RESULTS

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

1.1. BBP is present in CC2, but not detectable in CC1

To follow the fate of BBP during spliceosome assembly we constructed a yeast strain expressing BBP tagged at its C-terminus with two IgG-binding domains of the *Staphylococcus aureus* Protein A. This construct was integrated in the genome to maintain the expression of the BBP-ProtA protein at its normal level (Puig *et al*., 1998). Splicing extracts were prepared from this BBP tagged strain, a strain carrying Mud2p tagged with protein A at its C-terminus (Mud2-ProtA, gift of M. Luukkonen) and, as a control, the isogenic wild type strain. These extracts were used in commitment complex assembly reactions (ATP had been depleted to prevent further spliceosome assembly). A pre-mRNA lacking the branchpoint region (ΔBP) was used to selectively accumulate CC1 while the wild type pre-mRNA (WT) was used to generate mainly CC2 (Séraphin and Rosbash, 1991). Following the incubation, rabbit IgG or buffer was added and the complexes formed were resolved in a non-denaturing gel. The two commitment complexes accumulated to similar levels in all three extracts (Figure 8, lanes 1, 2, 4, 6 and 8 and data not shown). However, while the addition of IgG had no effect on the mobility of CC2 formed in wild type extract (Figure 8, lane 1), it led to a mobility shift when the extract contained either BBP-ProtA or Mud2-ProtA (Figure 8, compare lanes 4, 8 and lanes 5, 9). In contrast, addition of IgG to the same extracts did not alter the mobility of CC1 assembled on the mutant pre-mRNA (Figure 8, lanes 2, 6 and 3, 7). These results confirm that Mud2p is present in CC2 but not detectable in CC1 (see also Abovich *et al*., 1994). More importantly, they indicate that BBP is a bona fide component of CC2 and suggest that it is not present in CC1.
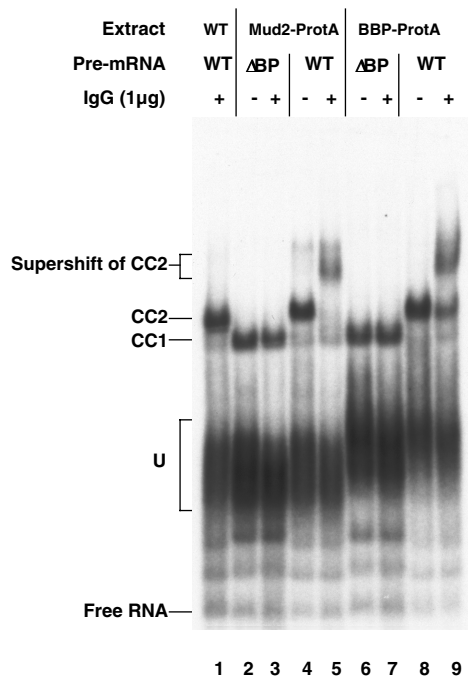
Extract WT | Mud2-ProtA | BBP-ProtA

Pre-mRNA WT | ΔBP | WT | ΔBP | WT

IgG (1µg) + | - + | - + | - + | - +

Supershift of CC2—

CC2—
CC1—

U

Free RNA—

1 2 3 4 5 6 7 8 9

Figure 8. BBP and Mud2p are present in CC2, but not detectable in CC1

Supershift experiments were performed with extracts from a wild type strain (lane 1) or strains carrying either BBP or Mud2p tagged with protein A (lanes 2-9). Extracts depleted for ATP were incubated with labeled wild type pre-mRNA (WT) or a pre-mRNA lacking the branchpoint region (ΔBP). After formation of complexes, rabbit IgG (lanes 1, 3, 5, 7, 9) or buffer (lanes 2, 4, 6, 8) were added to the reactions which were then stopped and analyzed in a non-denaturing gel. Positions of the two commitment complexes (CC1, CC2), the IgG-supershift of CC2, free RNA and unspecific complexes (U) are indicated on the left. Note that the shift of BBP-ProtA containing CC2 was reproducibly not complete, probably due to partial inaccessibility of the tag (lane 9).

1.2. Co-precipitation of BBP/ScSF1 with CC2 and not with CC1

To further analyze the presence of BBP in CC1 and CC2, a specific selection method for these complexes was developed (Figure 9). Biotinylated pre-mRNA was generated by *in vitro* transcription in the presence of biotinylated UTP (bio-UTP, see Materials and Methods). The plasmids encoding the different pre-mRNAs were cut with an restriction enzyme (*Pvu*II) so that about 300 bp of template were present after the intron. This should ensure that statistically most pre-mRNAs were biotinylated in the second exon to prevent effects of the biotinylation on the efficiency of splicing complex assembly. The amount of bio-UTP was titrated to incorporate the lowest amount of bio-UTP that still allowed for the precipitation of more than 95 % of the RNA on streptavidine-agarose beads (as measured by liquid scintillation counting, data not shown). Splicing complexes were assembled with extracts from a strain that contained BBP tagged at its C-terminus with ProtA (BSY576) to enable the detection of the co-precipitated protein. The pre-mRNAs used for assembly of splicing complexes contained the wild type intron (WT, pBS195), the same intron without the branchpoint region (ΔBP, pBS199) or a 5' splice site mutation together with a deleted branchpoint region (mt5'SS+ΔBP, pBS345). While the wild type pre-mRNA can assemble CC2 and, in the presence of ATP, also pre-spliceosomes and spliceosomes, the pre-mRNA lacking the branchpoint region is only capable to form CC1. The pre-mRNA containing in addition a mutation in the 5' splice site was used as a control

because it does not assemble any specific complexes. The extract was incubated with the different pre-mRNAs in the absence or presence of ATP (only for the wild type pre-mRNA) under splicing conditions. Afterwards, the reaction was incubated with streptavidin coated beads and input, supernatant and pellet were analyzed by western blotting for their protein content and by primer extension for the presence of U snRNAs.
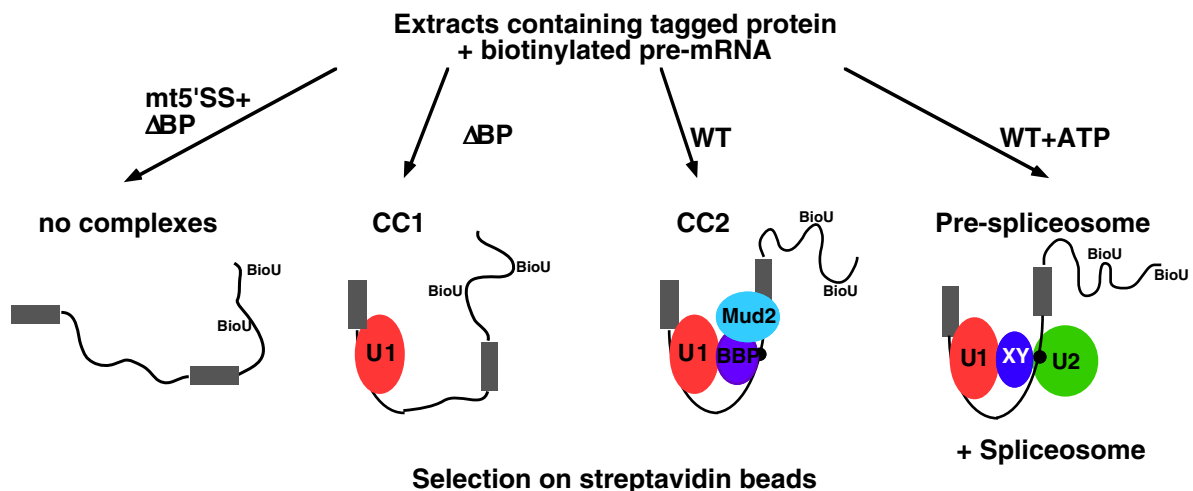

Figure 9. Selection of commitment complexes with biotinylated pre-mRNA

Complexes are assembled in the absence or presence of ATP on biotinylated pre-mRNA containing a mutated 5'SS and a deleted branchpoint region (mt5'SS+ΔBP), only a deletion of the branchpoint region (ΔBP) or no mutation (WT). SnRNPs are depicted as coloured spheres with the name of the snRNP (U1, U2). The proteins Mud2, BBP and as yet unidentified factors (XY) are also depicted as coloured spheres. Exons are shown as grey boxes, introns are shown as black lines.

Both, the wild type and the pre-mRNA lacking the branchpoint, precipitated U1 snRNA (Figure 10A, lanes 10, 11). This indicated that CC1 had assembled in the latter case and CC1 and CC2 had assembled on the wild type pre-mRNA. In contrast a pre-mRNA containing a 5' splice site mutation and a deletion of the branchpoint did not precipitate U1 snRNA showing that the precipitation was specific for commitment complexes (Figure 10A, lane 9). The other four U snRNAs were only detectable in the presence of ATP (Figure 10A, lane 12) indicating the co-precipitation of pre-spliceosome and spliceosome. However, in this pellet U2 snRNA was more abundant than the U4, U5 and U6 snRNAs indicating a high proportion of pre-spliceosome in this fraction. BBP was only co-precipitated in the reaction containing the wild type pre-mRNA (Figure 10B, lanes 11, 12) and not with complexes assembled on a pre-mRNA lacking the branchpoint region or being mutated in the 5' splice site and lacking the branchpoint region (Figure 10B, lane 9, 10). This data strongly suggests that BBP is absent from CC1. We conclude that BBP joins the

commitment complex during the transition from CC1 to CC2. Moreover, in the reaction containing ATP and showing co-precipitiation of U2, U4, U5 and U6 snRNA the level of co-precipitated BBP was significantly reduced (Figure 10B, lane 12). This suggests that BBP was absent from or less stable associated with pre-spliceosome and spliceosome.
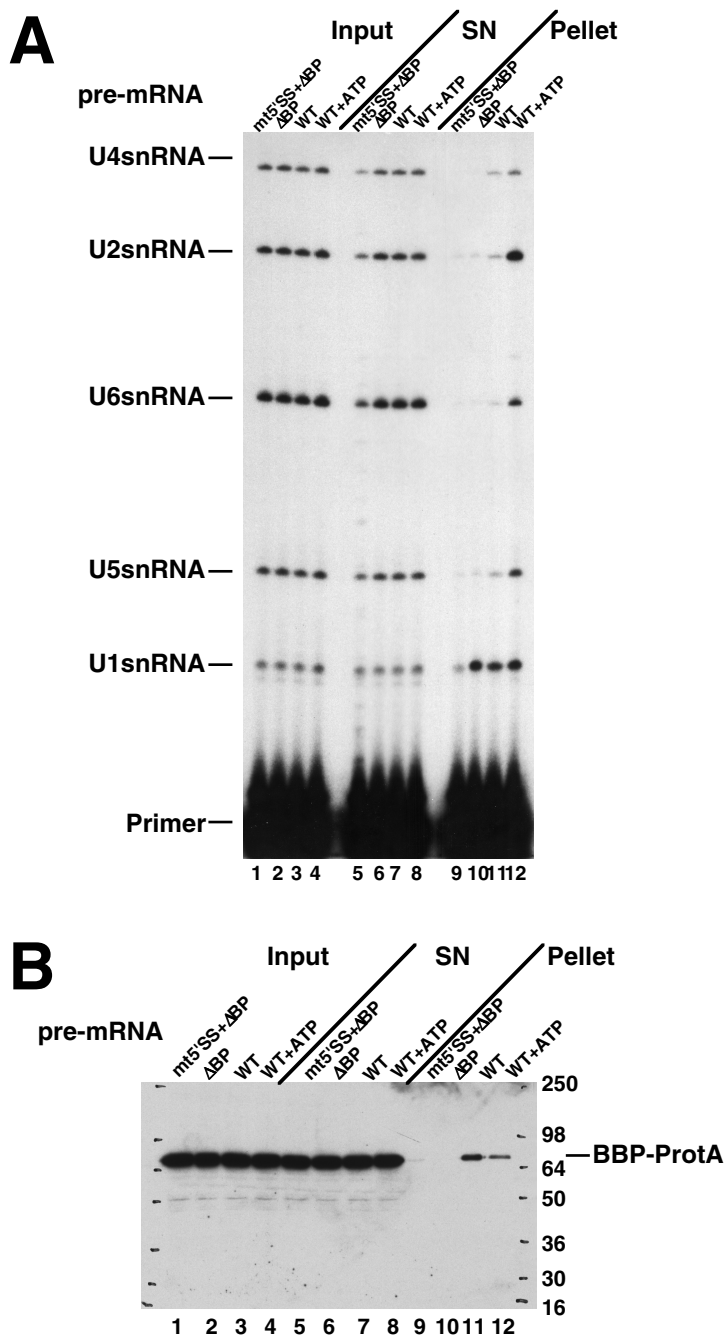

Figure 10. BBP/ScSF1 is co-precipitated with CC2

A: Primer extension analysis of U snRNAs in precipitated splicing complexes.

Biotinylated pre-mRNA containing a mutant 5' splice site and a deleted branchpoint (mt5'SS+ΔBP), only a deleted branchpoint (ΔBP) or no mutation (WT) were incubated with extract containing BBP-ProtA. All reactions were performed in the absence of ATP unless otherwise indicated (WT+ATP). After the selection on streptavidin beads RNA from input, supernatants (SN) and pellets were extracted and analyzed by primer extension for the presence of spliceosomal U snRNAs. The positions of the different U snRNAs are indicated on the left.

B: Western blot analysis of BBP-ProtA in precipitated splicing complexes.

Aliquots of the reactions in panel A were analyzed by SDS-PAGE, transferred to a membrane and developed with PAP. The molecular weight marker and the position of BBP-ProtA are indicated on the right.

1.3. BBP is required for the formation of CC2

We next analyzed the requirement of BBP for CC2 formation. For this purpose, we constructed a strain containing the single chromosomal copy of the gene coding for BBP under the control of a *GAL10* promoter. In this strain, a protein A tag was fused to the N-terminus of the BBP protein (ProtA-BBP). Twenty hours following the transfer of this strain to glucose containing media (repressing conditions) its growth rate decreased compared to a wild-type control (Figure 11). This suggested that the ProtA-BBP level becomes limiting for growth in these cells, consistent with the essential nature of the gene encoding BBP (Abovich and Rosbash, 1997).
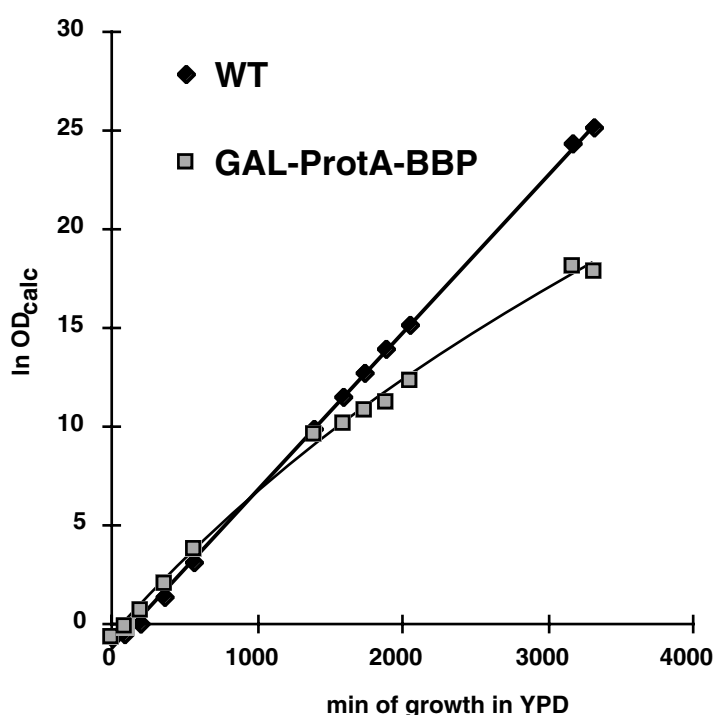

Figure 11. Growth curve of BBP depletion

The GAL-ProtA-BBP strain and an isogenic wild type strain (WT) were grown in glucose containing medium for more than 50 hours. The cultures were diluted several times with fresh medium to keep growth in the logarithmic phase. The OD_{600} of the cells was measured and a cumulative OD_{calc} was calculated (see Materials and Methods). The natural logarithm of this calculated OD_{calc} was plotted against the time of growth.

Western blot analysis indicated that the level of ProtA-BBP was reduced to less than 1 % of its starting level after 21 hours of depletion (Figure 20). This strain was used to prepare ProtA-BBP depleted extracts. Equal numbers of cells were harvested at two time points after glucose addition (17 and 21 hours) and extracts were prepared. These extracts were used in splicing complex assembly reactions followed by analysis in a non-denaturing gel. In the reactions containing wild type extracts in the absence of ATP (Figure 12, lanes 1, 5) both commitment complexes (CC1 and CC2) were detected. In contrast the reaction containing extract depleted for BBP showed reproducibly only trace levels of CC2 and accumulation of CC1 at 17 hours growth in glucose (Figure 12, lane 3). After 21 hours

growth in glucose CC2 was no longer detectable while CC1 clearly accumulated (Figure 12, lane 7). The formation of CC1 indicated that the extracts were active in complex assembly and not unspecifically inactivated. This conclusion was also supported by efficient (pre)-spliceosome formation and splicing in these extracts in the presence of ATP (Figure 12, lanes 2, 4, 6, 8 and Figure 20). The biochemical depletion of BBP-ProtA from extracts using IgG-beads also resulted in the absence of CC2 (data not shown). Taken together the results of these depletion experiments indicate that BBP is specifically required for the formation of CC2, but not of CC1.
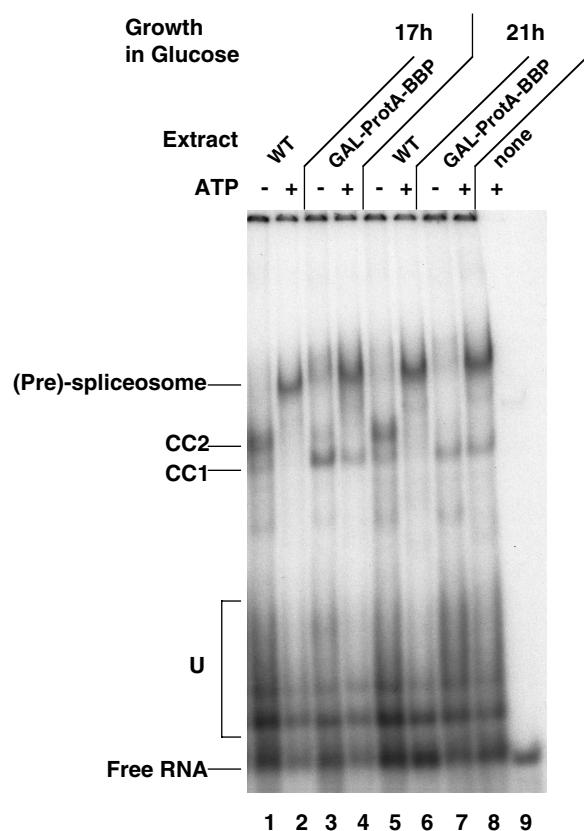

Figure 12. **BBP is required for the formation of CC2**

Extracts from a wild type strain (WT, lanes 1, 2, 5, 6) or a GAL-ProtA-BBP strain (lanes 3, 4, 7, 8) grown for 17 (lanes 1-4) or 21 hours (lanes 5-8) in glucose medium were analyzed for splicing complex formation with or without additional ATP using labeled wild type pre-mRNA. Samples of the reactions were fractionated in a non-denaturing gel. A reaction containing no extract was analyzed in parallel (lane 9). Positions of commitment complexes (CC1, CC2), (pre)-spliceosomes, unspecific complexes (U) and the free pre-mRNA are indicated on the left. Note that residual levels of ATP in the extracts led to some formation of (pre)-spliceosomes even when no ATP was added (lanes 1, 3, 7). Note that spliceosomes and pre-spliceosomes are not separated in the native gel used for commitment complex analysis. Therefore these complexes are referred to as (pre)-spliceosomes.

1.4. CC1 is a functional precursor of CC2 and (pre)-spliceosome

The BBP/ScSF1 depleted extracts were specifically blocked after CC1 formation. This feature was used to investigate the relationship between CC1, CC2 and (pre)-spliceosome in more detail. We analyzed if CC1 was a true precursor of CC2 by performing chase experiments with excess of unlabeled pre-mRNA. Extracts depleted for BBP-ProtA were used to assemble CC1 on labeled pre-mRNA. This reaction was then incubated with excess of unlabeled pre-mRNA and extract that had been metabolically depleted for U2 snRNA.

This should ensure that any CC2 formed in this reaction could not further advance to form pre-spliceosomes, because the latter require U2 snRNA for their formation (Séraphin and Rosbash, 1989). Finally a wild type extract was added to the reaction together with ATP to drive the formation of (pre)-spliceosomes. Each step of the reaction was analyzed by removing an aliquot from the reaction which was migrated in a native gel (Figure 13).
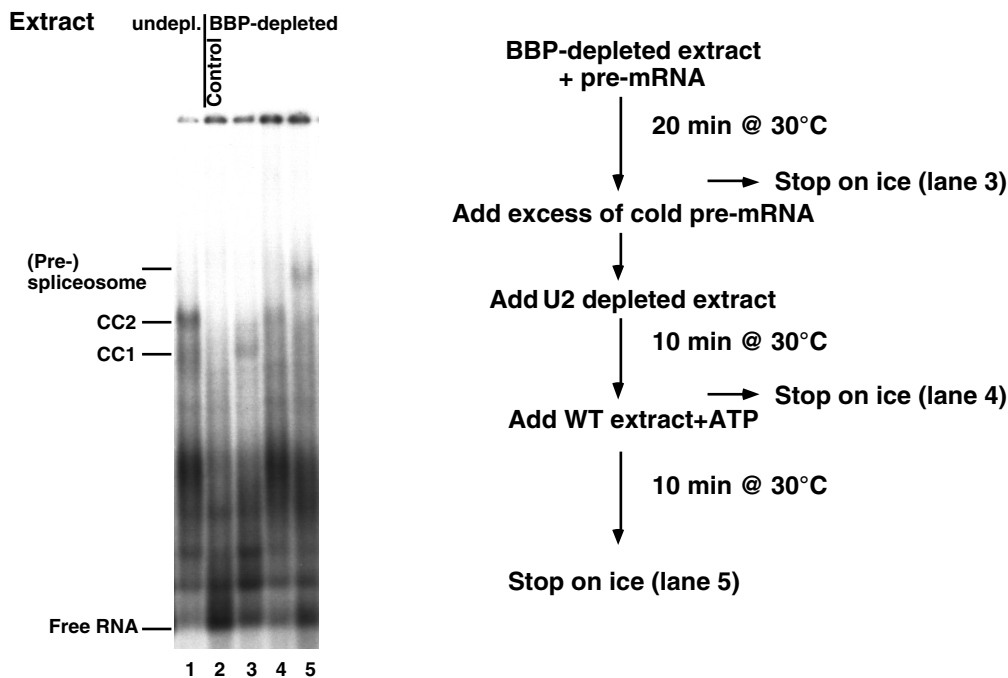

Figure 13. Chase of CC1 into CC2 and (pre)-spliceosomes

Extracts depleted for BBP-ProtA with IgG-beads were incubated with labeled pre-mRNA for 20 min at 30°C. After removal of an aliquot for native gel analysis (lane 3), a 100-fold excess of unlabeled pre-mRNA was added and then an equal amount of an extract genetically depleted for U2 snRNA was added. The reaction was continued for 10 min at 30°C. Again an aliquot was removed (lane 4) and an extract from a wild type strain was added to the remaining reaction together with ATP. After 10 min of incubation at 30°C the reaction was stopped on ice (lane 5). Equal relative amounts of all steps of the reaction were analyzed in a native gel. As controls, a reaction with undepleted extract in the absence of ATP was performed (lane 1) and a reaction where excess of unlabeled pre-mRNA was added at the beginning of the reaction (lane 2).

We observed that CC1 was chased nearly quantitatively into CC2 after the addition of a 100-fold excess of unlabeled pre-mRNA together with an U2 snRNA depleted extract. In contrast, if the excess of unlabeled pre-mRNA was added from the beginning of the reaction no complexes were detectable (lane 2). After addition of the wild type extract together with ATP, CC2 was chased nearly quantitatively into pre-spliceosomes and spliceosomes. This shows that CC1 is a functional precursor of CC2 and that pre-mRNA bound in CC1 is committed to the splicing pathway. Furthermore it demonstrates that the block of CC2

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formation induced by depletion of BBP/ScSF1 can be reversed by the addition of extract containing BBP/ScSF1.

1.5. BBP is not detectable in pre-spliceosomes and spliceosomes

Since BBP and Mud2p were shown to be present in CC2 (see 1.1.;1.2. and Abovich *et al.*, 1994), we next asked if they were also present in pre-spliceosome and spliceosome or whether they were released upon addition of U2 snRNP. To address this question, we first performed a mobility shift experiment. Wild type extract or extracts containing either BBP-ProtA or Mud2-ProtA were incubated in a splicing reaction with wild type pre-mRNA (WT) with or without addition of ATP. After complex formation, rabbit IgG or buffer was added to the reactions. The complexes formed were fractionated by non-denaturing gel analysis (Figure 14). In contrast to CC2 which is shifted upon addition of IgG in extracts containing BBP-ProtA or Mud2-ProtA (Figure 14, lane 3, 4 and 7, 8 and Figure 8) pre-spliceosomes and spliceosomes (which comigrate in this type of gel, Séraphin & Rosbash, 1989, and are therefore indicated as (pre)-spliceosome) are not shifted upon addition of IgG (Figure 14, lanes 5, 6 and lanes 9, 10). The same experiment showed a major shift of (pre)-spliceosomes when protein A-tagged SmB or Lea1, a component of U2 snRNP, were present in the extract (data not shown and F. Caspary pers. comm.). This suggests that BBP and Mud2p are not present in pre-spliceosome and/or spliceosome. However, we could not rule out that the protein A tag is not accessible in these complexes.
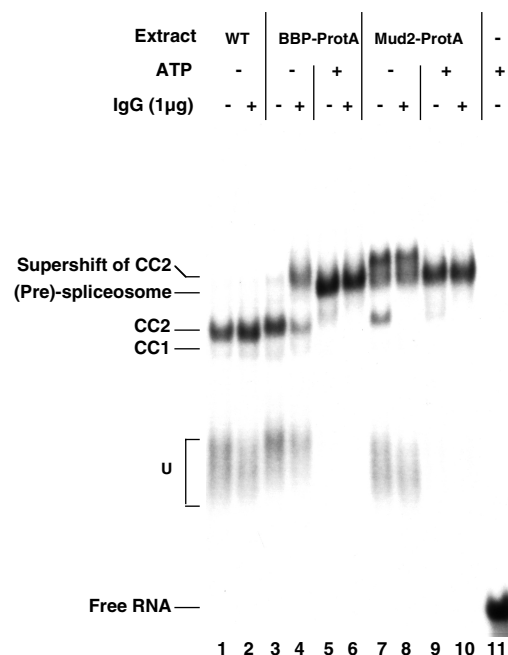

Figure 14. **(Pre)-spliceosomes are not supershifted with BBP-ProtA**

Splicing complexes were assembled with (lanes 5, 6, 9, 10) or without additional ATP (lanes 1-4, 7, 8) using extracts from either wild type (WT, lanes 1, 2), BBP-ProtA (lanes 3-6) or Mud2-ProtA strains (lanes 7-10) and labeled wild type pre-mRNA. After formation of complexes, either rabbit IgG or buffer were added to the reactions. Reactions were stopped and analyzed in a non-denaturing gel. As a control a reaction containing no extract was analyzed in parallel (lane 11). Positions of the two commitment complexes (CC1, CC2), (pre)-spliceosome, the IgG-supershift of CC2, free RNA and unspecific complexes (U) are indicated on the left. Note that the Mud2-ProtA extract contained residual levels of ATP leading to the formation of (pre)-spliceosomes without addition of ATP (lane 8).

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To analyze the presence of BBP and Mud2p in the pre-spliceosome more directly, we designed a specific selection strategy for this complex (Figure 15). First, to prevent assembly of complexes following pre-spliceosome, extracts were incubated with a DNA oligonucleotide complementary to U6 snRNA. Together with the RNase H activity present in yeast extracts, this specifically degrades the U6 snRNA that is required for the formation of mature spliceosomes (Fabrizio *et al.*, 1989). In these U6 depleted extracts, the only U2 snRNP containing splicing complex will be the pre-spliceosome, as the other early splicing complexes present, namely commitment complexes, do not contain the U2 snRNP (Séraphin and Rosbash, 1989; Séraphin and Rosbash, 1991). To recover only pre-spliceosomes, we specifically co-precipitated all U2 snRNP containing complexes present in U6 depleted extracts using tagged Lea1, the yeast homologue of human U2A' protein (Caspary and Séraphin, 1998).
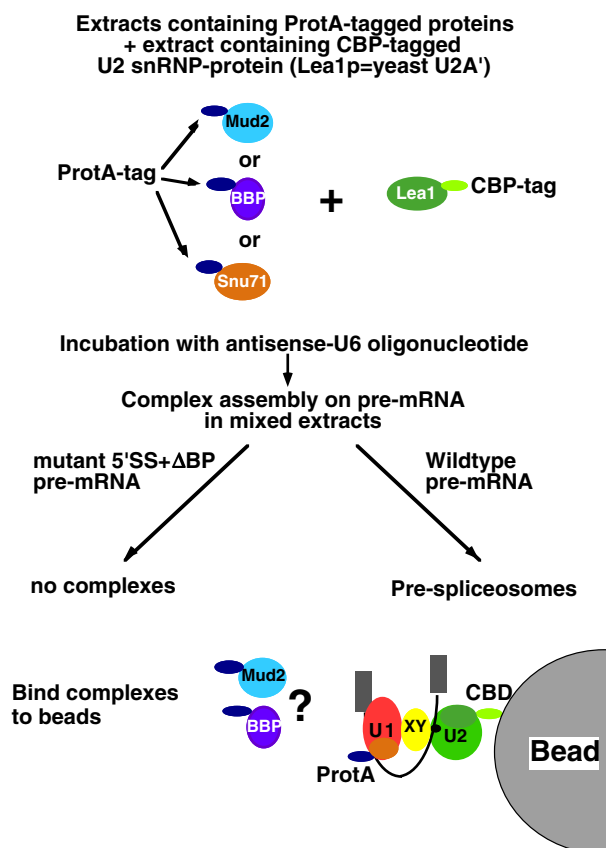

Figure 15. Pre-spliceosome selection with Lea1-CBP

Proteins and snRNPs are depicted as coloured spheres with their names. The ProtA-tag is indicated by a black ellipse, while the CBP-tag is indicated by a light green ellipse. Exons are shown as grey boxes, the intron is shown as a black line.

Extract containing both Lea1 tagged at the C-terminus with a CBP-tag (Calmodulin Binding Peptide, Stratagene) and the core snRNP protein SmB tagged at the C-terminus with a protein A tag (gift of F. Caspary) was mixed with an extract containing either BBP-ProtA,

Mud2-ProtA or, as a control, Snu71-ProtA (a component of the U1 snRNP, Gottschalk et al., 1998, gift of O. Puig). The tagged SmB protein served as a control for the precipitation of snRNP containing complexes. The U6 complementary oligonucleotide was added and the extract mixes were incubated to allow U6 snRNA degradation. Primer extension analysis of the levels of U6 snRNA before and after treatment indicated that it was quantitatively degraded while the other spliceosomal snRNAs were not affected (Figure 16A, lanes 1-6). A wild type (WT) or a mutant pre-mRNA lacking the branchpoint region and carrying a mutation at the 5' splice site (5'SS+ΔBP) were added to these extracts followed by incubation to allow for the assembly of splicing complexes. Splicing complexes can not assemble on the latter pre-mRNA, which served as a control for unspecific binding of RNA and proteins. Lea1-CBP associated complexes were selectively precipitated with calmodulin-agarose beads and specifically eluted from the beads with EGTA. RNA extracted from aliquots of the input extract mixtures before and after incubation with the oligonucleotide, supernatants and eluates were analyzed by primer extension for the presence of spliceosomal U snRNAs (Figure 16A). U2 snRNA was present at similar levels in all eluate fractions (Figure 16A, lanes 13-18) showing the specific precipitation of U2 snRNP containing complexes (including the free U2 snRNP) with the Lea1-CBP fusion. U1 snRNA was specifically recovered in the reactions containing the wild type pre-mRNA, but not the mutant pre-mRNA (Figure 16A, compare lanes 13, 15, 17 with lanes 14, 16, 18 respectively). This indicates the presence of pre-spliceosomes containing the U1 and U2 snRNPs associated on a pre-mRNA. Indeed, although original studies suggested that U1 snRNP was absent from pre-splicesosomes resolved by native gel electrophoresis (Konarska & Sharp, 1986; Pikielny *et al.*, 1986; Cheng and Abelson, 1987), use of biotin-avidin affinity chromatography to isolate splicing complexes (Bindereif and Green, 1987) and the development of new gel conditions demonstrated that U1 snRNP was present in this splicing complex (Séraphin and Rosbash, 1989; Colot *et al.*, 1996; Ruby, 1997 and B. Séraphin, pers. communication). This suggests that it had been lost in the original studies because its interaction with the spliceosome is easily disrupted during manipulation.. Our analysis revealed that the U4 and U5 snRNAs were only present at background levels in the selected complexes confirming that no fully assembled spliceosomes were formed in these reactions (Figure 16A, lanes 13-18). The presence of BBP-ProtA, Mud2-ProtA, Snu71-ProtA and SmB-ProtA was analyzed by western blotting and immunodetection of protein A (Figure 16B). Similar levels of SmB-ProtA could be detected in all eluate lanes indicating consistent precipitation (Figure 16B, lanes 4, 5, 9, 10, 14 and 15). The U1 snRNP protein Snu71 was clearly co-precipitated with Lea1, but only in the presence of the wild-type pre-mRNA (Figure 16B, compare lanes 4 and 5). This confirms the result obtained by RNA

analysis (see above) and indicates that our selection conditions did not disrupt weak interactions occurring in the pre-spliceosome. In contrast, BBP and Mud2p were not detected in pre-spliceosomes (Figure 16B, lanes 9, 10 and 14, 15). This can not be attributed to different detection sensitivities for these proteins because all proteins are recognized through the protein A tag. These data support the result of the native gel analysis, namely that BBP and Mud2p are not components of the pre-spliceosome, but rather leave the complex when U2 snRNP is added. However, we cannot rule out that BBP and Mud2p are extremely weakly associated with the pre-spliceosome or present in these complexes with a stochiometry that is much lower than that of the U1 snRNP Snu71 protein.

To demonstrate that the failure to detect BBP in pre-spliceosomes was significant and not a technical artefact, we assayed for its presence in commitment complexes using the same methodology. Extract containing the U1 snRNP protein Snu71 tagged with both CBP and protein A (TAP tag, Rigaut *et al*., 1999) was mixed with extract containing BBP-ProtA. This mixture was depleted of ATP and incubated with the pre-mRNA in the absence of ATP for 30 min at 30°C. Complexes were co-precipitated with calmodulin beads under the conditions described for pre-spliceosomes above. The western blot analysis (Figure 16C) showed that the tagged Snu71 protein is precipitated independent of the pre-mRNA (lanes 4, 5) as expected. A significant amount of BBP-ProtA is precipitated in the presence of wild type pre-mRNA, significantly less protein was detectable with mutant pre-mRNA (compare lanes 4, 5). This background might be attributed to the direct interaction of BBP with the U1 snRNP reported earlier (Abovich and Rosbash, 1997). This showed that even under very stringent conditions for the stability of commitment complexes (300 mM NaCl) BBP-ProtA was specifically co-precipitated in commitment complexes. This result confirms that the failure to detect BBP in pre-spliceosome is significant and indicates therefore that BBP dissociates from splicing complexes during the transition from CC2 to pre-spliceosome.
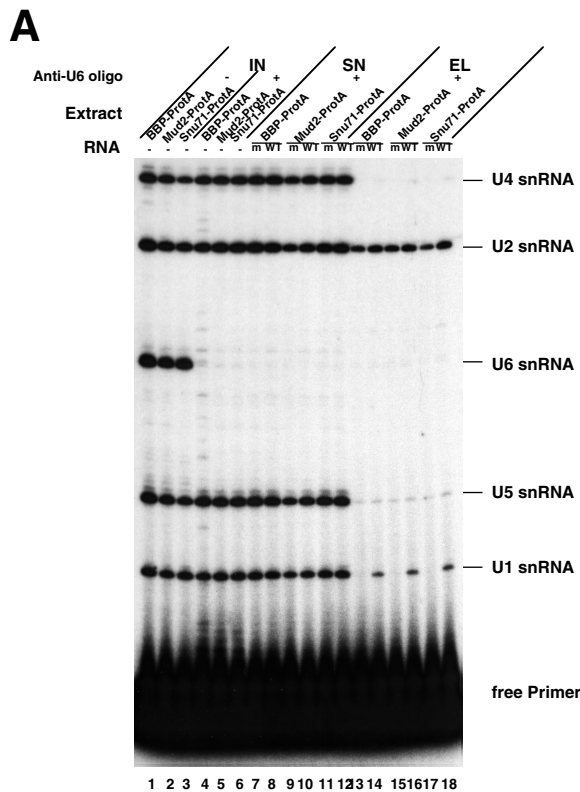
A

B

C

Figure 16. BBP and Mud2p are not detectable in pre-spliceosomes

A: U snRNA content of co-precipitated pre-spliceosomes.

Extract from a strain carrying the U2 snRNP protein Lea1 tagged with CBP (calmodulin binding peptide) and the SmB protein tagged with protein A was mixed to equal parts with either extract from a BBP-ProtA strain, a Mud2-ProtA strain, or a Snu71-ProtA strain. The extract mixture was incubated with an oligonucleotide directed against U6 snRNA. Splicing reactions in the presence of ATP were performed using either wild type pre-mRNA (WT) or mutant pre-mRNA lacking the branchpoint region and carrying a mutation in the 5' splice site (m, see above). Reactions were incubated with calmodulin-agarose beads and RNA was extracted from input before (lanes 1-3) and after (lanes 4-6) oligonucleotide treatment and from supernatant (SN, lanes 7-12) and eluate (EL, lanes 13-18). Spliceosomal U snRNAs present in the fractions were analyzed by primer extension and denaturing gel electrophoresis. Positions of the U snRNAs are indicated on the right. Note that the eluate was loaded 33 times more concentrated than input and supernatant.

B: Western blot analysis of co-precipitated pre-spliceosomes.

Proteins in the input (IN), supernatant (SN) and eluate (EL) of the experiment presented in panel A were analyzed by SDS-PAGE followed by western blotting and immunodetection of protein A. Positions of SmB-ProtA, BBP-ProtA, Snu71-ProtA, Mud2-ProtA are indicated on the left and right. Note that the elution fractions are loaded 67 times more concentrated than input and supernatant.
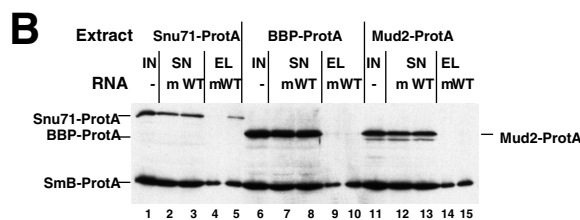
C: Western blot analysis of co-precipitated commitment complexes.

Extract containing Snu71-CBP-ProtA was mixed with extract containing BBP-ProtA. Complexes were formed in the absence of ATP on wild type (WT) or mutant pre-mRNA lacking the branchpoint region and carrying a mutation in the 5' splice site (m, see above). After binding to calmodulin beads input (IN), supernatant (SN) and eluate (EL) fractions were analyzed by SDS-PAGE followed by western blotting and immunodetection of protein A. Positions of BBP-ProtA and Snu71-CBP-ProtA are indicated on the left. Note that the elution fractions are loaded 67 times more concentrated than input and supernatant.

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When the same co-precipitation experiments were performed with extracts that had not been pre-treated with the U6 complementary oligonucleotide, fully assembled spliceosomes were recovered in addition to pre-spliceosomes, as indicated by the presence of all spliceosomal snRNAs in the eluate (Figure 17A, lanes 11, 13, 15). The co-precipitation was specific because in the presence of a mutated pre-mRNA only background levels of the U snRNAs (except for U2) were detectable (lanes 10, 12, 14). BBP and Mud2p could not be detected in the eluate fractions while Snu71 was still specifically co-precipitated (Figure 17B compare lanes 5, 10, 15). This result suggests that BBP and Mud2p are absent from both, pre-spliceosomes and mature spliceosomes.
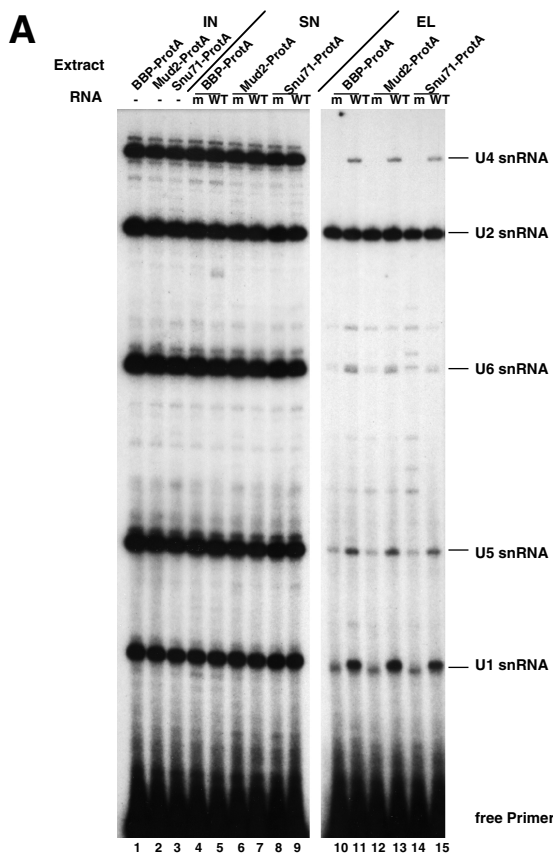

Figure 17. BBP and Mud2p are not detectable in spliceosomes

A: U snRNA content of co-precipitated (pre)-spliceosomes.

The experiment was performed as in Figure 16, but without addition of the oligonucleotide complementary to U6 snRNA. Labeling of the gel is like in Figure 16A.

B: Western blot analysis of co-precipitated (pre)-spliceosomes.

Labeling of the gel is as in Figure 16B.

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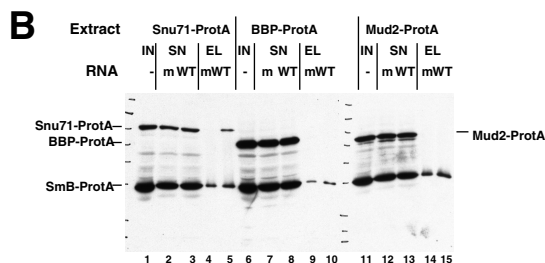
1.6. BBP and Mud2p are associated in a complex

The results described above and observations made by other groups (Abovich *et al*., 1994; Abovich and Rosbash, 1997; Rain and Legrain, 1997) indicated that BBP and Mud2p are involved in the same step of spliceosome assembly, namely the transition from CC1 to CC2, and seem to interact with each other (Abovich and Rosbash, 1997; Fromont-Racine *et al*., 1997; Rain *et al*., 1998). More specifically, BBP and Mud2p were absent from CC1, present in CC2 and absent from pre-spliceosome and spliceosome. The depletion of BBP or a disruption of the *MUD2* gene both led to a block in CC2 formation (see 1.3. and Abovich *et al*., 1994). We therefore asked if both proteins might be associated in a complex. To this aim we made use of a new purification strategy developed in our laboratory (Rigaut *et al*., 1999). This Tandem-Affinty-Purification (TAP) method is based on the fusion of a short affinity tag to the protein of interest. The tag contains the Calmodulin-Binding-Peptide (CBP, Stratagene) connected to two IgG binding domains of protein A from *Staphylococcus aureus* by a few amino acids that constitute the cleavage site for the tobacco etch virus (TEV) protease (Figure 18 and Materials and Methods).
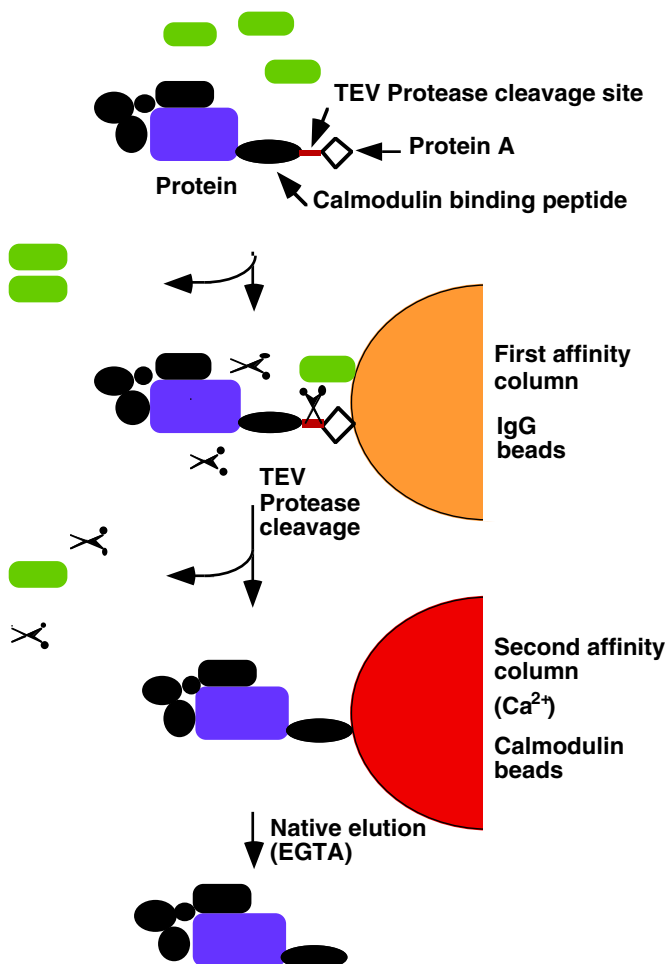

Figure 18. **The TAP method**

Proteins are shown as small objects of different sizes and shapes, beads are indicated as large half-spheres. The TEV-enzyme is depicted as a pair of scissors. See text for description.

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This arrangement allows for a first purification step through the binding of the protein A moiety to IgG coated beads. The bound material is released by the cleavage of the tag by TEV protease. In a second purifcation step the eluted material is incubated with beads coated with calmodulin. The tag can be fused to the C-terminus or N-terminus (F. Caspary, pers. communication) of the protein, either by cloning in an expression vector or by homologous recombination in the genome. For the latter purpose we constructed a plasmid where the TAP tag is present together with a heterologous selectable marker (*Kluyveromyces lactis TRP1*). Using a PCR strategy (Puig *et al.*, 1998) linear fragments containing the tag, the marker and ends homologous to the gene of interest can be genenerated. These DNA fragments are transformed into yeast and integration is selected by the expression of the marker. The C-terminal fusion of the tag ensures that expression remains at its natural level, because the endogenous promoter is not changed and also contributions of the chromosomal location of the gene are maintained.

We constructed a Mud2-TAP and a BBP-TAP strain (both proteins tagged at the C-terminus) and checked their correct expression and size by western blot detection of the ProtA tag (data not shown). Extracts from two liters of culture of each strain were prepared with a French Press (see Materials and Methods) and used for two independent purifications with the TAP method. The fractions obtained after the second purification step (Figure 19, lanes 1-4 and lanes 8-10) were analyzed in a gradient SDS-PAGE together with an aliquot of the TEV enzyme (lane 6) to distinguish protein bands resulting from this preparation. Three main bands could be detected in the BBP-TAP purification by coomassie staining (data not shown) and several additional minor bands were detectable in the subsequent silver staining of the same gel (lane 3). These three bands were analyzed by MALDI-mass spectrometry (Anna Shevchenko and Matthias Wilm, EMBL). Several peptides could be identified for each band leading to the identification of BBP, Mud2p and the gag coat protein of the yeast L-A virus. This showed that BBP/ScSF1 and Mud2p are associated together in a complex. The presence of the virus coat protein could not be explained so far, but could reflect an interaction of this protein that binds covalently to the cap structure of mRNA (Masison *et al.*, 1995) with RNA present in the purification. The purification of Mud2-TAP revealed two major bands (lane 9) which could be identified by western-blot using antibodies against Mud2p or BBP (data not shown). The upper band represented Mud2p while the lower band was BBP (Figure 19A). The migration of the two proteins had shifted compared to the BBP-TAP purification because the remaining part of the tag (CBP) changed the mobility of the tagged protein. This confirmed the result obtained with the BBP-TAP purification.

We conclude that BBP/ScSF1 and Mud2p are present in a binary complex in yeast cell extracts. This indicates that they join CC1 in a preformed complex to form CC2.
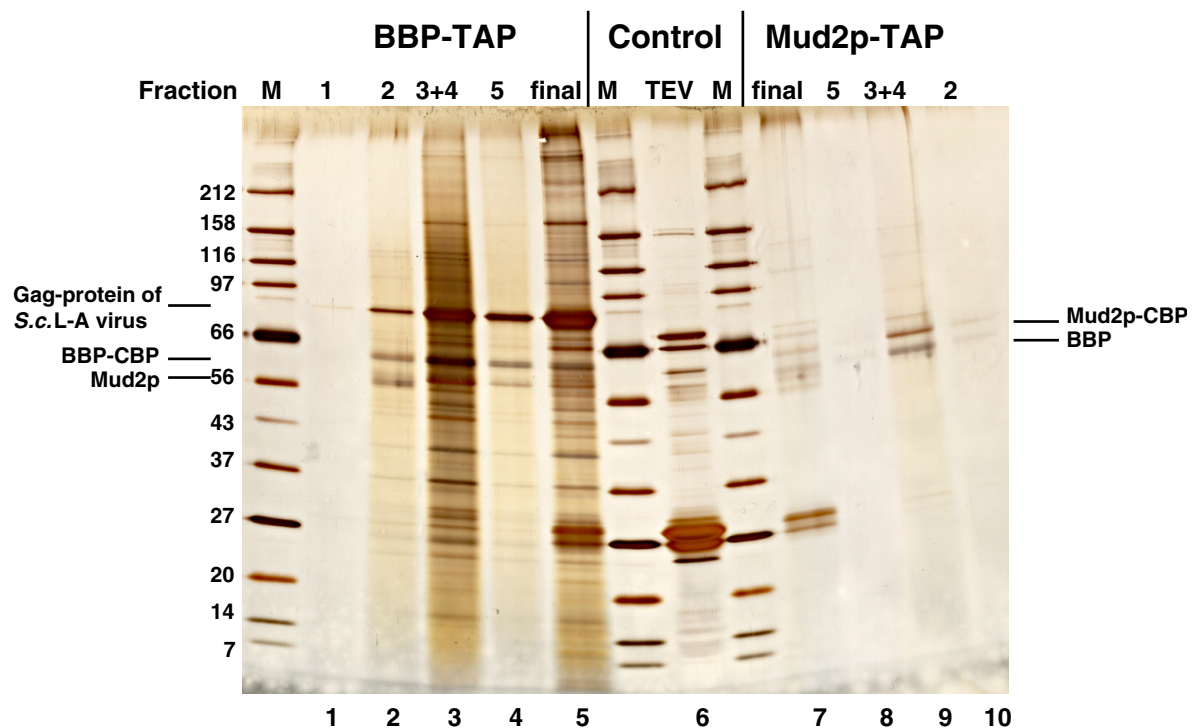

Figure 19. **Purification of a BBP/Mud2p complex**

Proteins associated with BBP-ProtA or Mud2-ProtA were purified with the TAP-method from extracts containing the appropriate tagged protein. Fractions of the CBP elution step were concentrated and analyzed in a 7-25% SDS-gel. As a control an aliquot of the TEV enzyme was loaded (TEV). Protein bands were visualized by silver staining. The positions of the identified proteins are indicated on both sides of the gel.

1.7. Depletion of BBP does not affect pre-spliceosome formation and splicing

We next investigated the effect of BBP depletion on pre-spliceosome formation. The strain carrying the GAL-ProtA-BBP construct and an isogenic wild type strain were grown for up to 30 hours in glucose medium. Cells were removed at several time points to prepare extracts from equal numbers of cells. The levels of ProtA-BBP in these extracts were analyzed by western blot analysis (Figure 20A). This showed that after 30 hours of depletion, less than 1% of the starting ProtA-BBP amount remained in the extract (Figure 20A, compare lane 1-5 and lane 9). However, since ProtA-BBP in this strain might be overexpressed because it is under the control of a *GAL10* promoter, we compared the ProtA-BBP levels under non-repressive conditions to those found in a strain where the BBP-ProtA fusion is expressed from its endogenous promoter. Protein levels in strains

grown to the same density were determined by western blotting. The expression levels of BBP under the control of its endogenous promoter or under the *GAL10* promoter were found to be very similar (Figure 20A, compare lane 10-14 and 15). We conclude that our depleted extract contains less than 1% of the normal BBP level (see also below).

Either the depleted extract or the control wild type extract were mixed with labeled pre-mRNA and splicing buffer, the reactions were incubated and splicing complex formation was analyzed on a native gel (Figure 20B). As observed previously, CC2 accumulated in a reaction containing the wild-type extract in the absence of ATP (Figure 20B, lane 2), while CC1 accumulated under the same conditions in the depleted extract (Figure 20B, lane 4; compare with the mobility of CC1 formed by incubation of a branchpoint mutant pre-mRNA (ΔBP) in a wild-type extract, lane 1). These data confirm that the amount of functional BBP present in the depleted extract was very low. This also rules out the presence of significant levels of undetectable, partially truncated, but nevertheless functional BBP in our depleted extracts. Surprisingly, however, (pre)-spliceosome formation was not affected by the BBP depletion (Figure 20B, compare lanes 3 and 5). Interestingly, a significant level of CC1 was detected in the presence of ATP in the BBP depleted reaction (Figure 20B, lane 5) compared to the control reaction where it is undetectable (Figure 20B, lane 3).

To test if the (pre)-spliceosomes observed in BBP depleted extracts were functional, we analyzed the splicing of the RP51A pre-mRNA in these extracts (Séraphin *et al*., 1988). The two steps of the splicing reaction were monitored by following the level of lariat intermediate and intron lariat as detected in a denaturing gel (Figure 20C). The detected bands appear to be bona fide splicing products because of their specific retarded migration and their dependence on ATP or its analog cordycepin. The wild type and the depleted extract spliced with similar efficiencies (Figure 20C, compare lanes 2, 3 and 4, 5).

Surprisingly, these results show that BBP depletion to more than 99% did not have an effect on spliceosome formation and splicing *in vitro*.
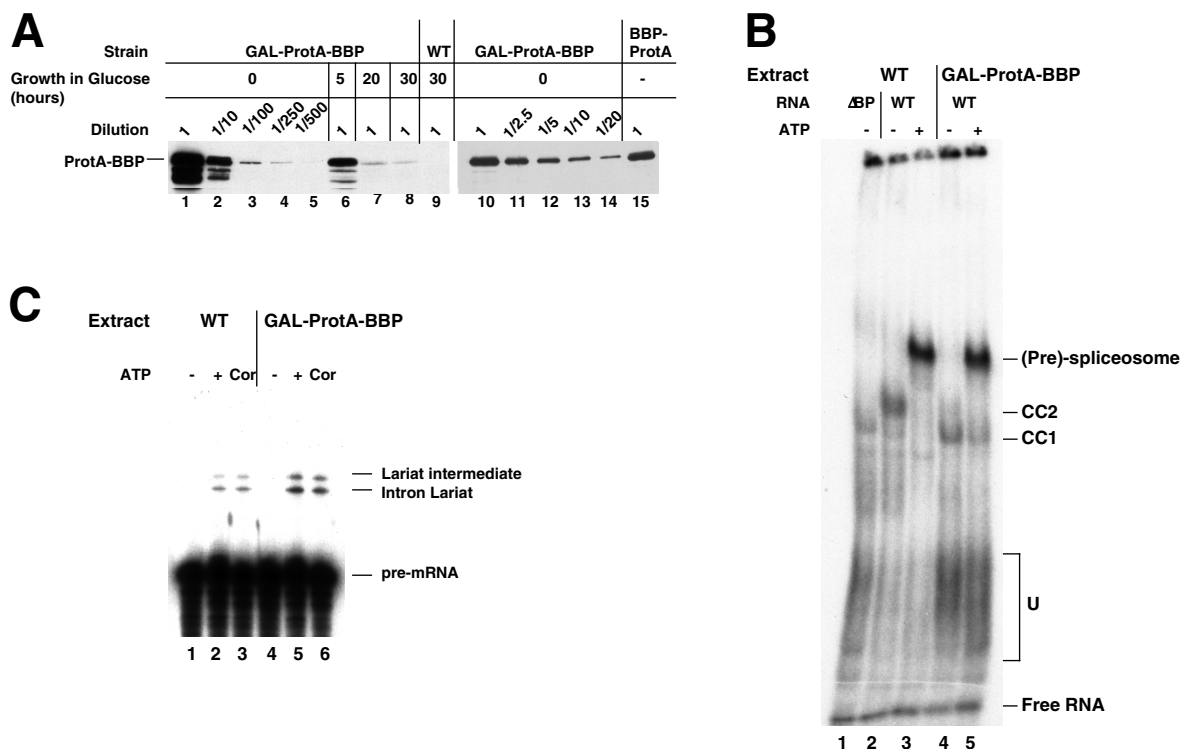
A

B

C

Figure 20. **Depletion of BBP does not affect (pre)-spliceosome formation and splicing**

A: Depletion of ProtA-BBP by growth in glucose medium.

Yeast cells carrying GAL-ProtA-BBP and an isogenic wild type strain were switched from galactose to glucose medium at time 0. Growth was continued and equal amounts of cells were removed at the indicated time points. Splicing extracts were prepared as described previously (Séraphin and Rosbash, 1989). Protein concentrations of the extracts were determined and found to vary not more than 20%. Aliquots were analyzed in an SDS-PAGE followed by western blotting and detection of ProtA-BBP. The extract of time 0 was loaded at several dilutions (lanes 1-5). Extract from the wild type strain was loaded as a control (WT, lane 6). A strain containing the C-terminal protein A tagged BBP (lane 15) was analyzed for BBP-ProtA expression in parallel with the GAL-ProtA-BBP strain grown in galactose (dilutions, lanes 10-14).

B: Depletion of BBP has no affect on (pre)-spliceosome formation.

Extracts from either a wild type (WT, lanes 1-3) or a GAL-ProtA-BBP strain (lanes 4, 5) grown for 30 hours in glucose medium (see panel A) were analyzed for complex formation with either labeled wild type pre-mRNA (WT, lanes 2-5) or a pre-mRNA lacking the branchpoint region (ΔBP, lane 1) in the presence (lanes 3, 5) or absence (lanes 1, 2, 4) of ATP. Samples of the reactions were fractionated in a non-denaturing gel. Positions of commitment complexes (CC1, CC2), (pre)-spliceosomes, free RNA and unspecific complexes (U) are indicated on the left.

C: Depletion of BBP does not affect pre-mRNA splicing.

Extracts from a wild type strain (WT) or the GAL-ProtA-BBP strain grown for 30 hours in glucose medium (see panel A and B) were used to analyze splicing *in vitro*. Splicing reactions using labeled pre-mRNA were performed in the presence or absence of ATP or cordycepin. RNAs were extracted and analyzed in a denaturing gel. Positions of lariat intermediate, intron lariat and pre-mRNA are indicated on the right. As described previously, with these extracts, the mRNA and exon 1 signals were partially obscured by degraded pre-mRNA. This could not be circumvented by the use of cordycepin, an ATP analogue which prevents the formation of heterogenous mRNA species by polyadenylation (data not shown, see also Séraphin and Rosbash, 1989).

We attempted to deplete this extract further by performing successive genetic and biochemical depletions. The GAL-ProtA-BBP strain was grown together with an isogenic wild type strain for 21 h in glucose medium and extracts were prepared. Those were then subjected to a further depletion of ProtA-BBP by incubation with IgG beads. The level of ProtA-BBP in these extracts was reduced by more than 99.9 % as determined by comparison in a western blot with a serial dilution of the starting extract (Figure 21A). These extracts were then analyzed for their ability to form commitment complexes and/or (pre-)splicesosomes as described above. While the formation of CC2 was blocked as observed before for the extracts depleted once, spliceosome formation was still not affected and CC1 accumulated in the presence or absence of ATP (Figure 21B, compare lanes 5, 6 and 9, 10). This reinforced the conclusion that depletion of BBP/ScSF1 to undetectable levels does not block spliceosome formation.
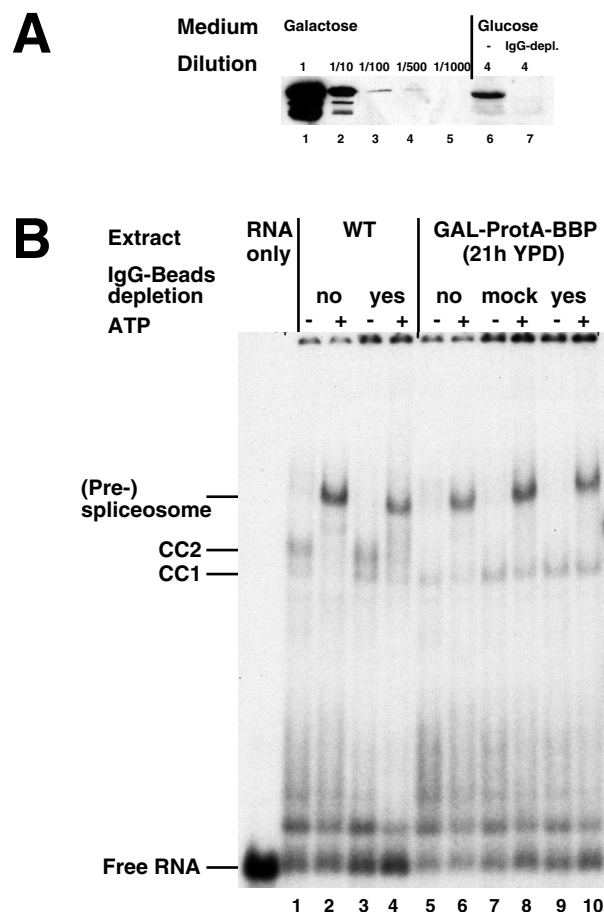

Figure 21. Combined genetic and biochemical depletion of BBP/ScSF1

A: Western blot analysis of depletion levels of BBP/ScSF1.

Extracts of the GAL-ProtA-BBP strain grown in galactose containing medium (dilution as indicated above the lanes 1-5) were compared with extracts from the same strain grown for 21h in glucose medium (lanes 6 and 7) by western blot and detection with PAP. The extract from cells grown in glucose was further depleted by incubation with IgG-beads (lane 7). Note that samples from the strain grown in glucose are loaded four times more concentrated.

B: Splicing complex formation in double depleted extracts.

Labeled pre-mRNA was incubated in the presence or absence of ATP with extracts from a wild type strain (WT) or the GAL-ProtA-BBP strain. Both strains had been grown for 21h in glucose containing medium to deplete BBP in the tagged strain. Before the incubation aliquots of the extracts were depleted with IgG-beads (yes) or with GST-beads (mock). The positions of (pre-)spliceosomes and commitment complexes are indicated on the left.

In addition, the effect of a BBP/ScSF1 depletion in the background of a disruption of the *MUD2* gene was tested. To this aim a strain was constructed that contained BBP tagged at

the C-terminus with the TAP tag in the background of a *MUD2* disruption (BSY740). Extract prepared from this strain was depleted using IgG beads or as a control GST beads. The depletion levels were checked on western blots and BBP was found to be reduced to more than 98% while the mock depletion showed no significant reduction in protein levels (Figure 22A, compare lanes 7 and 9). The assembly of (pre)-spliceosomes in the different extract preparations was analyzed in a native gel. No difference could be observed for the depleted compared to a mock depleted extract (Figure 22B, compare lanes 4 and 5). However, as observed before accumulation of CC1 was detectable in all reactions. The analysis of splicing of an RP51A intron was performed in extracts from the BBP-TAP strain or the *mud2*-Δ + BBP-TAP strain (Figure 22C). Although a difference between the untreated extracts (lane 1, 4) and the treated extracts (lanes 2, 3, 5, 6) was detectable, no difference was observed comparing the mock depleted extracts with the IgG depleted extracts (lanes 2 and 3, 5 and 6). This indicated a general partial inactivation of the splicing extracts due to the depletion procedure, but no specific inhibition of splicing by the depletion of BBP/ScSF1. We conclude that even in a *mud2*-Δ background depletion of BBP/ScSF1 has no detectable effect on (pre)-spliceosome formation and splicing *in vitro*.
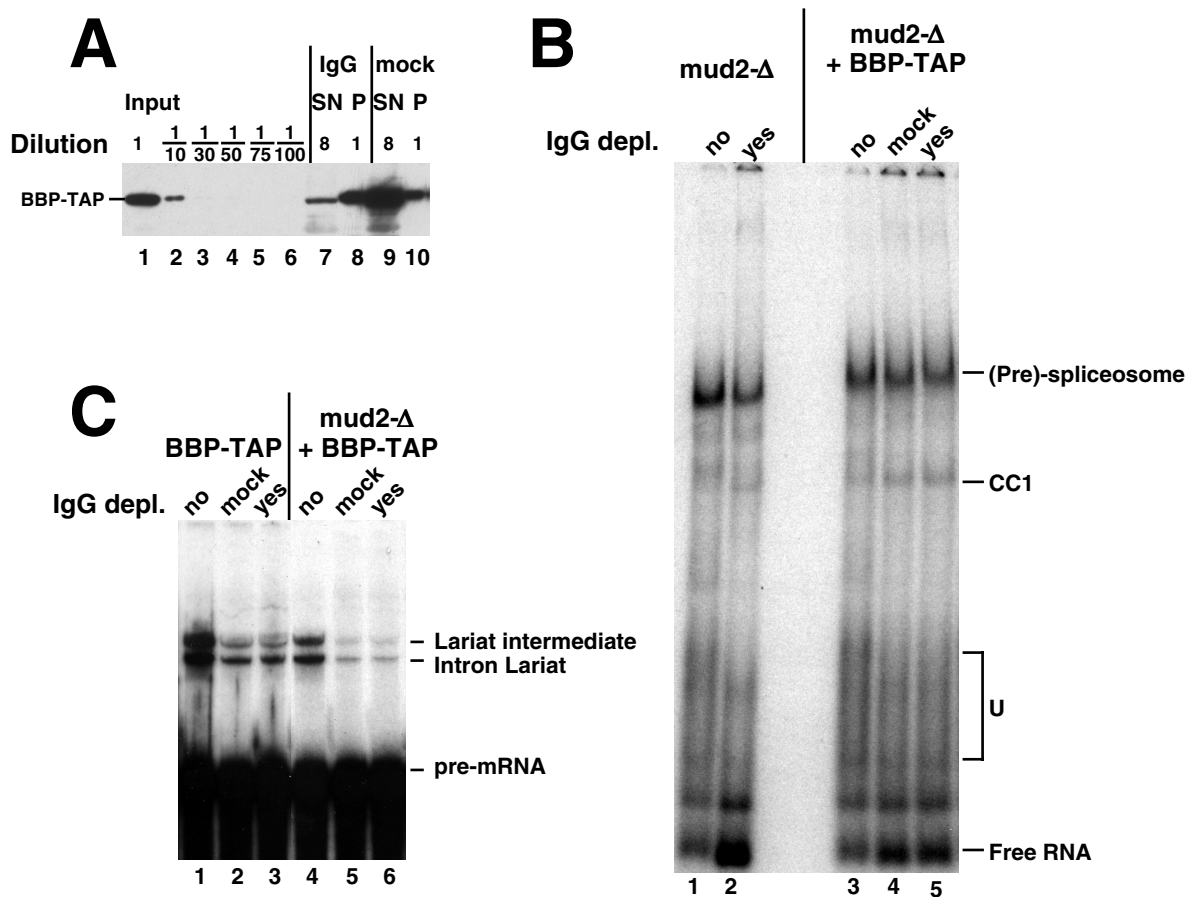
Figure 22. Depletion of BBP/ScSF1 in a *mud2*-Δ strain

A: Quantification of depletion of BBP.

Extracts from yeast cells containing BBP-TAP in a *mud2*-Δ background were depleted for BBP by incubation with IgG-beads (IgG) or with GST-beads (mock). Aliquots were analyzed in an SDS-PAGE followed by western blotting and detection of BBP-TAP. The extract before depletion (Input) was loaded at several dilutions (lanes 1-6). The supernatant after depletion (SN) and the beads pellet (P) of both depletion reactions were analyzed in parallel (landes 7-10). Note that the supernatants were loaded eight times more concentrated.

B: Spliceosome formation in *mud2*−Δ extracts depleted for BBP.

Extracts from either a *mud2*-Δ (mud2-Δ, lanes 1 and 2) or a *mud2*-Δ + BBP-TAP strain (mud2-Δ+BBP-TAP, lanes 3-5) that were untreated (no), depleted with IgG-beads (yes) or depleted with GST-beads (mock) were analyzed for complex formation with labeled wild type pre-mRNA in the presence of ATP. Samples of the reactions were fractionated in a non-denaturing gel. Positions of commitment complex 1 (CC1), (pre)-spliceosomes, free RNA and unspecific complexes (U) are indicated on the left.

C: Splicing in *mud2*−Δ extracts depleted for BBP.

Extracts from the BBP-TAP strain (BBP-TAP, lanes 1-3) or the *mud2*-Δ + BBP-TAP strain (mud2-Δ+BBP-TAP, lanes 4-6) that were untreated (no), depleted with IgG-beads (yes) or depleted with GST-beads (mock) were used to analyze splicing *in vitro*. Splicing reactions using labeled pre-mRNA were performed in the presence of ATP. RNAs were extracted and analyzed in a denaturing gel. Positions of lariat intermediate, intron lariat and pre-mRNA are indicated on the right.

1.8. *In vivo* splicing analysis in BBP depleted cells

We next asked if the lack of an effect of the BBP depletion on pre-spliceosome formation and splicing was restricted to the *in vitro* situation and if we could observe a phenotype (apart from the reduced growth rate) in living cells. To answer this question we analyzed RNA extracted from cells of the GAL-ProtA-BBP strain that had been depleted for ProtA-BBP by growth in glucose containing medium. First, we performed primer extensions with primers against the RP51A gene and the gene encoding the U3 snoRNA both of which contain an intron. The primer was located so that pre-mRNA and mRNA gave rise to extension products of different size. However, we could not detect significant differences in the mRNA to pre-mRNA ratios in a depleted compared to a undepleted control strain (data not shown). We therefore searched the yeast genome for introns with particular weak splicing signals. Because BBP/SF1 has been shown to bind to the branchpoint (Berglund *et al.*, 1997) and to be a component of CC2 (see 1.1.) we chose the intron of the *YRA1* gene which has a non-consensus branchpoint sequence (gACUAAC) and is the second longest intron in yeast (766 nt, Lopez and Séraphin, 2000). These features were likely to make the requirement for BBP more stringent.
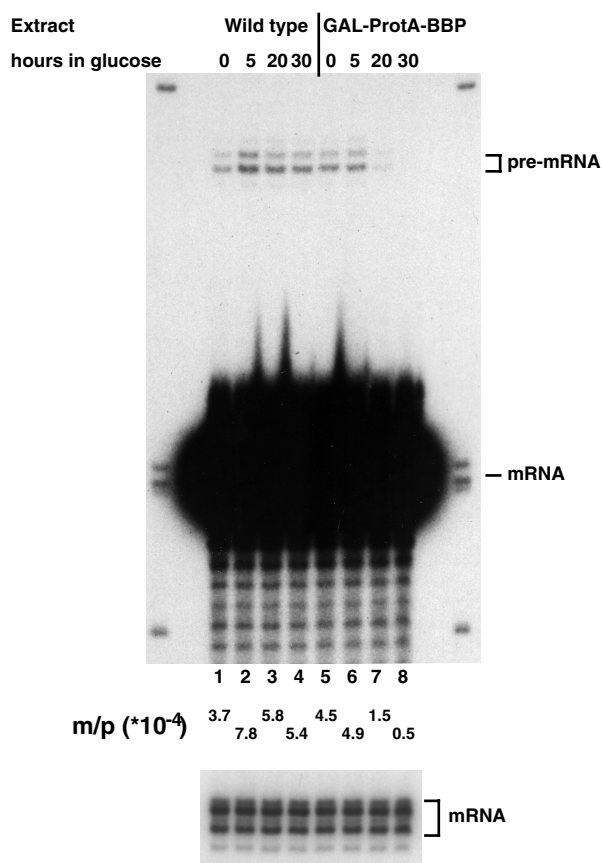

Figure 23. *In vivo* splicing analysis of *YRA1*

The GAL-ProtA -BBP strain and an isogenic wild type strain were grown for 30 hours in glucose containing medium to deplete BBP-ProtA (see Figure 20). RNA was extracted from aliquots of the culture removed at the indicated times and analyzed by primer extension with a primer complementary to the second exon of *YRA1* in the presence of ddGTP. This leads to the synthesis of differently sized products from the pre-mRNA and the mRNA. Primer extension products are indicated on the right side of the gel. The small picture at the bottom shows a shorter exposure of the same gel. Signals corresponding to pre-mRNA and mRNA were quantitated using a Fluor Image Analyzer (Fuji) and the mRNA to pre-mRNA ratio is shown below the gel for each lane.

This analysis showed that although mRNA levels were not decreased in the depleted strain compared to the undepleted control strain, pre-mRNA levels were reduced already after five hours of growth in glucose containing medium (Figure 23, compare lanes 2 and 6) and the pre-mRNA signal disappeared completely after 30 hours (lane 8). In contrast, the amount of mRNA remained unchanged (lower panel). The mRNA/pre-mRNA ratio of the quantified signals clearly confirmed this observation. Two explanations are possible for this phenotype: either splicing is more efficient in a BBP depleted strain or the pre-mRNA levels are decreased by other means (e. g. degradation). The latter possibility would implicate that pre-mRNA becomes more subsceptible to degradation when little BBP is available (see 2.7., synthetic interaction of BBP with the nonsense-mediated decay pathway).

1.9. Depletion of BBP leads to accumulation of CC1 during spliceosome formation

The results above show that BBP is a component of CC2 and is required for its formation. Nevertheless its depletion did not affect spliceosome formation and splicing. To detect even minor changes in spliceosome formation we performed a kinetic analysis comparing extracts from the wild type and the GAL-ProtA-BBP strains prepared after growth for 30 h in glucose medium. Complexes were assembled in the presence of ATP and aliquots were removed at different time points and migrated in a non-denaturing gel (Figure 24A) Signals corresponding to splicing complexes were analyzed on a Fluoresence Image Analyzer. Spliceosomes accumulated to slightly higher levels in the wild type extract, but the kinetics of their formation were comparable in both extracts (Figure 24A, compare lanes 1-5 and 6-10, see also Figure 24B). However, while spliceosomes accumulated without the appearance of significant levels of intermediate complexes in the wild type extract (Figure 24A, lanes 1-5 and Figure 24B), we reproducibly detected a transient accumulation of CC1 that preceded spliceosome formation in the BBP depleted extract (Figure 24A, lanes 6-10 and Figure 24B). These results strongly suggest that CC1 is a precursor of spliceosomes in this reaction. In addition they indicate a role for BBP in the kinetics of spliceosome formation.
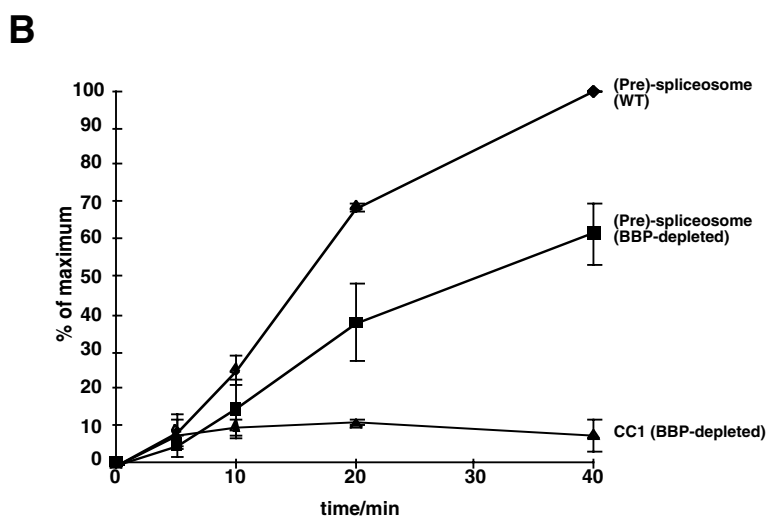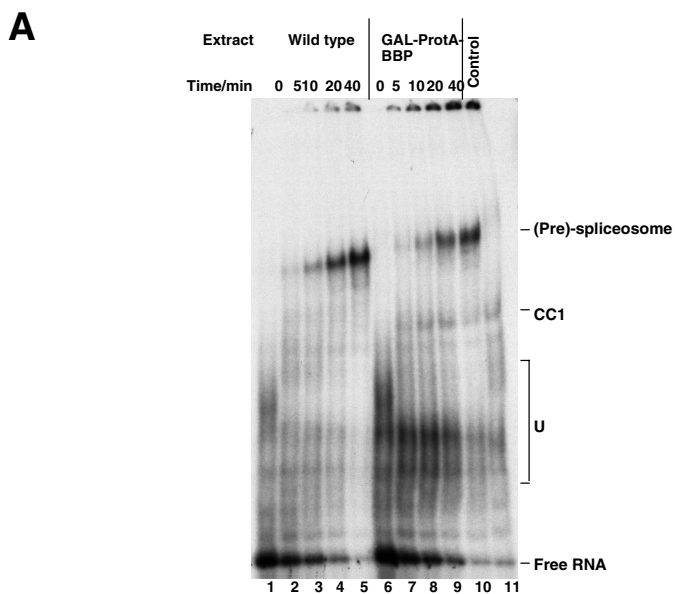
A

B

Figure 24. Depletion of BBP affects the kinetics of spliceosome formation

A: Time-course of complex formation in BBP depleted versus undepleted extracts.

Extracts from a wild type or a GAL-ProtA-BBP strain grown for 30 hours in glucose medium were incubated in the presence of ATP with labeled pre-mRNA. Samples were removed at the indicated times and stopped on ice. Reactions were analyzed in a non-denaturing gel. As a control for the migration of CC1 a reaction containing extract from the wild type strain and labeled pre-mRNA lacking the branchpoint region (ΔBP) was loaded (control, lane 11). Positions of the commitment complex (CC1), (pre)-spliceosome, free RNA and unspecific complexes (U) are indicated on the right.

B: Quantification of complex formation.

The gel shown in panel A and gels of two other independent experiments were scanned using a Fluorescent Image Analyzer (Fujifilm) and bands containing (pre)-spliceosome or CC1 were quantified. The diagram shows the average of the signals of the three experiments over the time of the reaction. Signals were calculated as the percentage of the band with the highest intensity (40 min time point of spliceosome in the wild type extract) Error bars indicate the standard deviation of the data points.

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