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A BBP–Mud2p heterodimer mediates branchpoint recognition and influences splicing substrate abundance in budding yeast

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ABSTRACT

The 3' end of mammalian introns is marked by the branchpoint binding protein, SF1, and the U2AF65-U2AF35 heterodimer bound at an adjacent sequence. Baker's yeast has equivalent proteins, branchpoint binding protein (BBP) (SF1) and Mud2p (U2AF65), but lacks an obvious U2AF35 homolog, leaving open the question of whether another protein substitutes during spliceosome assembly. Gel filtration, affinity selection and mass spectrometry were used to show that rather than a U2AF65/U2AF35-like heterodimer, Mud2p forms a complex with BBP without a third (U2AF35-like) factor. Using mutants of MUD2 and BBP, we show that the BBP–Mud2p complex bridges partner-specific Prp39p, Mer1p, Clf1p and Smy2p two-hybrid interactions. In addition to inhibiting Mud2p association, the bbpΔ56 mutation impairs splicing, enhances pre-mRNA release from the nucleus, and similar to a mud2::KAN knockout, suppresses a lethal sub2::KAN mutation. Unexpectedly, rather than exacerbating bbpΔ56, the mud2::KAN mutation partially suppresses a pre-mRNA accumulation defect observed with bbpΔ56. We propose that a BBP–Mud2p heterodimer binds as a unit to the branchpoint in vivo and serves as a target for the Sub2p-DExD/H-box ATPase and for other splicing factors during spliceosome assembly. In addition, our results suggest that the Mud2p may enhance the turnover of pre-mRNA with impaired BBP-branchpoint association.

INTRODUCTION

Pre-mRNA splice site consensus sequences are sampled multiple times during spliceosome assembly (1). For example, early in assembly the yeast 5' splice site is bound by the U1 snRNP particle through protein and snRNA-based contacts. U1 snRNP also interacts with the branchpoint region through association with the branchpoint binding protein (BBP), a homolog of the mammalian SF1 protein (2,3). Subsequent recruitment of the U2 snRNP particle displaces BBP and its binding partner Mud2p (the homolog of mammalian U2AF65) from the branchpoint region to permit base pairing between the U2 snRNA and the pre-mRNA branchpoint consensus (4). The displacement of BBP and Mud2p from the branchpoint region correlates with a weakening of the U1 snRNP association with the splicing apparatus, an apparent requirement for subsequent binding of U6 snRNA at the 5' splice site. Although U2 snRNA basepairs with the pre-mRNA substrate, initial U2 snRNP contact is likely protein-mediated and, in mammals, may be accomplished in part through U2AF65 interaction with the SAP155 subunit of the U2 snRNP particle (also called SF3b155) (5). Two-hybrid interaction between Mud2p and the Saccharomyces cerevisiae SAP155 homolog, Hsh155p, suggest that this interaction is conserved in yeast, implicating Mud2p function after the U1-snRNP-dependent step of commitment complex formation (6). While U1 displacement appears mediated by Prp28p, stable U2 snRNP addition requires participation of the Prp5p and Sub2 DExD/H-box proteins. Other DExD/H-box proteins promote subsequent steps in spliceosome assembly (Brr2p, Prp2, Prp16p) and product release (Prp22p and Prp43p) (7,8).

U2AF65 is recovered from mammalian cells as a heterodimer with U2AF35, a protein that crosslinks to the 3' splice site during spliceosome assembly (9–11). Homologs of U2AF35 are broadly represented in nature and have been shown to function in splicing in man, flies, nematodes and fission yeast (11–14). Since no U2AF35 homolog is obvious in budding yeast, Mud2p may have diverged to the degree that it acts independently or simply no longer provides an anchor for 3' splice site recognition factors. Alternatively, a structurally distinct...
but functionally related protein may bind Mud2p to serve this purpose or otherwise facilitate co-transcriptional assembly of the yeast mRNA (15).

BBP interacts with at least two essential U1 snRNP proteins, Prp39p and Prp40p, in addition to Mud2p in a step that commits the pre-mRNA to the splicing pathway (3,16). While mammalian U2AF65 binds a critical polypyrimidine stretch upstream of the 3′ splice site and is required for splicing, yeast splicing does not require either a polypyrimidine element or Mud2p (4,17,18). The yeast branchpoint motif is more rigorously conserved than the mammalian sequence, however, and BBP is necessary for yeast cell viability. The loss of either BBP or Mud2p increases pre-mRNA abundance in the cytoplasm (19,20), thereby linking both proteins to the nuclear retention of newly transcribed RNA (21). While BBP and Mud2p bind one another in vitro, the degree to which these proteins act in a concerted or independent function during spliceosome assembly or nuclear pre-mRNA retention in vivo is less clear.

Here, we report the results of experiments designed to investigate the composition and function of cellular Mud2p and BBP complexes. Like U2AF65, Mud2p is found to exist as a heterodimer. However, rather than interacting with a U2AF35 counterpart, Mud2p, in a concerted or independent function during spliceosome assembly or nuclear pre-mRNA retention in vivo is less clear.

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MATERIALS AND METHODS

Yeast strains and assays

Yeast strains BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), mud2::KAN (MATa, his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mud2::KAN), smy2::KAN (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 smy2::KAN), BBP-TAP (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, MSL5-TAP) and Mud2-TAP (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, MUD2-TAP) were obtained from Open Biosystems (Huntsville, AL, USA). The hbpΔ56 mutation was introduced by inverse PCR (5TAGTGATCCCCAATAGGAAACAAT A3′ and 5TAGGGATCCATCAACATTGTTTTT ATTGTG3′) on plasmid pRS414-BBP (obtained from Michael Rosbash). The deletion derivative was then subcloned into YlpLac 211 (24), linearized with BglIII and introduced into the genome by two-step replacement into wild-type yeast to generate the hbpΔ56 strain. FOA selected colonies were scored for excision of the wild-type BBP allele by PCR. The double mutant strains were created using standard yeast genetic techniques (25). The wild-type MUD2 and BBP genes with approximately 300 bp of flanking sequence were subcloned by PCR into YCplac22 (24) for complementation studies. The structures of all novel constructs were confirmed by DNA sequencing prior to use. Plasmids containing the RPS17A-based Acc (splicing), Nde/Acc (export) and SD5 (no intron) lacZ reporters genes were previously described in detail (26,27). The lacZ reporter strains were induced with 2% galactose in complete medium at 30°C for 4 h prior to β-galactosidase measurements. Each assay was replicated 4–10 times and pair wise comparisons of the normalized enzyme units were made using a two-tailed t-test of significance.

Yeast two hybrid studies were conducted with full-length PCR products (primer sequences available upon request) fused to the GAL4 DNA binding (DB) and activation domains (AD) on vectors pACT and pAS2, respectively, in yeast strain pIJ69-4A (28). The yeast transformants were scored for transactivation of the endogenous reporter genes by colony growth at 30°C on medium lacking adenine or on medium lacking histidine and containing 20 mM 3-amino triazole. Autostimulation of the chromosomal reporter genes was scored in the recombinant pAS2 and pACT transformants in the absence of the binding partner.

RNA analysis

RNA was recovered by breaking yeast on a vortex mixer with sterile glass beads followed by multiple extractions using phenol/chloroform/isomyl alcohol (25:24:1) and ethanol precipitation. Primer extension was performed on 12–24 μg of total RNA using a 32P end-labeled RPS17A exon II primer RB1 (5′CGCTTGACGGTGCGTTTGGTTCC3′) and Superscript reverse transcriptase (Invitrogen) at 37°C for 1 h (29). The cDNA products were resolved on a denaturing 7 M urea 5% polyacrylamide gel. Northern blot analysis was performed with the RPS17A and ADE3 hybridization probes on RNA resolved by electrophoresis on a 1% agarose formaldehyde gel and transferred to a charged nylon membrane (30,31). Band intensities were visualized by Typhoon 9600 phosphorimagetor and quantified with Image Quant software GE Healthcare (Piscataway, NJ, USA).

Protein analysis

Conditions for metabolically labeling yeast proteins with Trans 35S-label (ICN) were previously described in detail (32). In short, yeast were labeled for 4 h at
To screen for proteins that interact with Mud2p, Mud2p and BBP bind one another

**RESULTS**

To screen for proteins that interact with Mud2p, we metabolically labeled yeast with $^{35}$S-amino acids for 4 h and then used tandem affinity purification [TAP (33)] to identify co-purifying factors (Figure 1A, lane 1). In contrast to snRNP or spliceosome preparations previously assayed in this way (32–35), the pattern of $^{35}$S-labeled proteins recovered with Mud2-TAP at 200 mM NaCl is quite simple, with the 65 kDa fusion protein derivative, Mud2-CBP, and a single additional band that migrates as a 57 kDa protein. Mud2-CBP consists of the native Mud2 protein joined to a 5 kDa of CBP sequence that persists after TAP (33). Confirmation of the Mud2-CBP assignment was made by western blot using an anti-CBP antibody (see Materials and methods section). A few minor bands are also seen in this preparation, several which appear as background in the untagged control sample (lane 4 and see Figure 3D). RNase A is included at levels sufficient to degrade all detectable full-length rRNA and splicing-relevant snRNA (Figure 1C) although the pattern of recovered proteins appears equivalent without RNase addition (e.g. compare Figure 1A lanes 3 and 6).

In a parallel culture, yeast that express a TAP-tagged BBP derivative were similarly scored for the pattern of recovered proteins. Here also two prominent proteins, 63 and 60 kDa in apparent molecular weight were recovered (Figure 1A, lane 3). Western analysis shows that the 63 kDa band is BBP–CBP, which migrates more slowly than predicted for this 55 kDa fusion protein (data not shown). The 57 kDa and 60 kDa proteins that co-purify with Mud2–TAP and BBP–TAP, respectively, are roughly the predicted sizes for native BBP (50 kDa) and Mud2p (60 kDa). In agreement with this assignment, when Mud2p and BBP are both expressed as TAP-tagged derivatives, the pattern of recovered proteins is equivalent except that the bands corresponding to the untagged BBP and Mud2p are now absent (lane 2).

Although the background is somewhat higher, a similar protein pattern is seen when the isolation is scaled up and unlabeled Mud2–TAP or BBP–TAP complexes are visualized with Coomassie blue (Figure 1B; data not shown). Mass spectrometry was used to confirm the p60-Mud2p assignment and identifies BBP and ribosomal protein Rpl4p in the preparation (see Materials and methods section). While not ruling out the possibility of functional association, we note that the Rpl4p band appears to be of lower abundance than Mud2p or BBP in the preparative (Coomassie blue-stained) sample and that it co-migrates with a background band in the radiolabeled preparations, suggesting that Rpl4p may be a contaminant of the preparation. Lower scoring mass analysis signatures were sometimes observed for splicing factors Prp40, Prp8, Prp28, Brr2p, Prp6 and Isy1 as well as for the heat shock protein, Ssb2, and for the Ded1p and Dhp2p DExD/H-box factors. Since none of these proteins correspond to prominent $^{35}$S-labeled bands or Coomassie stained proteins, the associations likely reflect weak or transient interactions with the BBP–Mud2 complex or the presence of a small amount of a more elaborate (multi-subunit) complex.

The BBP–Mud2 complex is predominantly a heterodimer

The cellular levels of BBP and Mud2p appear equivalent (36) and the Coomassie blue stained BBP and Mud2p bands are of similar intensity, suggesting a 1:1 stoichiometry in the recovered sample. In support of this, after normalization for the greater sulfur content of Mud2p, the 35:S ratio of the metabolically labeled proteins was calculated to be approximately 1.15 when Mud2-TAP is used as bait and 0.87 when BBP–TAP is used as bait. While supportive of equimolar recovery, these experiments do not distinguish between a BBP–Mud2p heterodimer and higher order complexes. To address this, we resolved the calmodulin agarose purified Mud2–BBP

**Mud2p and BBP bind one another in vivo**

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Figure 1. Tandem affinity purification of BBP–TAP and Mud2–TAP. (A) Autoradiogram of a 7% polyacrylamide gel of metabolically labeled proteins selected by sequential IgG agarose and calmodulin agarose chromatography from yeast that express the indicated TAP-tagged gene constructs as genomic integrants. The untagged lane shows background proteins (asterisks). Samples in lanes 1–4 were pre-treated with RNase A prior to TAP selection while samples 5–6 were not. The numbers at the left indicate the positions of unlabeled protein molecular weight markers. Bands corresponding to the untagged Mud2p and BBP proteins and the proteins with the residual CBP tags are indicated. (B) Coomassie blue stain of unlabeled Mud2-TAP and co-purifying proteins. Lane 2 shows the migration of protein molecular weight markers. (C) Yeast used in panel a treated (+) or not (−) with RNase A hybridized with probes specific for the spliceosomal snRNAs (lanes 1–4) or stained with ethidium bromide for the 25S and 18S ribosomal RNAs (lanes 5–8). (D) Single-step recovery of BBP–TAP and Mud2–TAP by TAP by calmodulin agarose chromatography. To avoid band distortion due to sample overloading, the unfractionated (total) protein lanes contain 1/3 the equivalent amount of sample. Untagged = calmodulin agarose recovered material recovered from an untagged extract.
complex by tandem HPLC with 2000Å and 300Å sepharose columns. Complex recovery is generally quite efficient, typically 50–75% (Figure 1D). In contrast to the sharp focus of the molecular weight standards (Figure 2A), the BBP–TAP selected complex resolves as a broad peak with the greatest recovery between fractions 18–20 (Figure 2B, upper panel, note that here the protein is visualized with an antibody against the TAP tag, consequently only a single protein is visible). An equivalent distribution is seen when Mud-TAP is used for selection (Figure 2B, lower panel). The highest signals for BBP–TAP and Mud2–TAP correspond to fractions overlapping the γ globulin (158 kDa) and ovalbumin (44 kDa) protein standards. As we previously found no evidence for homodimerization of either Mud2p or BBP (6), this pattern indicates the presence of significant amounts of a BBP–Mud2p heterodimer, which has a predicted mass of ~130 kDa (including the full TAP epitope). Given the spread of signal and the somewhat greater recovery of the TAP tagged partner indicated by the metabolic labeling experiment, monomeric BBP–TAP (73 kDa) and Mud2–TAP (80 kDa) are likely present as well. While the existence of higher order structures (e.g. tetrameric) cannot be ruled out, such complexes are not abundant under these isolation conditions.

**Bridging interactions contribute to the previously reported two hybrid interactions of BBP and Mud2p**

Four proteins, splicing factors Clf1p, Prp39p and Mer1p, and the largely uncharacterized Smy2 protein have been reported to interact with both BBP and Mud2p by the two-hybrid assay (3,6,37–39). These interactions presumably reflect protein-based contacts made by BBP and Mud2p during spliceosome assembly or in other functional contexts (see Discussion section). Since BBP and Mud2p bind one another we thought it is possible that indirect (i.e. bridging) interactions might contribute to the observed two hybrid pattern. As a first step in addressing this issue, we sought confirmation of the reported interactions using full-length protein constructs. Although differences exist in the extent of host cell reporter gene transactivation, each two-hybrid pair shows detectable activity in the wild-type yeast (Figure 3A). For most constructs, the two hybrid
interactions are reciprocal when the GAL4 DNA-binding and transactivation domains are swapped. Autostimulation by CLF1 and PRP39 in the DNA binding cassette prevented this determination in these two cases.

Since MUD2 is not essential, the BBP associations can be assayed in the mud2::KAN null background to learn which, if any, is Mud2p-dependent. The Prp39p, Mer1p and Smy2p signals all persist in the mud2::KAN mutant, showing that none of these proteins require Mud2p for BBP association (Figure 3A). In contrast, the Clf1p-BBP signal is lost in the mud2::KAN mutant, strongly suggesting that binding of this essential splicing factor with BBP is indirect and bridged through Mud2p.

The two-hybrid results presented earlier, support direct association of BBP with Prp39p, Mer1p and Smy2p but do not rule out independent interactions of these proteins with Mud2p. As BBP is required for cell viability, we cannot assay for Mud2p-based interactions in its absence. We reasoned, however, that mutation of the Mud2p interaction domain of BBP would not be a lethal and would allow us to score for bridged two-hybrid associations. Previous studies localized the Mud2p binding site to the N-terminal half of BBP [see (19), (40) and references within]. We created a BBP derivative, bbpΔ56, from which only the most N-terminal region of the proposed interaction domain was deleted and tested this derivative for biological activity. The bbpΔ56 allele complements the lethality of bbp::KAN although the mutant grows more slowly at both 30°C and 37°C than wild-type yeast or the mud2::KAN mutant (Figure 3B). While BBP is readily affinity purified with Mud2-TAP, no band unique to the bbpΔ56 sample can be recovered with Mud2-TAP (Figure 3C compare lane 1 with lanes 2 and 3). Thus, unlike the full length protein, BbpΔ56p does not stably bind Mud2p.

Since BbpΔ56p impairs or blocks Mud2p binding, proteins which interact with Mud2p through a BBP-dependent bridge were anticipated to show reduced or abolished two-hybrid signals in the bbpΔ56 background. As seen in Figure 3A, Mud2p continues to interact with

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**Figure 3.** Bridged interactions of the BBP-Mud2p heterodimer. (A) Two hybrid interactions conducted in yeast wild-type for both genes (MUD2, BBP), deleted for MUD2 (mud2::KAN, BBP), or containing a non-lethal mutation within BBP (MUD2, bbpΔ56). Reporter gene activity was scored after 3 days at 30°C. (B) Direct growth assay in the absence of 2-hybrid plasmids of yeast with wild-type MUD2 and BBP alleles (WT), deleted for MUD2 (mud2::KAN, BBP), containing a non-lethal mutation within BBP (MUD2, bbpΔ56) or both mutations (mud2::KAN, bbpΔ56) at 23 and 37°C. (C) Tandem affinity selection of metabolically labeled proteins with Mud2–TAP from yeast that express the bbpΔ56 allele (lane 1) and wild-type BBP allele (lane 2). Lane 3 shows background proteins isolated from an untagged strain. The positions of unlabeled protein markers are shown on the right.
Clf1p under these conditions while the Prp39p, Smy2p and Mer1p two-hybrid interactions are lost. Based on this, we propose that Clf1p most likely binds Mud2p directly and that Prp39p, Mer1p and Smy2p interact with Mud2p through a BBP-dependent bridge.

Previously, Guthrie and colleagues (41) showed that deletion of MUD2 suppresses the lethality (or near lethality) of a sub2 loss-of-function allele, suggesting that the dissociation of Mud2p from pre-mRNA is an important function of Sub2p. Our results show that Mud2p and BBP bind one another in vivo, raising the possibility that the BBP–Mud2p heterodimer is the target of Sub2p activity. To investigate this further we tested whether the bbpΔ56 mutation likewise compensates for the loss of Sub2p. Here a URA3-marked plasmid copy of SUB2 [i.e. pSUB2(URA3)], was introduced into yeast bearing a chromosomal sub2::KAN null allele in a wild-type background or in the presence of either the mud2::KAN or the bbpΔ56 mutation (Figure 4A). When selected for plasmid loss on five fluoroarotic acid (FOA) medium, no visible colonies form after 3 days at 30°C with the sub2::KAN mutant in an otherwise wild-type background (streaks 4 and 8) confirming the vital requirement for Sub2p function. Similar results are seen with a control strain in which a lethal ntr2::KAN mutation is complemented by a URA3-linked plasmid that expresses a functional NTR2 allele (streak 2). In contrast, the mud2::KAN, sub2::KAN double mutant (streak 5) and the bbpΔ56, sub2::KAN double mutant (streaks, 6 and 7) are viable in the absence of the pSUB2(URA3) plasmid. Importantly, re-introduction of a plasmid-based wild-type copy of BBP on a TRP1-marked plasmid in the bbpΔ56, sub2::KAN double mutant background restores the essentiality of pSUB2(URA3) (i.e. p414-BBP, TRP1; Figure 4B, streak 2). As expected, this same strain transformed an empty vector (i.e. p414-TRP; streak 3) or a wild-type yeast strain transformed with the p414-BBP, TRP1 plasmid (streak 1) continue to form colonies after pSUB2(URA3) loss. The ability of both mud2::KAN and bbpΔ56 to suppress sub2::KAN lethality bolsters the view that BBP and Mud2p functionally interact in vivo and strongly suggests that the BBP–Mud2p heterodimer is a substrate for Sub2p.

Deletion of MUD2 suppresses pre-mRNA loss in the bbpΔ56 background

Since BBP and Mud2p are believed to act cooperatively in branchpoint binding, yeast with the mud2::KAN or bbpΔ56 mutations might be expected to show similar growth characteristics if the only consequence of bbpΔ56 was the loss of Mud2p interaction. However, the bbpΔ56 mutant and the mud2::KAN, bbpΔ56 double mutant grow more slowly than the mud2::KAN strain, suggesting that some additional function of BBP is impaired by the bbpΔ56 lesion (Figure 3B). When pre-mRNA is assayed at 23°C, the mRNA to pre-mRNA ratio (a measure of splicing efficiency) of the RPS17A transcript appears almost equivalent in the wild-type, single and double
mutant backgrounds (Figure 5A; the intronless ADE3 mRNA is presented as a sample loading control). At 37°C, splicing is more impaired in the mud2::KAN mutant when compared to the wild-type strain although it remains better than what is observed after inactivation of the essential U4/U6.U5 tri-snRNP protein, Prp38-1p (30). The bbpD56 mutant has a somewhat stronger splicing defect than that seen with mud2::KAN mutant at 37°C, consistent with the lower vitality of this strain. We see no exacerbation of this splicing defect in the mud2::KAN, bbpD56 double mutant background (Figure 5A and see below), however. While there is some experimental variability in splicing efficiency with temperature shift, we see no clear splicing defect with the smy2::KAN knockout mutant and no reproducible exacerbation of the mud2::KAN or bbpD56 splicing defects by this mutation (Figure 5B).

We next investigated the impact of the mud2::KAN and bbpD56 deletions on RPS17A reporter gene transcripts with splice site consensus mutations (26). For wild yeast, the pre-mRNA to mRNA ratio increases only modestly when the branchpoint consensus, UACUAAC, is changed to UAUAAC (HZ8) or UCCUAAC (HZ3) while splicing is essentially abolished when the branchpoint nucleotide is changed in UACUAAC (HZ10) [Figure 5C and (26)]. A 5’ splice site mutant, GUAUGU->GUUAU (HZ12), also shows a decrease in splicing efficiency in wild-type yeast. In the mud2::KAN

Figure 5. Splicing inhibition by the mud2::KAN and bbpD56 mutations. (A) Northern analysis of RNA extracted from wild-type yeast (WT), the ts splicing mutant, prp38-1, and yeast with the bbpD56 and mud2::KAN single mutations and with the combined bbpD56 plus mud2::KAN double mutant background. RNA harvested from yeast grown continuously at 23°C (-) and after a 2 h shift to 37°C (+) was hybridized with radiolabeled probes to detect the intronless ADE3 mRNA and the RPS17A pre-mRNA and mRNA. The relative abundance of mRNA and pre-mRNA (M/P ratio) is presented below the image. (B) Northern analysis as in panel A with added samples smy2::KAN and smy2::KAN combined with bbpD56 or mud2::KAN. (C) Primer extension analysis of RNA isolated from the indicated yeast backgrounds that express RPS17A reporter gene constructs with the wild-type intron (WT), branchpoint mutants (HZ8, HZ3, HZ10) and 5’ splice site mutant (HZ12). (D) Primer extension of RNA from the bbpD56, mud2::KAN double mutant before (left) and after (right) add back of MUD2.
background, these splicing defects are modestly exacerbated with the greatest impact seen with HZ3 where the pre-mRNA/mRNA ratio increases ~2-fold compared to the wild-type strain.

Reporter transcript splicing in the bbpΔ56 background showed two distinctive features. First, although this lesion is outside the RNA binding KH domain core (approximately amino acids 149–133), splicing is much more sensitive to mutations at the branchpoint consensus when compared with wild-type yeast or the mud2::KAN mutant; splicing of the 5′ splice site mutant appears largely unaffected. Second, even as mRNA levels drop, pre-mRNA levels do not accumulate to the degree observed with wild-type or mud2::KAN yeast. This characteristic is especially obvious with the HZ3 and HZ10 substrate mutations and is consistent with the observation that diminished BBP activity results in leakage of unprocessed pre-mRNA into the cytoplasm where it is sensitive to turnover by nonsense-mediated decay (42). Surprisingly, the pre-mRNA accumulation defect of bbpΔ56 is largely suppressed in the bbpΔ56, mud2::KAN double mutant background. This is most easily seen with the HZ3 substrate where pre-mRNA levels are 2- to 3-fold higher than with bbpΔ56 alone. Although a more subtle change; spliced mRNA also appears to increase (here by ~35% when normalized to background bands) in the bbpΔ56, mud2::KAN double mutant. Importantly, transformation with a MUD2-containing plasmid reverses this effect, resulting in lowered pre-mRNA levels mostly clearly seen with the HZ3 and HZ10 branchpoint motif mutants (Figure 5D).

Since pre-mRNA is degraded by nonsense-mediated decay in the cytoplasm (42), a reduction in nuclear export of pre-mRNA in the bbpΔ56, mud2::KAN background might contribute to the increased pre-mRNA recovery. To learn if this is the case, we used two previously described splice-sensitive reporters in which the lacZ gene is inserted into exon 2 in frame with either the upstream exon (to monitor splicing) or with the intron (to monitor translation of pre-mRNA exported from the nucleus) (27). Unlike the reporter genes described before (Figure 5), the intron of this export reporter contains no pre-mature translational termination codons and remains stable in the cytoplasm (27). To better control genetic variability, β-galactosidase measurements were made in the bbpΔ56, mud2::KAN double mutant before and after reintroduction of either MUD2 or BBP (see Materials and methods section). The β-galactosidase values are adjusted for variation in gene induction by normalization with an intronless reporter cassette (19,20).

Consistent with the earlier studies (19,20), deletion of MUD2 enhances nuclear pre-mRNA export about 2-fold and results in reduced expression from the inherently inefficiently spliced mRNA reporter (Figure 6). The bbpΔ56 mutant shows a greater reduction in spliced mRNA expression and a corresponding increase in signal from the unprocessed pre-mRNA. Rather than decreasing, β-galactosidase levels increase with export reporter in the bbpΔ56, mud2::KAN double mutant background compared to either single mutant. Based on this, we conclude that the increase in pre-mRNA abundance noted before for the bbpΔ56, mud2::KAN double mutant (Figure 5C and D) cannot be due to a simple reduction in the amount of pre-mRNA exported to the cytoplasm. We note that the signal from the inefficiently spliced reporter also appears to increase slightly in the bbpΔ56, mud2::KAN double mutant when compared with the bbpΔ56 alone (P = 0.0008). While other models are conceivable, these results are consistent with the deletion of MUD2 increasing the synthesis or stability of premRNA in the bbpΔ56 background (see Discussion section). Finally, while normal levels of splicing and export are seen with the smy2::KAN mutant or with the smy2::KAN, bbp Δ56 double mutant, when smy2::KAN is combined with mud2::KAN expression from the export reporter increases significantly (P = 0.0004). This genetic interaction suggests a common intracellular function of Smy2p and Mud2p.

**DISCUSSION**

Specific interactions between BBP and the branchpoint motif occur early in spliceosome assembly that together with the U1 snRNP particle bound to the 5′ splice site contribute to the formation of a nuclear retained structure called the commitment complex (43). In vitro, Mud2p binds BBP to enhance substrate association (23) and recent chromatin immunoprecipitation studies suggest an early-acting role for this protein during spliceosome assembly in vivo (44). In the mammalian system U2AF65 binds tightly to a smaller U2AF subunit, U2AF35 (45), which, together with DEK(46) and PUF60 (47,48) act in 3′ splice site selection. The Schizosaccharomyces pombe (fission yeast) Mud2p/U2AF65 homolog also binds a smaller U2AF subunit, U2AF23, as well as stably interacting with the BBP (22). In contrast to the mammalian and fission yeast systems, our studies identify
a BBP–Mud2p heterodimer as the prominent form of these commitment complex proteins in budding yeast and rule out stable association of a U2A/F35-like third factor.

Previous two-hybrid and protein-interaction studies localized the Mud2p binding domain between residues 41–141 of BBP (37,40). We have refined the map of critical residues by showing that deletion of BBP residues 2–55 significantly weakens or prevents Mud2p association. The BBP–Mud2p heterodimer bridges at least four common two-hybrid interactions. Of these, the BBP-binding partners Prp39p and Mer1p have established roles in early spliceosome assembly consistent with functional associations with the BBP–Mud2p heterodimer. Prp39p is an essential U1 snRNP protein and extracts where Prp39p is inactive or absent fail to form commitment complexes (49). As such, in addition to BBP–Prp40p (3), BBP–Prp39p bolsters the cross-intron association of U1 snRNP with the 5′ splice site and branchpoint region of the pre-mRNA. Mer1p is a meiosis-specific splicing factor that interacts with the Nam8 and Bud13 proteins to promote early spliceosome assembly [(39,50,51) and references within]. The BBP–Mer1p interaction may help integrate early spliceosome assembly [(39,50,51) and references within]. The BBP–Mer1p interaction may help integrate predicted U1 snRNP–Nam8p contacts near the 5′0 within]. The BBP–Mer1p interaction may help integrate early spliceosome assembly consistent with functional associations with the BBP–Mud2p heterodimer. Prp39p is an essential U1 snRNP protein and extracts where Prp39p is inactive or absent fail to form commitment complexes (49). As such, in addition to BBP–Prp40p (3), BBP–Prp39p bolsters the cross-intron association of U1 snRNP with the 5′ splice site and branchpoint region of the pre-mRNA. Mer1p is a meiosis-specific splicing factor that interacts with the Nam8 and Bud13 proteins to promote early spliceosome assembly [(39,50,51) and references within]. The BBP–Mer1p interaction may help integrate early spliceosome assembly [(39,50,51) and references within]. The BBP–Mer1p interaction may help integrate predicted U1 snRNP–Nam8p contacts near the 5′ splice site and branchpoint region of the pre-mRNA. Mer1p is a meiosis-specific splicing factor that interacts with the Nam8 and Bud13 proteins to promote early spliceosome assembly [(39,50,51) and references within].

The biological relevance of the Smy2p-BBP interaction to splicing is less clear. Smy2p was originally identified as a dosage suppressor of a mutation in MYO2, a gene encoding the heavy chain of the yeast type V myosin (57). Smy2p contains a GYF motif (58) (amino acids 205–261), a domain that binds proline-rich sequences and that can be found in other splicing factors, including the U5 snRNP-associated CD2BP2 protein (59,60) and yeast Lin1 (61,62). Although Smy2p is predicted to interact with the proline-rich domains of BBP and Prp8p (63), we detect no reduction in splicing efficiency in the smy2::KAN mutant or evidence for smy2::KAN exacerbating the mud2::KAN or bbpΔ56 splice defects. We note, however, that Smy2p is also predicted to bind a number of cytoplasmic mRNA decay factors including Eap1p, Pat1p, Kcm1p, Mot1p, Ccr4p, Pop2p (64) and that it localizes to cytoplasmic P-bodies enriched in these factors (64). While requiring additional study, the increased RNA export reporter signal in the mud2::KAN, smy2::KAN double mutant might reflect stabilization of cytoplasmic pre-mRNA in the absence of Smy2p and Mud2p. Pre-mRNA stabilization was not obvious for natural transcripts in this background (Figure 5) although this might be explained by the normally rapid degradation of pre-RNAs with premature translational termination codons.

The hypersensitivity to branchpoint mutations observed for BbpΔ56p has been with other BBP mutations (19) and for each mutant assayed impaired splicing is correlated with enhanced pre-mRNA export from the nucleus. Increased nuclear export of pre-mRNA is not limited to mutants of BBP and Mud2p, however, as mutations in a number of early acting splicing factors and other nuclear proteins also show this defect (37,65–67), indicating considerable complexity to the nuclear retained mRNP.

We observe that reduced pre-mRNA recovery with branchpoint motif mutants in the bbpΔ56 background might be partially suppressed by deletion of MUD2, apparently without reducing pre-mRNA export. All else being equal, stabilizing nuclear pre-mRNA in the mud2::KAN, bbpΔ56 background might be expected to provide increased opportunity both for the precursor to be spliced or exported, both possibilities consistent with the results of our reporter gene assays (Figures 5 and 6).

Nuclear pre-mRNA is subject to surveillance by the exosome (68). While the details of exosome target selection are not well understood (69), it seems reasonable that pre-mRNA assembled into spliceosomes will be less sensitive to turnover. The suppression of sub2::KAN lethality by bbpΔ56 and mud::KAN argues for the bypass of the Sub2p-dependent step, possibly enhancing spliceosome assembly. A second but not mutually exclusive possibility for the enhanced pre-mRNA recovery in the mud2::KAN, bbpΔ56 double mutant is that the presence of Mud2p may normally enhance the turnover of intron-bearing pre-mRNA in the cytoplasm. As mutations in the pre-mRNA branchpoint motif increase the level of cytoplasmic pre-mRNA (27), suppression by mud2::KAN might reasonably be most pronounced with branchpoint mutants in the bbpΔ56 background. A cytoplasmic function for Mud2p would be consistent with its localization in both the nucleus and cytoplasm (70), the association of the BBP–Mud2p heterodimer with the P-body linked Smy2p, and with the observation that the metazoan U2AF can be recovered bound to spliced mRNA and to the transcripts of intronless genes (71,72). Finally, given the functional coupling of transcription, splicing, mRNA export, translation and decay [see (73,74) and references within], it is conceivable that Mud2p acts elsewhere in the gene expression pathway to alter cellular pre-mRNA levels.

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