that are not components of the U1 snRNP (FBP11 and FBP21; Bedford *et al*., 1998) have been implicated together with the U1C protein in bridging. In addition, a bridging model involving SR proteins (Wu and Maniatis, 1993) has been proposed earlier. The question how the crosstalk betwen 5' and 3' splice site is taking place during spliceosome formation remains to be answered.

Another important point that is still obscured is the replacement of BBP/SF1 by the U2 snRNP at the branchpoint. What are the interactions leading to this substitution? Are there energy dependent steps involved as suggested by the requirement of ATP and the involvement of two ATPases at this step?

So far SF1 has not been implicated in the splicing of U12 dependent introns. However, the consensus branchpoint sequence of these introns varies only in one position from the vertebrate consensus sequence (UUAAC instead of CURAY; the branchpoint is underlined). This position seems not to be crucial for binding of mBBP/SF1 *in vitro* (Berglund *et al*., 1997). Moreover, the U2 snRNP SF3b complex (consisting of four subunits) is also part of the U11/U12 di-snRNP (Will *et al*., 1999). Since this complex functions during the recruitment of U2 snRNP to the branchpoint, this could suggest that also mBBP/SF1 is shared by both classes of spliceosomes. U11/U12 snRNP exists as a pre-formed di-snRNP and seems to bind to the intron in this form, therefore it is also possible that a BBP/SF1 like activity is not required in the minor class of spliceosomes. Further studies using for example crosslinking methods should answer this question.

Another variation of the "classical" splicing theme is the *trans*-splicing mechanism found in trypanosomes and nematodes. The branchpoint region of trypanosomal introns is quite divergent from the *cis*-splicing consensus sequence in yeast or vertebrates and base pairing with U2 snRNA has been proposed, but has not been demonstrated yet (Mottram *et al*., 1989; Patzelt *et al*., 1989). A putative homologue of SF1 has not been found so far in trypanosomes. The question how the branchpoint is recognized in trypanosomes (in addition to U2 snRNA binding) remains to be elucidated.

Given the existing reports about functions of SF1 other than splicing (e.g. transcription repression, Zhang and Childs, 1998, and macrophage inherent functions, Wrehlke *et al*., 1999) it seems possible that at least in higher eukaryotes SF1 is implicated in additional processes. To get further insight, it will be necessary to perform functional assays, analyze the genome wide effects of mutations in SF1 on gene expression and study the localization of SF1 in the cell and inside the nucleus.

Interestingly, all BBP/ScSF1 mutants analyzed were defective in pre-mRNA retention in the nucleus. We could further strengthen this point by the finding of a synthetic phenotype of three different *msl5* mutants with a disruption of the NMD pathway. This indicates an important role for SF1 in the decision if a given pre-mRNA is exported to the cytoplasm or spliced in the nucleus. Also for this function of SF1 the different isoforms of the protein in higher eukaryotes could confer substrate specificity. Our results do not allow us to conclude how splicing and pre-mRNA retention are linked. It is possible that these two aspects of pre-mRNA metabolism are two faces of a single process with a kinetic advantage for the splicing process preventing leakage of most pre-mRNA. Alternatively, two separate processes could exist implicating the presence of a dedicated "retention machinery". Further experiments will be required to understand the integration of pre-mRNA splicing and pre-mRNA retention in eukaryotic nuclei.