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The Splicing Factor BBP Interacts Specifically with the Pre-mRNA Branchpoint Sequence UACUAAC

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Summary

The yeast splicing factor BBP (branchpoint bridging protein) interacts directly with pre-mRNA at or very near the highly conserved branchpoint sequence UACUAAC within the commitment complex. We also show that the recombinant protein recognizes the UACUAAC sequence. Therefore, BBP is also an acronym for branchpoint binding protein. The mammalian splicing factor SF1 is a BBP ortholog (mBBP) and an E complex component, and also has branchpoint sequence specificity. The relative conservation of this region in yeast and mammals correlates well with the RNA-binding differences between BBP and mBBP, suggesting that BBP contributes to branchpoint sequence definition in both systems.

Introduction

The branchpoint sequence is conserved in both yeast and mammalian pre-mRNAs. In yeast (S. cerevisiae), this sequence is UACUAAC and almost invariant. In contrast, in mammals the branchpoint sequence is degenerate, with a consensus sequence of YNCURAY (Y = pyrimidine; R = purine; N = any nucleotide) (Moore et al., 1993). The 2′-OH of the underlined adenosines forms the lariat intermediate during the first step of splicing. In addition to participating in the chemistry of splicing, the branchpoint sequence is important for splicing-complex formation (Pikielny et al., 1986; Champion-Arnaud et al., 1995; Chiara et al., 1996; Query et al., 1996). This is due in part to a base-pairing interaction between this sequence and a highly conserved region of U2 snRNA (Parker et al., 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989). The yeast branchpoint region is also necessary for an earlier step of splicing, i.e., commitment-complex formation. The commitment complex is the substrate for U2 snRNP addition and contains pre-mRNA, U1 snRNP, the protein Mud2p, and other unidentified protein factors (Abovich et al., 1994). One of these proteins, BBP (branchpoint bridging protein), was recently identified in a Mud2p-dependent synthetic lethal screen (Abovich and Rosbash, 1997). Genetic and biochemical evidence showed that BBP interacts with Mud2p and the yeast U1 snRNP protein PRP40p. Therefore, BBP is involved in protein–protein interactions that bridge the 3′ and 5′ splice-site ends of the intron during the early steps of yeast pre-mRNA splicing (Abovich and Rosbash, 1997).

BBP, as well as its mammalian ortholog, SF1, contains RNA-binding motifs, and SF1 has been shown to possess general RNA-binding properties in vitro (Arning et al., 1996; Abovich and Rosbash, 1997). Based on primary sequence conservation (Arning et al., 1996; Abovich and Rosbash, 1997), the similar functional properties of BBP and SF1 (Abovich and Rosbash, 1997; this work), and the fact that two different splicing activities named SF1 have been reported (Krainer and Maniatis, 1985; Kramer, 1992), we refer to SF1 (Kramer, 1992) as mBBP.

Here, we show that BBP UV cross-links to pre-mRNA at or very near the pre-mRNA branchpoint sequence during commitment-complex assembly. We made a recombinant form of BBP and tested its in vitro RNA-binding properties with small synthetic RNA substrates. Remarkably, BBP has striking sequence specificity for the branchpoint sequence, indicating that its interaction with the branchpoint region is not solely dependent on protein–protein interactions. Recombinant mBBP also recognizes the UACUAAC sequence but with less specificity than that of BBP. This parallels the differences in branchpoint-sequence conservation between yeast and mammals, suggesting that the earliest definition of the branchpoint region in both systems involves a specific interaction between BBP and the pre-mRNA branchpoint sequence during commitment-complex formation.

Results

We first used UV cross-linking to address a possible direct association between BBP and pre-mRNA within commitment complexes. RNase T1 digestion and specific immunoprecipitation of a radioactive BBP–HA-tagged protein indicates close contact between BBP and radioactive pre-mRNA (Figure 1A). The absence of signal from a nontagged extract (Figure 1A, lane 1) verified the specificity of the immunoprecipitation. Importantly, BBP did not cross-link to a pre-mRNA substrate with a deletion of its UACUAAC sequence (Figure 1A, lanes 3 and 4).

To determine whether BBP contacts pre-mRNA in the vicinity of the branchpoint sequence, we generated a nearly wild-type pre-mRNA substrate that was ^{32}P-labeled at a single position 3 nt downstream of the UACUAAC sequence (Figure 1B). Digestion with RNase T1 prior to gel electrophoresis indicates that the site of the protein–RNA cross-link is at or very close to the branchpoint sequence (Figures 1B and 1C). This is supported by a significant reduction in signal with RNase A digestion (data not shown), which further delimits the cross-link site to the branchpoint region. Since these experiments were done under commitment-complex conditions (no ATP and/or oligonucleotide-mediated RNase H digestion of U2 snRNA), they suggest a simple

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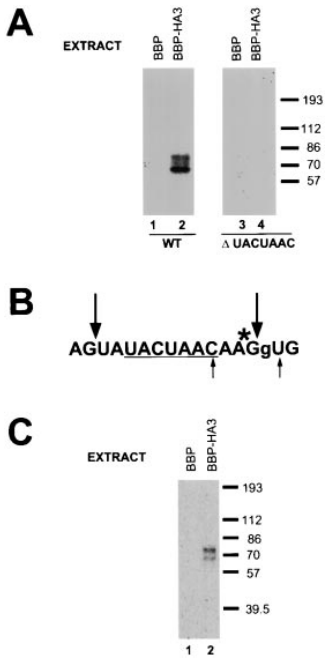

Figure 1. UV Cross-Linking of BBP to Pre-mRNA in Commitment Complexes

(A) Commitment complexes assembled with the indicated extracts and uniformly labeled wild type (lanes 1 and 2), or a 7 bp deletion of the UACUAAC sequence (lanes 3 and 4), were UV irradiated. After RNase T1 digestion and immunoprecipitation with 12CA5 antibody, the radiolabeled proteins were separated on an 8% acrylamide–SDS gel.

(B) Sequence of the pre-mRNA in the branchpoint region used in the site specific cross-linking experiment with the branchpoint sequence underlined. The asterisk represents the radiolabeled guanosine. The lower case guanosine was changed from a uridine for transcriptional reasons. The large arrows above the sequence represent RNase T1 cleavage sites, and the small arrows represent RNase A cleavage sites.

(C) Same as in (A) except that the substrate (wild-type pre-mRNA) contained a single labeled phosphate, such that after T1 digestion, a 12 nt fragment containing the UACUAAC sequence is radiolabeled.

explanation for the UACUAAC requirement for BBP–pre-mRNA contact (Figure 1A): BBP interacts directly with the branchpoint sequence during the earliest steps of splicing-complex formation.

BBP interacts with the splicing factor Mud2p and with the U1 snRNP protein PRP40p (Abovich and Rosbash, 1997), and we suspected that these protein–protein interactions serve to position BBP at or near the branchpoint sequence. Branchpoint binding and branchpoint recognition would then be dependent on these interactions. However, we considered the possibility that BBP and even its mammalian ortholog, mBBP, might have some specificity for the UACUAAC sequence. BBP contains three RNA-binding motifs: a KH domain (Siomi et al., 1993; Musco et al., 1996) and two retroviral Zn knuckle domains (Darlix et al., 1995); its mammalian ortholog, mBBP, has a KH domain and a single Zn knuckle domain (Figure 2) (Arning et al., 1996; Abovich and Rosbash, 1997). The two proteins share extensive sequence similarity, and mBBP has been shown to possess general RNA-binding properties (Arning et al., 1996).

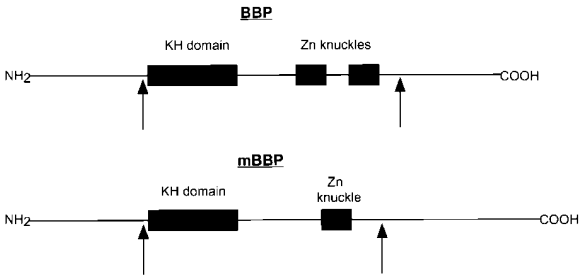

Figure 2. Schematic Representation of mBBP and BBP

The KH domain and Zn knuckle domains are boxed. The arrows represent the start and end sites of the recombinant proteins BBP(193) and mBBP(181).

To test the possibility of specific binding to the UACUAAC sequence, the putative RNA-binding regions of both proteins were purified after expression in E. coli (see Experimental Procedures). For BBP, the expressed region consists of amino acids 145–330 plus a 6His-tag at the N-terminus, i.e., 193 amino acids and an approximate molecular mass of 22 kDa. For mBBP, it is from amino acids 134–307, i.e., the same predicted molecular mass of 22 kDa (data not shown). To distinguish between the full-length natural proteins and these two recombinant fragments, we have named the latter BBP(193) and mBBP(181). The same purification scheme was used for both recombinant proteins: Ni-NTA agarose (Qiagen) followed by a CM–Sepharose column. The second column increased purity to approximately 90% and eliminated contaminating RNA binding and ribonuclease activities; both purified proteins have the same apparent molecular mass of 22 kDa (data not shown). At this point, the proteins were used for gel-shift and filter-binding assays. The wild-type RNA substrate was a 22 nt oligonucleotide containing the natural branchpoint sequence plus surrounding sequence from the yeast rp51A intron (Teem and Rosbash, 1983) (Table 1).

BBP(193) interacts with the oligonucleotide, and the apparent K_D is 500 nM (Figure 3A; Table 1). We tested for specific binding by using mutant versions of the 22 nt sequence. The gel-shift assay demonstrated that all mutants within the UACUAAC sequence had a negative effect on the apparent K_D (Figure 3; Table 1). Alteration of the branchpoint adenosine (to all three other possibilities) or the adjacent uridine (the fourth position within UACUAAC) eliminated detectable BBP(193) binding (Table 1 [−]). The other five point mutants within the branchpoint sequence reduced binding approximately 5- to 20-fold compared to the wild-type sequence (Table 1 [+]). All mutations outside the UACUAAC sequence were without effect (Table 1 [++]).

Because an interaction with RNAs mutant at the branchpoint adenosine or at the adjacent uridine was too weak to detect with the gel-shift assay, competition experiments were undertaken to estimate the K_D of binding to these mutant substrates. Nonradioactive wild type or mutant RNA was used to compete binding to radioactive wild-type RNA (Figure 3B).

For all three branchpoint mutations, a 125-fold excess of nonradioactive mutant RNA was necessary to observe comparable competition to that observed with

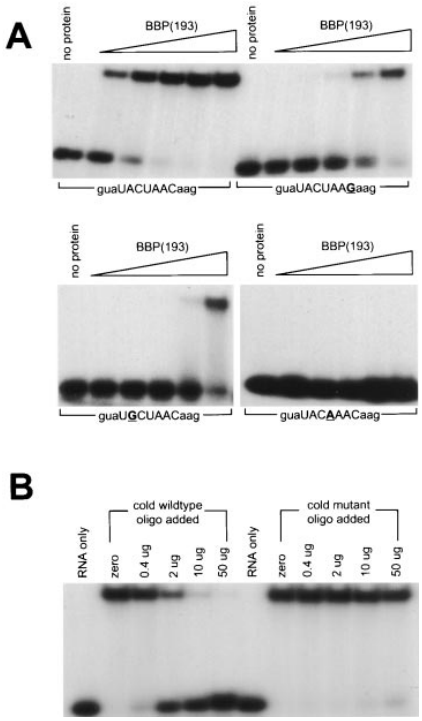

Figure 3. Gel Shift with BBP(193)

(A) Direct assay with four different oligonucleotides, as marked below each panel. The top left is wild type, and the other three are mutants within the UACUAAC sequence. Mutations are in bold and underlined. The range of BBP(193) concentration is from 230 nmol to 9 μmol.

(B) Competition experiment using radiolabeled wild-type branchpoint oligo and a constant amount of BBP(193), 2 μmol. Cold oligo was added in excess as labeled above the figure, and the mutant shown here is the branchpoint (lariat forming) adenosine changed to a cytidine. These experiments were repeated multiple times with different protein preparations, yielding the same results.

Table 1. Summary of Binding Data for BBP(193) and mBBP(181)

	BBP(193)	mBBP(181)
cagua↓UACUAACaaguugaauu	++	++
caguaUACUAACaa**c**uugaauu	++	++
caguaUACUAAC**g**aguugaauu	++	ND
caguaUACUAACa**g**guugaauu	++	++
cagu**g**UACUAACaaguugaauu	++	ND
cag**g**aUACUAACaaguugaauu	++	ND
caguaUACUAACaag**g**ugaauu	++	ND
caguaU**G**CUAACaaguugaauu	+	++
cagua**G**ACUAACaaguugaauu	+	++
caguaUACU**G**ACaaguugaauu	+	++
caguaUACUAA**G**aaguugaauu	+	++
caguaUA**G**UAACaaguugaauu	+	++
caguaUAC**A**AACaaguugaauu	-	-
caguaUACUA**C**Caaguugaauu	-	-
caguaUACUA**G**Caaguugaauu	-	-
caguaUACUA**U**Caaguugaauu	-	-

The arrow marks the position of the branchpoint adenosine. Two plus signs (++) indicate wild-type binding with an approximate K_D of 500 nM for BBP(193) and 30 μM for mBBP(181). One plus sign (+) indicates a decrease of approximately of 5- to 20-fold in binding as assayed by gel shift. The minus sign (−) indicates no binding by gel shift for BBP(193). In the case of mBBP(181), the minus sign (−) indicates little or no binding by gel shift.

wild-type RNA. This indicates that a single nucleotide change at the branchpoint adenosine (to C, U, or G) decreases the binding affinity of BBP(193) by at least 100-fold (Figure 3B). Mutations at position 4 were less dramatic, as only a 25-fold excess of mutant RNA matched the competition profile of wild-type RNA (data not shown). Mutations at other branchpoint positions were also examined in this way, and they were 5- to 20-fold less efficient than wild-type RNA (data not shown). These competition results support those obtained in the direct assay (Table 1). Both approaches indicate that the branchpoint adenosine and adjacent uridine are most important for specificity. Mutations at all other branchpoint locations have detectable but less severe effects.

To verify the direct interaction between BBP and the UACUAAC branchpoint sequence, we also used a footprinting assay (Figure 4A). For this experiment, we used BBP(190) (see Experimental Procedures) and a larger substrate containing 35 nt from the rp51A intron branchpoint region (Figure 4B). The RNA was end labeled and digested with RNase T1 or T2 in the presence or absence of BBP(190). As predicted, there is a strong footprint over the UACUAAC branchpoint sequence; only two additional nucleotides on either side are protected from T2 digestion by BBP(190) (Figure 4A). Taken together with the binding data (Table 1), the additional nucleotides probably indicate nonspecific (e.g., backbone) binding to these positions or steric inhibition of RNase T2 digestion.

The mammalian branchpoint consensus is degenerate, but the highly conserved yeast sequence UACUAAC is also the optimal branchpoint sequence for mammals (Zhuang et al., 1989; Moore et al., 1993). Taken together with the extensive conservation between mBBP and BBP (Arning et al., 1996), we considered that mBBP might also manifest similar sequence-specific binding. We therefore assayed binding of mBBP(181) to the same wild type and mutant RNA oligonucleotides (Figure 5).

mBBP(181) binds to the wild-type oligonucleotide with a K_D of 30 μM, about 50-fold weaker than BBP(193). However, mBBP(181) still manifested substantial specificity for the branchpoint sequence: like BBP(193), mutations at the branchpoint adenosine and the upstream uridine had the strongest effects on mBBP(181) binding. In contrast to BBP(193), however, these were the only two positions to affect mBBP(181) binding (Table 1). The gel-shift assay indicated that mutating the upstream uridine reduced binding 10-fold compared to the wild-type sequence (Figure 5A). Because binding to the branchpoint adenosine mutants could not be detected by gel-shift, we used competition experiments to estimate the difference in affinity between wild-type RNA and the branchpoint adenosine mutants (Figure 5B). For all three mutants, the binding constant was reduced approximately 25-fold (Figure 5B; Table 1). This more

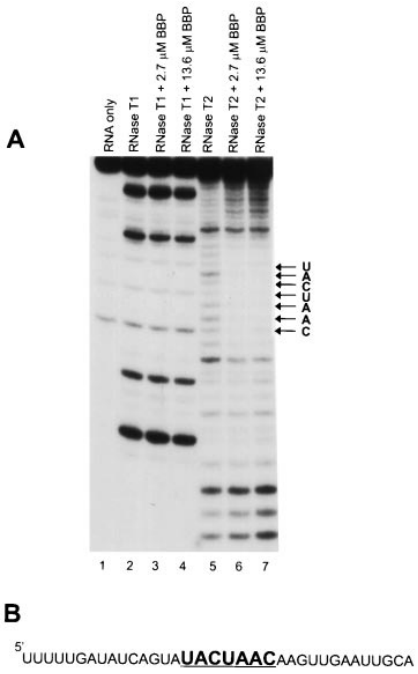

Figure 4. Footprint of BBP(190) on a 35 nt RNA Containing the Branchpoint Sequence

(A) The T1 cleavage sites allowed a precise determination of the branchpoint-sequence nucleotides. Only with RNase T2, in the presence of BBP(190) (lanes 6 and 7), is protection of the branchpoint sequence observed. There is also protection of two nucleotides to either side of the branchpoint sequence.

(B) The sequence of the 35 nt RNA, with the branchpoint sequence in larger case, bold, and underlined.

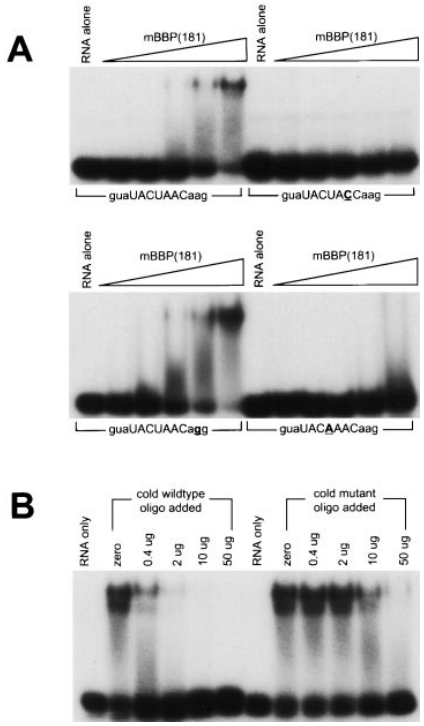

Figure 5. Gel Shift with mBBP(181)

(A) Direct assay with four different oligonucleotides. The wild-type sequence (top left), and the oligo with a mutation outside of the branchpoint (lower left), both bind mBBP(181) with similar affinity. While the mutations in the branchpoint (top right and lower right) show no or very little binding, mBBP(181) concentrations range from 4–65 μmol. These experiments were repeated multiple times with different protein preparations, yielding the same results.

(B) Competition experiment using radiolabeled wild-type branchpoint oligo and a constant amount of mBBP(181), 30 μmol. Cold oligo was added in excess as labeled above the figure, and the mutant shown here is the branchpoint (lariat forming) adenosine changed to a cytidine.

limited specificity probably reflects the relative evolutionary constraints on mammalian and yeast branchpoint sequences (see Discussion).

Discussion

Using purified recombinant forms of yeast BBP and mammalian mBBP, we show that both proteins interact with the branchpoint sequence. We have identified a yeast protein with sequence specificity for a pre-mRNA sequence element that in either system shows sequence specificity for the branchpoint (UACUAAC) sequence in a remarkable fashion.

Although the specificity of mBBP binding is weaker than that of BBP, the results suggest that mBBP is the 80 or 72 kDa protein that cross-links to the branchpoint sequence during the early steps of mammalian pre-mRNA splicing (MacMillan et al., 1994; Chiara et al., 1996, respectively). Our anti-mBBP antibodies do not efficiently immunoprecipitate this protein from splicing extracts, which precludes a comparable mammalian cross-linking experiment (Figure 1). Nonetheless, the properties of BBP fit with those of the 72 kDa protein, as it was shown to cross-link to the branchpoint sequence within a functional E complex but not in A complexes (Chiara et al., 1996). Although there are multiple forms of mBBP (Arning et al., 1996), we propose that this protein(s) and BBP are functional orthologs and recognize the branchpoint sequence in a similar manner. This is also based on the primary sequence conservation between mBBP and BBP (Arning et al., 1996), and the two nucleotides that affect mBBP(181) binding most strongly also have the strongest effect on BBP(193) binding (Table 1). Taken together with the recent characterization of BBP and mBBP (Abovich and Rosbash, 1997), the results suggest that the branchpoint sequence is recognized at least twice during spliceosome-complex formation, first by this protein and then by U2 snRNP.

We do not know which protein regions of BBP contribute to affinity and specificity. For the pre-mRNA branchpoint sequence, however, mutations at every position have a negative effect on BBP(193) binding. Although this correlates well with the poor formation of the CC2 commitment complex and the poor splicing of yeast substrates that carry these same mutations (Fouser and Friesen, 1986; Jacquier and Rosbash, 1986; Séraphin and Rosbash, 1991), it is impossible to distribute the quantitative effects on splicing to the BBP–branchpoint interaction, the U2 snRNP–branchpoint interaction, and to other as yet unidentified branchpoint-interacting factors. We also note that the failure to observe complete

suppression in the original U2 snRNA–UACUAAC pairing experiments suggested that other factors might be involved in branchpoint recognition (Parker et al., 1987).

Although the yeast protein has been previously characterized in vitro, full-length mBBP has been expressed and shown to bind RNA almost nonspecifically, with poly(G) and poly(U) being better than poly(A) and poly(C) (Arning et al., 1996). Our experiments are consistent with this report and indicate that mBBP has only modest branchpoint specificity. Even BBP specificity decreases in the context of much larger RNA substrates, owing to the large number of competing nonspecific binding sites (data not shown). The strategy of using small RNA substrates was therefore important to observe significant sequence specificity. We suspect that the small-substrate approach is also relevant to the biological context of splicing complexes: protein–protein contacts (e.g., Abovich and Rosbash, 1997) might position BBP and restrict binding to a narrow window of pre-mRNA that includes the branchpoint sequence. In addition, other pre-mRNA regions might be occupied with different RNA-binding proteins and therefore inaccessible.

The biological context of splicing complexes is probably also related to the issue of affinity. Neither BBP(193) nor mBBP(181) is a particularly good RNA-binding protein. BBP(193) has a K_D of 500 nM, and mBBP(181) has a K_D of 30 μM, whereas other RNA-binding proteins have K_Ds of 1 nM or even lower (Daly et al., 1989; Hall and Stump, 1992). We cannot rule out the possibility that apparent weak binding is an artifact of a large percentage of inactive molecules in both protein preparations, although there is no denaturation step in the purification protocol, and both proteins are chromatographically homogeneous. A more optimistic interpretation is that weak binding reflects the interactions of BBP with other splicing factors: they tether BBP (or mBBP) in close proximity to the branchpoint sequence (Abovich and Rosbash, 1997), obviating the need for single protein–high affinity binding. High affinity binding might even be incompatible with a multistep pathway: a low off-rate might be rate limiting and prevent the replacement of BBP by U2 snRNP during the assembly pathway (Abovich and Rosbash, 1997; Chiara et al., 1996). These considerations might be relevant to other in vitro situations where recombinant proteins bind RNA poorly or not at all (Lu and Hall, 1995).

The protein–RNA interactions suggest that sequence-specific RNA binding by both BBP and mBBP contributes to branchpoint recognition and possibly to branchpoint selection during early splicing-complex formation (CC2 for yeast and E complex for mammals). mBBP(181) binds RNA 50-fold weaker and is less UACUAAC sequence–specific than BBP. Although these differences could be due to the yeast substrate, they more likely reflect the relative conservation and importance of the branchpoint regions in the two systems. In the mammalian system, there is evidence that U2AF65, through its strong interaction with the polypyrimidine tract (Zamore and Green, 1991), makes a major contribution to branchpoint selection. Taken together, the data suggest that mBBP is positioned primarily through its strong protein–protein interaction with U2AF65 and only secondarily through its modest specificity for a branchpoint sequence (Figure 6). The more degenerate branchpoint sequences of mammals would then reflect the more prominent role of U2AF65 and the more relaxed sequence specificity of mBBP. In one study, mutation of the branchpoint sequence did not reveal a contribution of this sequence element to E complex formation (Champion-Arnaud et al., 1995). However, this experiment was done with a very strong pyrimidine tract, making it possible that the strong U2AF65–pyrimidine tract interaction masked the contribution of the mBBP–branchpoint sequence interaction.

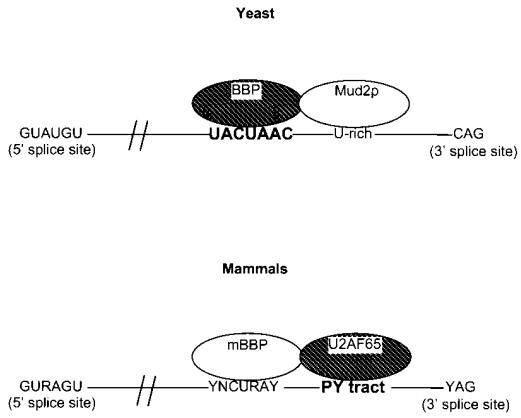

Figure 6. Model for Branchpoint Recognition in Yeast and Mammalian Pre-mRNA Splicing

See text for details.

There is a parallel yeast protein–protein interaction between Mud2p and BBP, and it would not be surprising were Mud2p to interact with intronic polypyrimidine tracts (Figure 6). Indeed, Mud2p also cross-links to the pre-mRNA site–specifically labeled 3 nt downstream of the branchpoint sequence (data not shown). The fact that Mud2p is inessential for viability, as well as for splicing, fits well with the relatively weak role and conservation of yeast polypyrimidine tracts (Abovich et al., 1994). In contrast, the yeast branchpoint is highly conserved, and BBP is an essential splicing factor. The tighter binding and greater sequence specificity of BBP suggest that it makes the larger contribution to branchpoint selection in the yeast system (Figure 6).

If BBP and mBBP are important for branchpoint selection, it should be possible to swap proteins or protein domains between the two systems. We are currently testing the prediction that this will lead to more stringent branchpoint selection in a mammalian system and less stringent selection in yeast.

Experimental Procedures

Cross-Linking

UV cross-linking and immunoprecipitations were done as previously described (Umen and Guthrie, 1995), except that irradiation was carried out in a stratalinker with 256 nm bulbs for 14 min. RNA substrates: plasmids Δ2–3 (wild type) and a 7 bp deletion (ΔUACUAAC) (Séraphin and Rosbash, 1991) were used as templates for in vitro transcription with T7 RNA Polymerase. [α-^{32}P]ATP and -UTP were included in the reactions to generate the uniformly labeled substrates. For the site specific–labeled pre-mRNA, the Δ2–3 plasmid

was used to generate templates by PCR for in vitro transcription. Oligonucleotide primers for the 5′ half molecule were a 17-mer containing the T7 promoter and 5′-TTGTTAGTATACTGA TAT-3′. Oligonucleotide primers for the 3′ half molecule were 5′-GACTAAT ACGACTCACTATAGGTGAATTGCATTTACAAACTTT-3′ and 5′-CCT TAGAAGCACGCTTGACGG-3′. Transcription, labeling of the 3′ half RNA with [γ-^{32}P]ATP, and ligation were done as previously described (Chiara et al., 1996). The bridging oligonucleotide was 5′-TAAATGCA ATTCACCTTGTTAGTATACTGATAT-3′.

Cloning and Protein Purification

The fragment for BBP(193) was amplified by PCR using two oligos, 5′-CGGGCCATG GCACATCATCATCATCATCACACCAAATTTCAG GA CAAGTAT-3′ and 5′-GGGGGATCCTCAAGAATTATTTACCGTGGC-3′. The template for the PCR was plasmid *MSL*-5 LEU containing the open reading frame of BBP (Abovich and Rosbash, 1997). This fragment was cut with NcoI and BamHI, inserted into pet11D (Novagen), and transformed into BL21(DE3) cells (Novagen) for overexpression. The 6His-tagged BBP(193) was purified using Ni-NTA agarose as recommended by Qiagen. Because the protein was expressed at low levels, and there were contaminating RNases and RNA-binding proteins after the Ni-NTA column, it was necessary to run a second column. The pooled fractions from the Ni-NTA column were dialyzed and bound to CM–Sepharose (Pharmacia). BBP(193) was eluted with an NaCl gradient of 50–1000 mM, and the peak fractions were pooled and concentrated using an Amicon ultrafiltration cell and then dialyzed against 25 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, and 15% glycerol.

For mBBP(181), the PCR product was amplified using 5′-CGGGC CATGGCACATCA TCATCATCATCACACACGTGTGAGTGATAAA-3′ and 5′-GGGGGATCCTCATGCTTTATCCTGAGCTGA-3′. The template was from cDNA as described (Abovich and Rosbash, 1997). The rest of the protocol is the same as that for BBP(193).

BBP(190) is slightly different from BBP(193). BBP(190) contains amino acids 145–330 of BBP plus 5 amino acids from the linker between GST and the BBP fragment cloned into pGEX-6P-1 (Pharmacia). The cloning was done using 5′-GGGGGAT CCAAATTTC AGGACAAGTATTAT-3′ and 5′-GGGGAATTCTCAAGAATTATTTAC CGTGGC-3′. The template used was the same as that for BBP(193) (Abovich and Rosbash, 1997). The purification and cleavage of the GST fusion using Prescission Protease was as recommended by Pharmacia. CM–Sepharose (Pharmacia) was also used to purify further BBP(190).

RNA Substrates

All RNA oligos were made using Perseptive Expedite RNA amidites on an Expedite 8909 Oligonucleotide Synthesizer. Oligos were kinased with [γ-^{32}P]ATP, gel purified, and further purified on a Bio–Rad 6 spin column. The substrate for the footprinting was labeled with [5′-^{32}P]PCP (England et al., 1980) and purified as the other substrates.

Gel-Shift Assay

RNA and protein–RNA complexes were separated in 0.5× TBE 7.5% Acrylamide gels run at 4°C for approximately 3 hr at 100 V. Binding buffer consisted of 25 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 30 μmol tRNA. Radiolabeled oligo was at an approximate concentration of 0.2 nmol. The mixture was allowed to incubate at room temperature for 15 min before loading.

Footprinting Assay

3′ end-labeled RNA (approximately 1 × 10^6 cpm per lane) was incubated in binding buffer (except that tRNA was at a final concentration of 800 nM) at 37°C for 5 min. RNase T1 (Ambion) was at a final concentration of 2 U/ml, and RNase T2 (GIBCO) at 0.05 U/ml. The reactions were stopped by adding an equal volume of phenol/chloroform.

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