Evidence for complex dynamics during U2 snRNP selection of the intron branchpoint

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ABSTRACT

Splicing of pre-mRNA is initiated by binding of U1 to the 5′ splice site and of Msl5-Mud2 heterodimer to the branch site (BS). Subsequent binding of U2 displaces Msl5-Mud2 from the BS to form the prespliceosome, a step governing branchpoint selection and hence 3′ splice site choice, and linking splicing to myelodysplasia and many cancers in human. Two DEAD-box proteins, Prp5 and Sub2, are required for this step, but neither is stably associated with the pre-mRNA during the reaction. Using BS-mutated *ACT1* pre-mRNA, we previously identified a splicing intermediate complex, FIC, which contains U2 and Prp5, but cannot bind the tri-snRNP. We show here that Msl5 remains associated with the upstream cryptic branch site (CBS) in the FIC, with U2 binding a few bases downstream of the BS. U2 mutants that restore U2-BS base pairing enable dissociation of Prp5 and allows splicing to proceed. The CBS is required for splicing rescue by compensatory U2 mutants, and for formation of FIC, demonstrating a role for Msl5 in directing U2 to the BS, and of U2-BS base pairing for release of Prp5 and Msl5-Mud2 to form the prespliceosome. Our results provide insights into how the prespliceosome may form in normal splicing reaction.

INTRODUCTION

The pre-mRNA splicing reaction takes place on the spliceosome, which consists of five small nuclear RNAs (snRNAs)—U1, U2, U4, U5 and U6—and many proteins. The snRNAs are associated with specific sets of proteins to form small nuclear ribonucleoprotein complexes (snRNPs), and they interact with the pre-mRNA in a sequential manner to assemble the spliceosome. Spliceosome assembly is initiated by binding of U1 to the 5′ splice site, together with binding of Msl5-Mud2 (SF1-U2AF in human) heterodimer to the branch site (BS), to form the commitment complex (CC or E-complex) (1–4). The interaction of Msl5 with the BS is then replaced by U2, which base pairs with the BS sequence, to form the prespliceosome (or A-complex) (5). Following addition of the U4/U6.U5 tri-snRNP, the spliceosome undergoes a major structural rearrangement, releasing U1 and U4 and forming new base pairs between U2 and U6, as well as between U6 and the 5′ splice site (for review, see 6,7). Concomitantly, the Prp19-associated complex (NTC) and several other protein factors are associated with the spliceosome in part to stabilize the interactions of U5 and U6 with the pre-mRNA during formation of the active spliceosome, which catalyzes the two-step transesterification reactions of splicing (8–12).

The spliceosome is a highly dynamic structure that undergoes continuous structural rearrangements throughout the entire splicing cycle (6,7). Structural changes of the spliceosome are mediated by eight DExD/H-box ATPases (13–16), among which Prp5 and Sub2 are involved in formation of the prespliceosome (17–20). The ATPase activity of Prp5 is required for remodeling of U2 snRNP, rendering it functional, and it is further required for binding of U2 to the pre-mRNA independently of ATP (21). Human and fission yeast Prp5 have been shown to interact with both U1 and U2, suggesting a role for Prp5 in bridging the 5′ splice site and the BS for formation of the prespliceosome (22). Prp5 interacts with U2 component SF3b1 (23,24) and with U2 snRNA, and it needs to be released upon binding of U2 to the pre-mRNA before the tri-snRNP can be integrated into the spliceosome (25).

Previous genetic studies identified a U2 branchpoint-interacting stem loop (BSL) structure that presents the U2 nucleotides for interaction with the BS sequence (26). The BSL was observed from cryo-EM analysis of human 17S U2 snRNP, which revealed U2 protein components surrounding the BSL, with the human Cus2 orthologue Tat-SF1 positioned near the loop (24). By UV-crosslinking, Prp5 was shown to contact U2 residues around the base of the BSL stem, suggesting a role for Prp5 in stabilizing or modulating the BSL structure or in promoting U2-BS base pairing during engagement of U2 with the intron (25).

Another DEAD-box protein, Sub2, is required for prespliceosome formation at or before U2 addition (18–20).

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Sub2 is required for formation of commitment complex 2 (CC2) and might be recruited to the pre-mRNA by Msl5-Mud2 (18). It has also been proposed to be responsible for removal of the Msl5-Mud2 heterodimer to enable base pairing between U2 and the BS (20). Msl5 binds the BS with high sequence specificity (27,28), and it interacts with the U1 component Prp40, so Msl5 likely plays a role in bringing the 5′ splice site and the BS into close proximity at the early stage of intron recognition (29,30).

Branch site recognition by U2 is crucial for 3′ splice site (3′SS) selection in human cells. Mutations in the U2 core component SF3b1 are frequently associated with myelodysplasia and many cancers (31–33). SF3b1/Hsh155 HEAT motif mutations can alter BS selection in yeast (23,34). How U2 docks on the BS to replace Msl5-Mud2 is not clear. Mud2 was shown to interact with the U2 component Prp11 by yeast two-hybrid assays, and it may play a role in recruiting U2 to the BS (35). Since Mud2 is dispensable for yeast vegetative growth and for the splicing reaction, the recruitment of U2 to the BS may involve additional factors. The human protein SUGP1 has recently been shown to play an important role in BS recognition (36). SUGP1 interacts with both SF3b1 and SF1-U2AF, and absence of SUGP1 or the presence of a mutation in SF3b1 that disrupts its interaction with SF1 results in aberrant selection of the BS and 3′SS. This suggests that SF1-U2AF are important in defining BS-3′SS specificity, but other factors might be required for ultimate selection of the branchpoint.

We have previously identified a Prp5-associated intermediate splicing complex, FIC (Prp <u>F</u>ive <u>I</u>ntermediate <u>C</u>omplex), using *ACT1* pre-mRNA carrying mutations at the -2 position of the BS (U257). When splicing is performed with pre-mRNA containing the U257 mutation, splicing is arrested before the tri-snRNP is added and Prp5 is still associated with the pre-mRNA (25). However, whether the Msl5-Mud2 heterodimer is still present on FIC or how Prp5 is retained on the spliceosome to prevent the binding of the tri-snRNP was not known. Here, we show that Msl5 and Mud2 remain associated with FIC, but neither Msl5 nor U2 is correctly positioned at the mutated BS. While Msl5 interacts with a cryptic branch site (CBS) upstream of the BS, we show via psoralen crosslinking that U2 is bound to sequences downstream of the BS in two regions. Deletion of the CBS greatly reduces FIC formation as well as splicing rescue by compensatory U2 mutations that restore U2-BS base pairings using synthetic U2 snRNA, indicating that the CBS contributes in critical ways to splicing through the authentic BS. Our results suggest that Msl5 is important for stable association of U2 with the pre-mRNA and helps U2 identify a region from which a branchpoint can be selected, but other factors are required for determining which branchpoint is ultimately selected.

MATERIALS AND METHODS

Yeast strains

The yeast strains used were BJ2168 (*MATa prc1 prb1 pep4 leu2 trp1 ura3*), YSCC024 (*MATa prc1 prb1 pep4 leu2 trp1 ura3 HSH155-HA*), YSCC026 (*MATa prc1 prb1 pep4 leu2 trp1 ura3 PRP5-V5*), YSCC040 (*MATa prc1 prb1 pep4 leu2 trp1 ura3 LSR1∆::LSR1-S*), YSCC041 (*MATa prc1 prb1 pep4 leu2 trp1 ura3 Msl5-HA*) and YSCC042 (*MATa prc1 prb1 pep4 leu2 trp1 ura3 Mud2-HA*).

Antibodies and reagents

Anti-HA antibody 8G5F is a monoclonal antibody produced by immunizing mice with a keyhole limpet hemocyanin-conjugated HA peptide. The anti-V5 antibody was purchased from Serotec Inc. Anti-Lea1, anti-Prp5, anti-Msl5 and anti-Prp8 are polyclonal antibodies produced by immunizing rabbits with full-length recombinant protein (Lea1) or a segment of the corresponding proteins (amino acid residues 510–480 for Prp5, 273–412 for Msl5 and 1–115 for Prp8) expressed in *Escherichia coli*. Protein A-Sepharose CL-4B was purchased from GE Healthcare, SuperScript III from Invitrogen, proteinase K from MD Bio Inc. and RNase H from Promega. Dinucleotide 4-thio-UpG was purchased from Dharmacon. SP6 RNA polymerase was purchased from Promega, RNase P1 from Sigma, and RNasin and T4 RNA ligase 2 were from Enzymatics.

Oligonucleotides

The following oligonucleotides were used for construction of the U2S strain:

U2-1: CAGCAGGATAGCGAA
U2-2: CCGGCATATGCGGAAAACAACTTGC TT
U2-3: GGCCCTCGAGACTAGCATGAGAAACGA
U2-4: CCGGCATATGACGCCTGTTTTTAAAGT

The following oligonucleotides were used for oligonucleotide-directed RNase H cleavage and primer extension:

I: TCTTACAGTTAAATGGGATGG
II: AGATCAGTCAATATAGGAGGTTATGGGAGA
III: CTATCACTTATCACGAA
IV: CCTCTAAACATATAA
V: ACCGGCTTTACACATACCAG
U2-B: GCCTCATTGAGGTCATTTCAG

The following 5′-biotinylated oligonucleotides were used for affinity selection:

VI$_{bio}$: GGAGGTTATGGGAGAGTG
U1$_{bio}$: CAGTAGGACTTCTTGATC
U2-A$_{bio}$: CCATTTTATTATTTTGGGTGCC

Construction of the U2S strain YSCC040

A 0.5 kilobase (kb) DNA fragment containing the 5′ part (1–175) of the U2 coding sequence and 340 nucleotides of the upstream sequence was generated by polymerase chain reaction (PCR) using primers U2-1 and U2-2, and then digested with *Hind*III and *Nde*I. Another 0.4 kb DNA fragment containing the 3′ part (1068–1175) of U2 and 335 nucleotides of the downstream sequence was generated by PCR using primers U2-3 and U2-4, and digested with NdeI and XhoI. The two fragments were ligated together with HindIII- and XhoI-digested pRS406. The U2S sequence was used to replace the full-length U2 gene in yeast strain BJ2168 by means of the pop-in and pop-out gene replacement method (37).

Preparation of splicing extracts and substrates

Yeast whole-cell extracts were prepared according to the method of Cheng *et al.* (38). Splicing substrates were synthesized by *in vitro* transcription with SP6 RNA polymerase using EcoRI-linearized plasmid pSPAct6-88 and its derivatives as templates. 4sU-labeled pre-mRNAs were prepared according to the procedure of Chung *et al.* (39).

In vitro splicing assay, immunodepletion and immunoprecipitation of the spliceosome

Splicing assays were carried out according to the procedure of Cheng *et al.* (38) at 25°C for 30 min using 40% (v/v) regular extracts or 50% (v/v) immunodepleted extracts unless otherwise indicated. Immunodepletion of Prp5 was performed as described by Liang *et al.* (25). Immunodepletion of Prp8 was performed by incubating 100 μl of splicing extracts with 100 μl anti-Prp8 antibody conjugated to 50 μl of protein A-Sepharose (PAS) at 4°C for 1 h. PAS was removed by centrifugation to recover Prp5- or Prp8-depleted extracts. Immunoprecipitation was performed as described by Tarn *et al.* (9) with anti-HA, anti-Lea1, anti-Prp5, anti-V5 or anti-Msl5 antibody. For each 20 μl of the splicing reaction, 30 μl of anti-HA, 5 μl of anti-Lea1, 3 μl of anti-Prp5, 1 μl of anti-V5 or 1 μl of anti-Msl5 antibody was used. Splicing reactions were incubated with antibody-conjugated PAS at 4°C for 1 h.

Crosslinking with 4sU-labeled pre-mRNA

Site-specific 4sU-crosslinking was performed according to published procedures (40) using actin pre-mRNA containing a single 4sU with ^{32}P labeled at its 5′-end. Splicing reactions were carried out in the Prp8-depleted extracts to accumulate the prespliceosome. The reaction mixtures were then placed as drops on a precooled Parafilm-covered aluminum block and irradiated (365 nm) for 10 min at a distance of ∼3 cm to the UV lamp in a CL-1000 Ultraviolet Crosslinker system (UVP). Each 10 μl of the irradiated mixture was precipitated with PAS conjugated with specific antibodies, and incubated with an equal volume of solution containing 0.06 U/μl Nuclease P1 and 6X Complete EDTA-free Protease Inhibitor Cocktail at 37°C for 30 min. Proteins were analyzed on SDS-PAGE. To identify specific proteins, the irradiated mixtures were treated with Nuclease P1 as described above and then denatured in the presence of 1% SDS, 1% Triton X-100 and 100 mM DTT in boiling water for 90 s. The mixtures were diluted 10-fold with NET-2 buffer containing 300 mM NaCl and subjected to immunoprecipitation. The precipitates were treated with Nuclease P1 again and analyzed by electrophoresis on SDS-PAGE.

Psoralen crosslinking and primer extension

Psoralen crosslinking was performed according to published procedures (41). AMT (4′-aminomethyl-4,5′,8-trimethyl) psoralen was added to splicing reaction mixtures at a final concentration of 20 μg/ml, and the mixtures were distributed as drops on a precooled Parafilm-covered aluminum block and covered with a 3 mm-thick glass plate to prevent photo-reversal of psoralen crosslinks. The

aluminum block was irradiated with UV$_{365 nm}$ for 10 min at a distance of 3–5 cm from the light source, and the irradiated mixtures were subjected to immunoprecipitation. RNAs were extracted and selected with biotinylated oligonucleotides according to the method of Chan *et al.* (12). Primer extension was performed with SuperScript III reverse transcriptase based on the method of Chan *et al.* (12) using primer U2-B. Extension products were analyzed by electrophoresis on 12% polyacrylamide/8M urea gels.

UV-crosslinking

Splicing reactions were carried out in the absence of ATP with 2 nM of pre-mRNA at 10-fold specific radioactivity in ATP-depleted extracts to accumulate Msl5-associated commitment complex. Reaction mixtures were placed in a 10-cm culture plate (pre-chilled on a bed of ice) and irradiated with UV$_{254 nm}$ in a UV Stratalinker 1800 (Stratagene) at a distance of ∼5 cm from the light source with an energy level of 0.8 J/cm^2. Irradiated mixtures were denatured in the presence of 1% SDS, 1% Triton X-100 and 100 mM DTT in boiling water for 3 min, then diluted 10-fold with NET-2 buffer and subjected to immunoprecipitation. To map the Msl5-binding site on the pre-mRNA, splicing reactions were carried out in Msl5-HA extracts with 2 nM pre-mRNA. After UV irradiation, denaturation and immunoprecipitation with anti-HA antibody, crosslinked products were subjected to primer extension analysis.

Quantification

Gels were exposed to GE Storage Phosphor Screen (GE Healthcare Life Sciences). RNA bands were quantified with a TyphoonTM FLA 9000 system (GE Healthcare Life Sciences) and analyzed using ImageQuant TL7.0 (GE Healthcare Life Sciences). To calculate the splicing efficiency in Figure 6, the values of quantified RNA bands were normalized to the number of uridine in each RNA species. Splicing efficiency was determined by the ratio of L + M to L + M + P (where L, P and M represent the molar amounts of lariat-intermediate, pre-mRNA and mRNA product, respectively).

RESULTS

Aberrant interactions of U2 snRNP with BS-mutated pre-mRNA

To understand how Prp5 is arrested on the pre-mRNA containing a single mutation at the branch site, we first examined whether the conformation of FIC resembles that of the prespliceosome by probing protein-pre-mRNA interactions in the BS region using a site-specific crosslinking technique. We synthesized *ACT1* pre-mRNAs containing a single 4-thiouridine (4sU) ^{32}P-labeled at its 5′-end at positions flanking the BS (39). Splicing reactions were carried out with wild-type (WT) or U257G pre-mRNA in Prp8-depleted extracts to arrest the reaction before tri-snRNP binding. Upon irradiation with UV$_{365nm}$, the reaction mixtures were immunoprecipitated with antibody against U2 component Lea1 to isolate U2-containing spliceosomes, and proteins crosslinked to the pre-mRNA were analyzed on SDS-PAGE

following digestion of the precipitates with ribonuclease P1 (Figure 1). The identities of crosslinked proteins were confirmed by immunoprecipitation of denaturant-treated reaction mixtures with specific antibodies (Supplementary Figure S1). The results show that the pattern of proteins crosslinked to U257G pre-mRNA around the BS is completely different from that of wild-type pre-mRNA. Hsh155 (yeast SF3b1) crosslinked most strongly to the + 3 position, and less so to the -7, +12 and + 18 positions of WT pre-mRNA as has previously been reported (39), yet crosslinks were only barely detected at these positions on U257G pre-mRNA. Crosslinking of U2 components Prp9, Cus1, Prp11 and Hsh49 observed on WT pre-mRNA at the -16 position was barely or not detected on U257G pre-mRNA. Instead, Hsh49 was crosslinked to the -7 position on the U257G pre-mRNA. Several unidentified crosslinks were also observed at the -7 position on the U257G pre-mRNA. Crosslinks at positions + 12, +18 and + 25 by proteins, likely to be components of the retention and splicing (RES) complex as observed on the B^{act} spliceosome (39), were also only observed for WT but not U257G pre-mRNA. In contrast, Msl5 and Mud2, both of which migrated as around ∼60 kD, were observed to crosslink more strongly to the U257G than WT pre-mRNA across the BS, suggesting either that Msl5-Mud2 are retained on FIC, or more commitment complex may accumulate with U257G than with WT pre-mRNA. These results suggest that the conformation of FIC is distinct from that of the prespliceosome in the BS region.

Failure to detect interactions of U2 components with BS flanking sequences suggests that U2 either does not interact with the pre-mRNA or does not interact with the BS on FIC. Next, we examined how U2 snRNA interacts with U257G pre-mRNA in comparison with WT pre-mRNA by psoralen crosslinking. A short form of U2 (U2S) containing only functional domains (42,43) was constructed to replace the long U2 snRNA to better facilitate crosslinking analysis (Supplementary Figure S2). Splicing was performed in Prp8-depleted extracts prepared from the U2S yeast strain, and the reaction mixtures were irradiated with UV$_{365nm}$ upon addition of AMT (4′-aminomethyl-4,5′,8-trimethyl) psoralen (40). RNA was extracted and annealed with a biotinylated oligonucleotide complementary to U2. U2-crosslinked products were then selected by binding to streptavidin agarose. Figure 2A shows that only one crosslinked product was recovered for both WT (lane 2) and U257G (lane 5) pre-mRNAs, indicating that U2 also directly interacts with U257G pre-mRNA. RNase H mapping of crosslinked sites revealed crosslinking of U2 to the same region of both pre-mRNAs within a segment of ∼100 nucleotides flanking the branchpoint (Supplementary Figure S3). Primer extension was performed to determine the crosslinked residues. A strong stop at -6 and two weaker ones at -7 and -8 positions were observed for WT pre-mRNA. These stops infer crosslinks at -7, -8 and -9 positions, respectively, upstream of the branchpoint (Figure 2B). Interestingly, major crosslinks on U257G pre-mRNA were detected at + 3 and + 8 positions downstream of the branchpoint, indicating that U2 was not positioned correctly at the BS. To map the crosslinked sites on U2, crosslinked products were selected with an oligonucleotide complementary to *ACT1* pre-mRNA for primer extension

analysis using a U2 primer. A single crosslinked site was identified at position 40 of U2 for both WT and U257G pre-mRNAs (Figure 2C). Based on these results, we propose a model for how U2 may interact with WT pre-mRNA on the prespliceosome (Figure 2D) and with U257G pre-mRNA on FIC in the BS region (Figure 2E and F), which shows U2 may base pair with the BS downstream region through sequences in the BSL loop or stem. This also suggests that U2 may fluctuate its structure by unfolding and refolding BSL in search for the BS.

Msl5 is associated with FIC

Although it only accumulates on mutant pre-mRNA substrates, FIC is presumed to reflect an unstable but normal precursor complex of the prespliceosome, containing U2 and Prp5 but not the tri-snRNP (25). It is not known whether Msl5 and Mud2 are still associated with the pre-mRNA or have already been displaced in this structure. The observation that U2 is not yet positioned on the BS suggests that Msl5-Mud2 might still occupy the BS on FIC. We assessed if Msl5 is associated with the pre-mRNA under the condition of FIC formation using U257G pre-mRNA (Figure 3A). Splicing was carried out in Msl5-HA extracts, and the reaction mixtures were precipitated with antibodies against Prp5, Lea1 for U2 snRNP and HA for Msl5. The results show that anti-HA antibody precipitated the pre-mRNA nearly as efficiently as anti-Lea1 or anti-Prp5 antibodies, suggesting that Msl5 is associated with the pre-mRNA under the condition of FIC formation.

Since Msl5 binds to the pre-mRNA to form the CC2 prior to binding of U2 (29), it is possible that coprecipitation of pre-mRNA with Msl5 represents the presence of CC2 instead of FIC in the reaction mixture. Accordingly, we investigated by co-immunoprecipitation if Msl5 is associated with U2 and Prp5 in the same splicing complex (Figure 3B). Splicing reactions carried out in Prp5-V5 extracts were precipitated with anti-Lea1, anti-V5 or anti-Msl5 antibodies and the precipitated proteins were analyzed by Western blotting. The results show that antibody against each protein coprecipitated the other two more markedly in the presence of U257G pre-mRNA than WT or no pre-mRNA, indicating that Msl5, Lea1 and Prp5 are associated in the same complex during splicing with U257G pre-mRNA. Since neither U2 nor Prp5 is associated with the CC, we conclude that Msl5 remains associated with FIC together with U2.

Interaction of Msl5 with an upstream cryptic branch site is required for FIC formation

Msl5 harbors an RNA-binding KH-QUA2 domain, which recognizes the conserved branch site sequence UACUAAC. Mutation of branchpoint A$_0$ or U$_{-2}$ abolishes RNA binding, and mutation of C$_{+1}$ or A$_{-4}$ weakens RNA binding by Msl5 fragments containing the KH-QUA2 domain (27,28). Since Msl5 is associated with U257G pre-mRNA during the splicing reaction, we assessed by UV-crosslinking if Msl5 binds BS-mutated pre-mRNA under splicing conditions. First, we targeted the CC by performing splicing in the absence of ATP with WT or U257 mutant pre-mRNA in Msl5-HA extracts. Following denaturation, the reaction mixtures
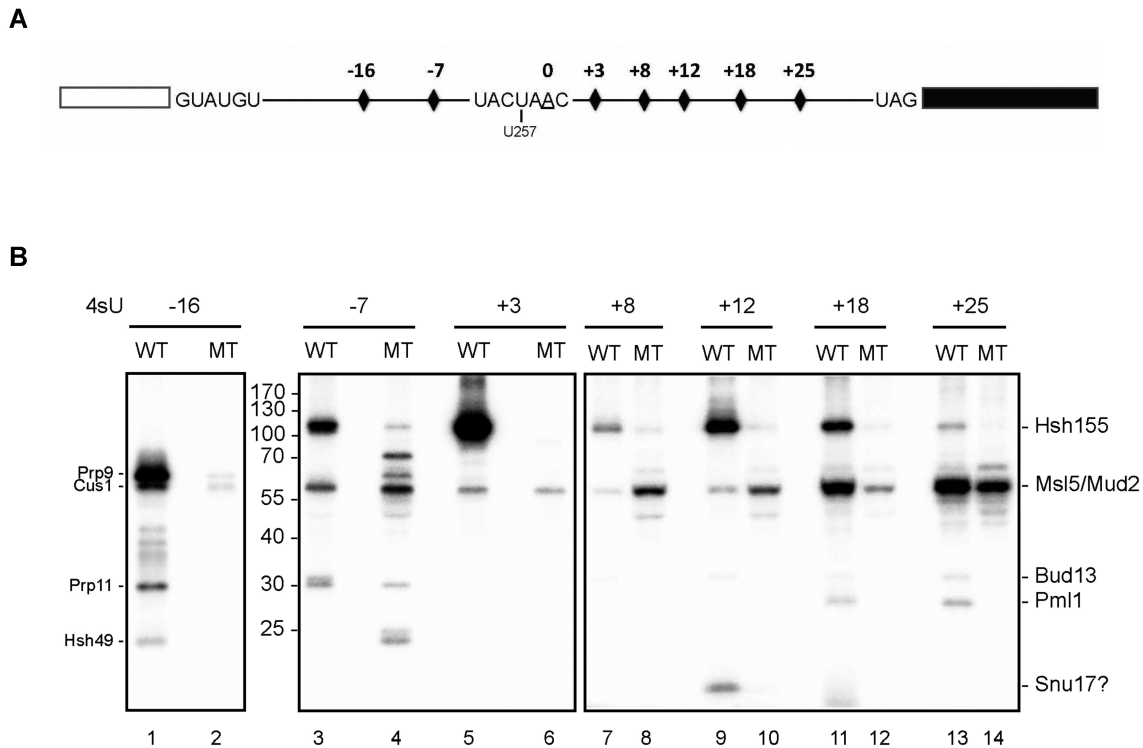
Figure 1. Analysis of proteins crosslinked in the BS-3′SS region of *ACT1* intron. (**A**) Positions of 4sU relative to the branchpoint adenosine (position 0). (**B**) Splicing reactions were performed using wild-type or U257G pre-mRNA with 4sU labeled in the branch site region at indicated positions in Prp8-depleted Hsh155-HA extracts. WT, wild-type; MT, U257G mutant.

were precipitated with anti-HA antibody, and RNA was extracted for analysis. We found that Msl5 bound WT pre-mRNA and, to a lesser extent, U257A pre-mRNA with or without crosslinking (Figure 4A, lanes 3 and 7). Primer extension analysis revealed that Msl5 crosslinked to WT pre-mRNA at U_{-5} within the BS sequence but to U257 mutant pre-mRNA at a site upstream of the BS at U_{-19} within the cryptic branch site (CBS) sequence UACUAAG. This outcome indicates that though Msl5 binds the BS sequence on WT pre-mRNA, its binding to the mutated BS was not detectable by crosslinking. Instead, Msl5 was detected to bind to the upstream CBS (Figure 4B, lanes 1 and 2). This observation is consistent with reported sequence specificity for RNA binding by Msl5-(KH-QUA2) (28).

To establish the importance of the CBS for Msl5 binding to the pre-mRNA, we analyzed crosslinking of Msl5 with WT and U257 mutant pre-mRNAs having six nucleotides UACUAA deleted from the CBS (CBSΔ). As expected, neither association of Msl5 with the pre-mRNA nor crosslinking of Msl5 to the pre-mRNA was greatly affected by deletion of the CBS from WT pre-mRNA, indicating preferential binding of Msl5 to the authentic BS. However, when we deleted the CBS from U257 mutant pre-mRNA, both association and crosslinking of Msl5 were compromised, suggesting that this CBS is the primary Msl5-binding site for U257 mutant pre-mRNA (Figure 4A, lanes 10 and 14). Primer extension revealed that Msl5 crosslinked to the same U_{-5} residue within the BS when the CBS was deleted from WT pre-mRNA. When the CBS was removed from U257 mutant pre-mRNA, no specific crosslinked site was detected

from the small amount of crosslinked product (Figure 4B, lanes 3 and 4), suggesting that the interaction of Msl5 with the CBS-deleted U257 mutant pre-mRNA might be dynamic and/or lacks sequence specificity. Deletion of the CBS also prevented FIC formation as revealed by failure to coprecipitate the pre-mRNA with Lea1 or Prp5 (Figure 4C).

The cryptic branch site is required for splicing rescue of U257 mutant pre-mRNA

We were intrigued that a single point mutation at U257 results in retention of Prp5 on the spliceosome, and the consequent inhibition of tri-snRNP binding. Since such mutations disrupt U2-BS base pairings, we queried if restoring U2-BS base pairings could release Prp5 arrest and splicing block. U2 can be depleted from splicing extracts via oligonucleotide-directed RNase H cleavage and be reconstituted with *in vitro* synthesized U2 snRNA (44). It has previously been shown that splicing of U257A pre-mRNA could be rescued by using the compensatory U2-A36U mutant, albeit with low efficiency (44). We performed reconstitution experiments to determine if restoring U2-BS base pairings would prevent Prp5 retention. We observed much better splicing of U257 mutant pre-mRNAs with U2 compensatory mutations than previously reported (Figure 5A). Whereas splicing was completely inhibited upon U2 depletion (lanes 1, 5 and 9), addition of *in vitro*-synthesized U2 RNA recovered the splicing activity in all reactions, but with much greater activities when U2-BS complementar-
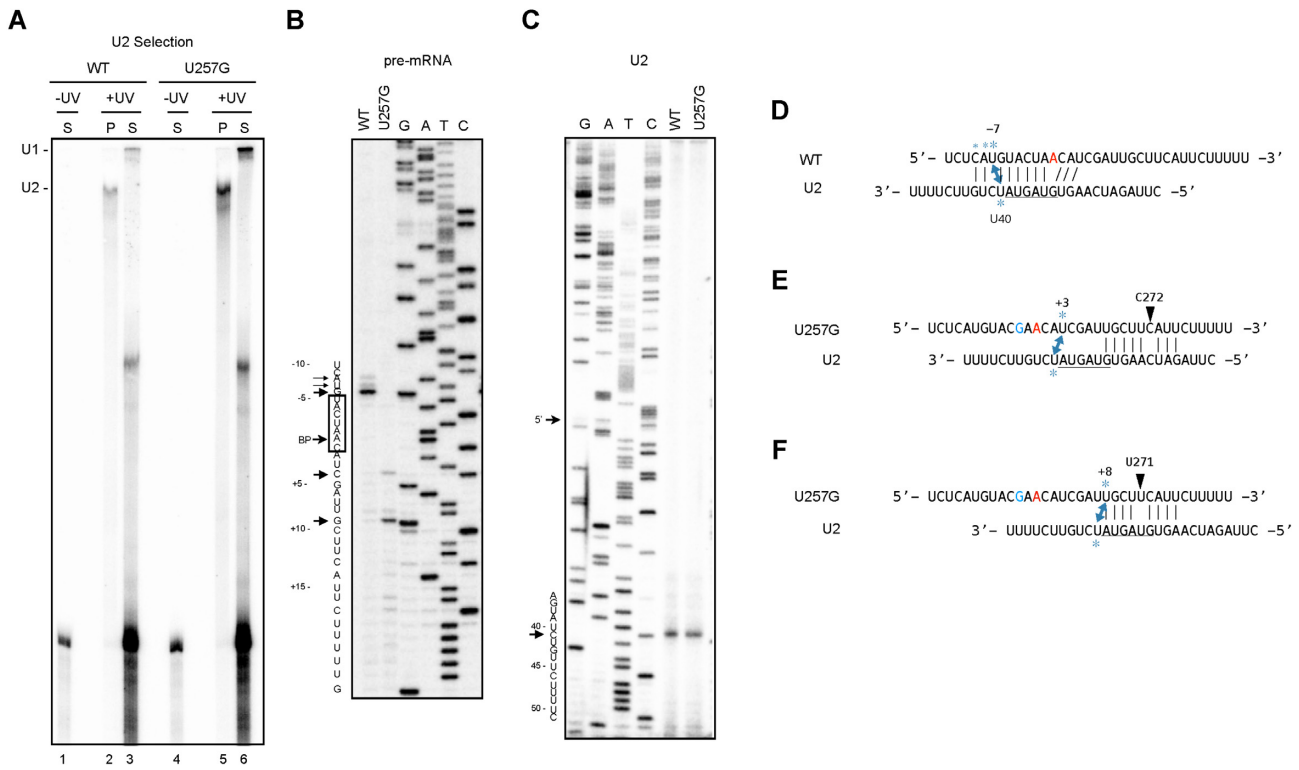
Figure 2. Dynamic interaction of U2 with the pre-mRNA on FIC. (**A**) Splicing reactions were performed with wild-type or U257G pre-mRNA in Prp8-depleted extracts, and the reaction mixtures were irradiated with UV_{365nm} in the presence of psoralen. The mixtures were then precipitated with anti-Lea1 antibody. RNA was extracted from the precipitates, and U2-crosslinked products were selected with a 5′-biotinylated U2 antisense oligonucleotide; S, supernatant; P, pellet. (**B**) Primer extension was performed to map U2-crosslinked sites on the pre-mRNA using an actin primer. The branch site (BS) is boxed with a solid line. BP, branchpoint. Arrows mark primer extension stops. (**C**) U2-pre-mRNA crosslinked products were isolated as in (A), and selected by a 5′-biotinylated actin oligonucleotide for primer extension to map crosslinked sites on U2. The arrow marks primer extension stop. (**D–F**) Proposed base pairing patterns between U2 and the WT (D) and U257G (E and F) pre-mRNAs near the branch site. The BS is indicated in red, and the BSL loop is indicated in green. * marks crosslinked residues, and arrows connect crosslinked residues.
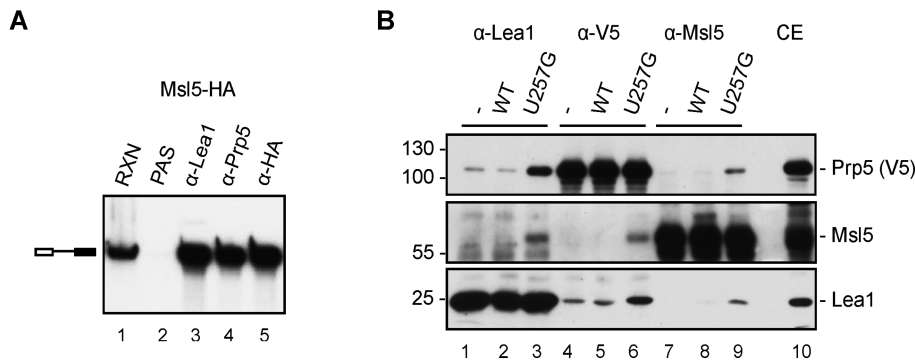

Figure 3. Association of Msl5 with FIC. (**A**) Splicing reactions were performed with U257G pre-mRNA in Msl5-HA extracts, and the reaction mixtures were precipitated with anti-Lea1, anti-Prp5 or anti-HA antibody. (**B**) Splicing reactions were performed with WT or U257G pre-mRNA in Prp5-V5 extracts, and the reaction mixtures were precipitated with anti-Lea1, anti-V5 or anti-Msl5 antibody. Precipitates were analyzed by Western blotting and probed with anti-V5, anti-Msl5 and anti-Lea1 antibodies.

ity was restored (lanes 2, 7 and 12). The fact that low levels of splicing occurred with mismatched U2-BS pairing (lanes 3, 4, 6, 10 and 11) implied that U2 might be using the CBS as the branch site. To map the branchpoint, we carried out splicing reactions with U257G pre-mRNA in Prp16-depleted extracts to accumulate greater amounts of lariat intron-exon 2 for primer extension analysis (Figure 5B). Interestingly, although a small amount of RNA used A_{-14} (A_0

being the branchpoint of the authentic BS) within the CBS as the branchpoint, the majority used A_{-1} of the mutated BS as the branchpoint (lane 2). This outcome suggests that despite being the Msl5-binding site and potentially also forming extensive base pairing with the BSL loop, the CBS is not favored for stable interaction with U2 during the splicing reaction although when chosen it is used correctly (Figure 5B). The correct branchpoint at the BS was used when
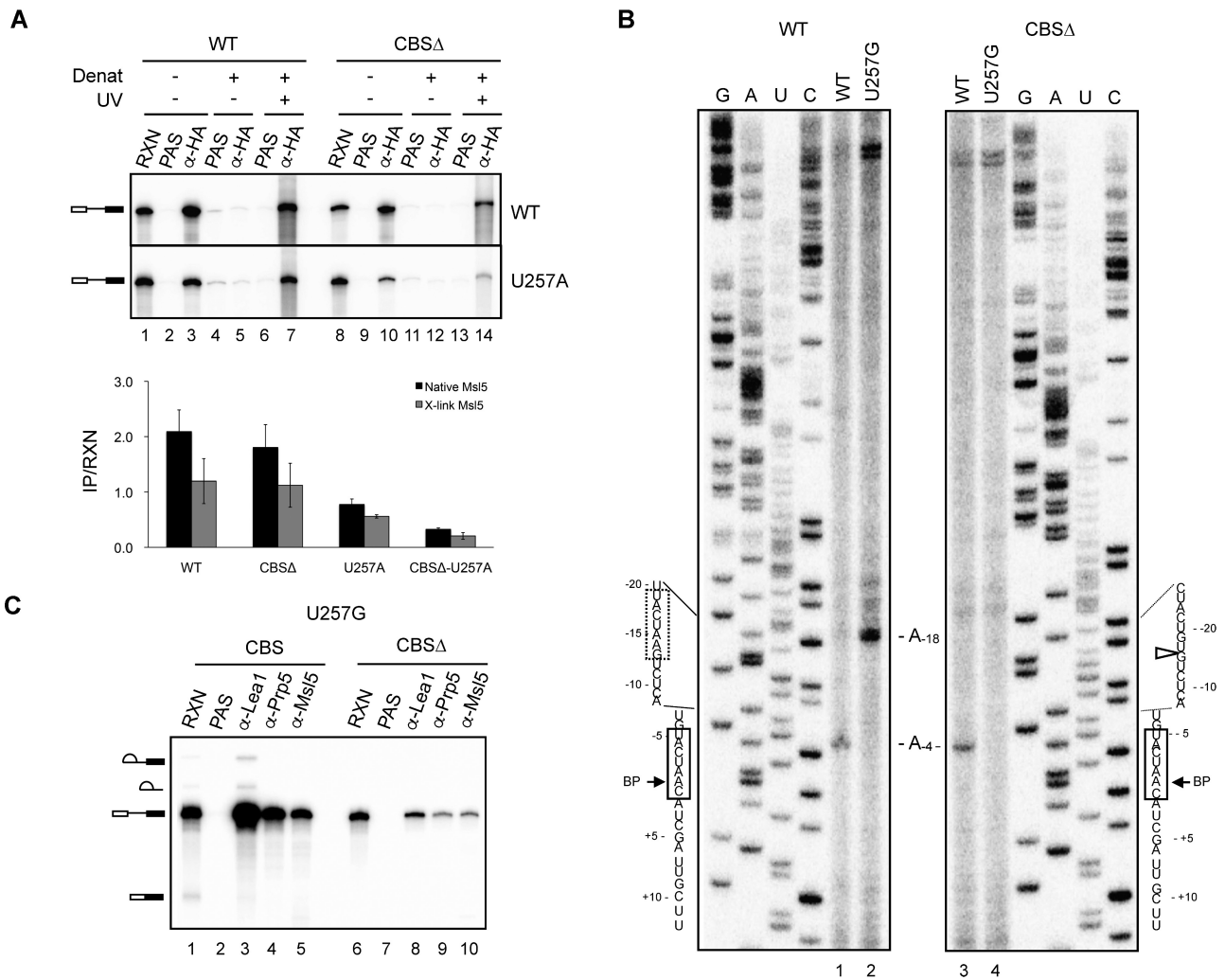
Figure 4. Msl5 binds on the CBS. (**A**) Splicing reactions were carried out in the absence of ATP with wild-type or U257A pre-mRNA with (lanes 1–7) or without (lanes 8–14) CBS deletion in Msl5-HA extracts. The reaction mixtures were irradiated with UV_{254nm}, and precipitated with anti-HA antibody without (lanes 2 and 3) or with (lanes 4–7) prior denaturant treatment. RNA was quantified by a PhosphorImager. The ratios of the pre-mRNA precipitated by antibodies to total reaction of each reaction set from three experiments are plotted in a bar graph. RXN, 1/10 of total reaction; PAS, protein A-Sepharose. (**B**) Msl5-crosslinked products were analyzed by primer extension to map crosslinking sites. The authentic BS is boxed with a solid line, and the CBS is boxed with a dotted line. The position of the deleted CBS is marked by the triangle. BP, branchpoint; A_{-4} and A_{-18}, primer extension stops. (**C**) Immunoprecipitation of the spliceosome formed with U257G pre-mRNA without (lanes 1–5) or with (lanes 6–10) CBS deletion.

U2-BS base pairing was restored (Figure 5B, lane 1). Accordingly, Prp5 did not accumulate on the U257G spliceosome when we used the U2-A36C mutant, as revealed by immunoprecipitation of the spliceosome with anti-Prp5 antibody (Figure 5C, compare lane 16 with lane 10). These results clearly demonstrate that proper U2-BS base pairing is critical for the release of Prp5 from the spliceosome.

Msl5 is essential for splicing and is required for FIC formation. Given that Msl5 binds on the CBS instead of the U257-mutated BS, the CBS is likely required for splicing rescue of U257 mutant pre-mRNA. We again employed CBSΔ U257G mutant pre-mRNA and found that splicing could not be rescued by U2-A36C (Figure 5D, lane 6). This outcome suggests that Msl5 plays a crucial role for recruiting U2 to a region where a potential branchpoint resides. The fact that U2 crosslinked to the BS downstream regions

instead of the BS on U257 mutant pre-mRNA led us to speculate that in normal splicing reactions, U2 may be recruited to the pre-mRNA by first interacting with U1 or Msl5-Mud2, and is then loaded onto the pre-mRNA in the BS downstream region, guided by interaction with Msl5-Mud2, to seek the BS. Upon base pairing with the BS, the BSL structure is totally disrupted if pairing between U2 and the BS is correct, and Prp5 is released. U2 is stalled on the pre-mRNA in the BS downstream region when mutation in the branch site causes a mismatch within the U2-BS base pairings. Conceivably, if the CBS is located downstream of the BS, targeting of U2 to the BS may be impaired due to structural hindrance by Msl5-Mud2 or due to conformational constraints. To test those possibilities, we moved the CBS to a position downstream of the branch site with putative branchpoint of the CBS at the + 16 position (CBS$_{+16}$,
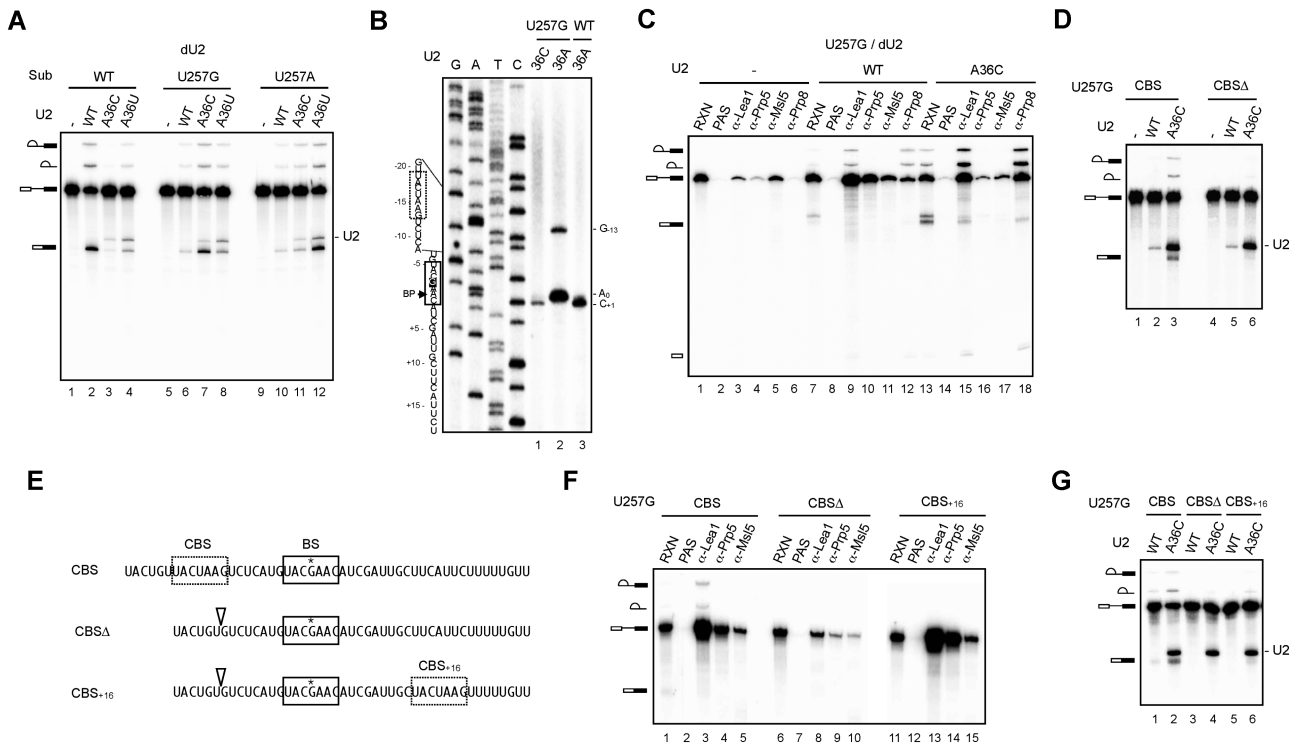
Figure 5. Restoring U2-BS base pairing and an upstream CBS are required for Prp5 release and progression of the splicing reaction. (**A**) Splicing reactions were performed with wild-type, U257G or U257A pre-mRNA in U2-depleted extracts supplemented without (lanes 1, 5 and 9) or with wild-type (lanes 2, 6 and 10), U2-A36C (lanes 3, 7 and 11) or U2-A36U mutant (lanes 4, 8 and 12). (**B**) Primer extension of purified lariat IVS-E2 to map the branchpoint of pre-mRNA performed in U2-depleted extracts supplemented with U2-A36C (lane 1) or U2-A36 (lane 2). Splicing with wild-type pre-mRNA was used as a reference (lane 3). The authentic BS is boxed with a solid line; the CBS is boxed with a dotted line, and 257G is shaded. BP, branchpoint; C_{+1}, A_0 and G_{-13}, primer extension stops. (**C**) Splicing reactions were performed with U257G pre-mRNA in U2-depleted extracts supplemented without (lanes 1–6), or with wild-type U2 (lanes 7–12) or U2-A36C (lanes 13–18) U2S. The reaction mixtures were precipitated by anti-Lea1, anti-Prp5, anti-Msl5 or anti-Prp8 antibody; RXN, 1/10 of total reaction; PAS, protein A-Sepharose. (**D**) Splicing reactions were performed with CBS (lanes 1–3) or CBSΔ (lanes 4–6) U257G pre-mRNA in U2-depleted extracts supplemented without (lanes 1 and 4) or with wild-type U2 (lanes 2 and 5) or U2-A36C (lanes 3 and 6). (**E**) U257G pre-mRNA sequences in the BS flanking region with the CBS upstream (CBS), deleted (CBSΔ) or downstream (CBS_{+16}) of the mutated BS. (**F**) Immunoprecipitation of the spliceosome formed with U257G pre-mRNA deleted of the CBS (CBSΔ, lanes 6–10), or with the CBS present upstream (CBS, lanes 1–5) or downstream (CBS_{+16}, lanes 11–15) of the BS. (**G**) Splicing reactions were performed with CBS (lanes 1 and 2), CBSΔ (lanes 3 and 4) or CBS_{+16} U257G pre-mRNA in U2-depleted extracts supplemented with wild-type U2 (lanes 1, 3 and 5) or U2-A36C (lanes 2, 4 and 6).

Figure 5E). Subsequent splicing reactions showed that, despite the pre-mRNA being capable of FIC formation (Figure 5F, lanes 13–15), the CBS_{+16} barely supported splicing of U257G pre-mRNA with U2-A36C (Figure 5G, lane 6). Crosslinking analysis confirms that U2 indeed interacts with the pre-mRNA in the BS downstream region similarly to when the CBS is upstream of the BS (Supplementary Figure S4). This result supports the notion that the position of the CBS is important for splicing rescue and suggests that in normal splicing, U2 scanning can be effective to reach the BS only when Mud2-Msl5 binds on or upstream of the BS.

Loading of U2 onto the pre-mRNA to probe for the branch site

We speculated that U2 crosslinked sites on U257G pre-mRNA (Figure 2) may represent sites that allow stable interactions of U2 with the pre-mRNA in the absence of a perfect branchpoint, and reasoned that increasing base pairings between U2 and the intron sequence in this region might further deter targeting of U2 to the mutant BS

and slow down the splicing reaction. We created single or double mutations at positions 270, 271 and 272 of the intron to increase complementarity between U2 and the intron sequence in the region where crosslinks were identified (Figure 6A) and assayed for splicing rescue of U257G pre-mRNA by U2-A36C. Indeed, single-mutant C272G or double-mutant C270G/U271A, both of which can potentially form nine consecutive base pairings with U2-A36C, showed slower kinetics than original pre-mRNA with mismatches within the complementary region (Figure 6B). Notably, although increasing base pairing in each interaction mode results in corresponding reduction of base pairing in the other mode, the reaction rate appeared to more significantly respond to enhanced base pairing. We then tested if the C272G mutant would also affect splicing of pre-mRNA with wild-type BS and U2 (Figure 6C) by performing splicing at 15°C to slow down the reaction so that smaller differences could be resolved. Indeed, the C272G mutation resulted in a noticeably slower kinetics (Figure 6D). These results suggest that increasing base pairings between U2 and the BS downstream sequences may deter targeting of U2 to the branch site.

A model for targeting U2 to the branch site

Based on the results of this work and previous reports, we propose a model for how U2 might be targeted to the BS (Figure 7). Human and fission yeast Prp5 have been shown to interact with U1 and U2 through distinct domains (22). SpPrp5 directly binds U2 component SF3b, and also interacts with U1 through SR protein Rsd1 in bridging U1 and U2 (45). Thus, U2 can be recruited to the CC by interacting with U1, and upon binding to CC2, is presented with a pre-mRNA region downstream of the Msl5-Mud2 binding site. U2 is then translocated to the pre-mRNA, and is stabilized on the complex through protein–protein, protein–RNA and RNA–RNA interactions. U2 then searches for sequences that can form proper base pairing by moving along the pre-mRNA or dynamically docking and undocking from the pre-mRNA until it reaches the BS. Msl5-Mud2 likely also interact with the pre-mRNA in a dynamic manner so that U2 can access the BS. Proper base pairing between U2 and the BS leads to displacement of Msl5 from the BS and of Prp5 from U2. When the branch site contains the U257 mutation and a CBS is present on the pre-mRNA upstream of the BS, Msl5 cannot bind to the mutated BS, and it instead binds to the CBS with lower affinity. Mutations in the BS weaken U2-BS interaction and impede displacement of Msl5 or Prp5. Being unable to anchor on the BS, U2 may keep moving along or getting on and off the pre-mRNA in probing for a suitable sequence. The two psoralen-crosslinked sites on U257G pre-mRNA may represent hot spots of stable U2-pre-mRNA interaction in circumstances where U2 cannot anchor to the branch site. Compensatory U2 mutation allows U2 to form correct base pairing with the BS, and consequently to promote the displacement of Msl5 and Prp5. When the CBS is present downstream of the BS, U2 cannot reach the BS due to structural hindrance by Msl5-Mud2 or conformational constraints.

DISCUSSION

In this work, we have elucidated the molecular mechanism of prespliceosome formation by characterizing in detail a previously identified Prp5-associated intermediate complex using branch site-mutated pre-mRNA. We show that the interaction of Msl5 with the intron is important for guiding U2 to the pre-mRNA region containing the BS, and that after being loaded onto the pre-mRNA in association with U2, Prp5 is released if and only if there is correct U2-BS base pairing to enable binding of the tri-snRNP to the spliceosome for the subsequent step.

It is known that nucleotides within the BS are important for branch site recognition by U2 for U2-BS base pairing and for interactions with U2 components (23,46,47). A genetic study of *PRP5* mutants that suppressed splicing defects of *ACT1* U257 mutants implicated Prp5 as proofreading the BS sequence for splicing fidelity control (48). We have shown that Prp5 is tightly associated with the spliceosome assembled on U257 mutant pre-mRNA, forming FIC, but is not detected on the spliceosome when splicing is performed with wild-type pre-mRNA (25). On FIC, Prp5 directly binds on the BSL region of U2, possibly to stabilize or modulate the BSL structure. Prp5 suppressor mutants

show lower affinity for the spliceosome and allow more binding of the tri-snRNP, suggesting that FIC is an intermediate complex for formation of the prespliceosome (25). Here, we show that splicing defects of the U257 mutant could be rescued by U2 compensatory mutations that restored base pairing between U2 and the mutated BS. The association of Prp5 with the spliceosome is destabilized and more tri-snRNP is recruited to the spliceosome upon restoration of U2-BS base pairing. A previous *in vivo* study demonstrated that the identity and position of the bulged nucleotide within the BS-U2 helix is more important than the sequence of the helix for the branching reaction (49). Pre-mRNAs with BS changed to GC-rich sequences supported splicing when U2 variants with corresponding sequence changes in the branch binding region were used. We have examined one such BS-CG mutant (CGCCG<u>A</u>CG) *in vitro* and could also detect splicing with compensatory U2-CGCGGCG mutant, but not with wild-type or A36C mutant U2 (Supplementary Figure S5). Yet the efficiency was much lower than U257G mutant for splicing rescue, suggesting that the sequence of the BS is important for efficient splicing, likely for interaction with U2 components. The CG mutant pre-mRNA also formed FIC as efficiently as U257G mutant in the presence of wild-type U2, indicating that FIC formation is independent of the BS sequence. Together, these results strengthen the notion that FIC is a true intermediate between the commitment complex and the prespliceosome, and mutations in the BS prevent transition from FIC to the prespliceosome.

We have previously shown that changing the branchpoint adenosine to cytidine also caused retention of Prp5 on the spliceosome, albeit to a lesser extent than U257 mutants (25). This finding indicates that the bulged branchpoint nucleotide, although not involved in base pairing with U2, may coordinate interactions with other splicing factors to maintain an appropriate configuration at the BS during prespliceosome formation. Full base pairing between U2 and the BS is required but not sufficient for release of Prp5 and for promoting the splicing reaction. Conceivably, U2-BS base pairing may be stabilized by U2 components that interact with the branch adenosine (50), and changing the branchpoint sequence could impair the interaction and consequently destabilize U2-BS base pairing. This suggests that Prp5 and other U2 components may coordinate to regulate the interaction of U2 with the BS in branchpoint selection for splicing fidelity control.

The presence of a CBS sequence upstream of the branch site on yeast *ACT1* pre-mRNA was previously reported and was shown not to affect splicing of pre-mRNAs with wild-type BS sequence (51). Yet deletion of this CBS dramatically reduced splicing when the BS contained mutations (47,52,53). We have uncovered the function of the CBS on *ACT1* pre-mRNA, showing its presence to be important for FIC formation and for targeting U2 to the authentic BS on BS-mutated pre-mRNAs. Deletion of the CBS from U257 mutant pre-mRNA greatly reduced the binding of Msl5 to the pre-mRNA. Despite a small amount of pre-mRNA being crosslinked to Msl5, we did not identify any specific crosslinked sites by primer extension, suggesting that Msl5 might bind the pre-mRNA nonspecifically and with low affinity. Under this condition, neither Prp5 nor U2 is as-
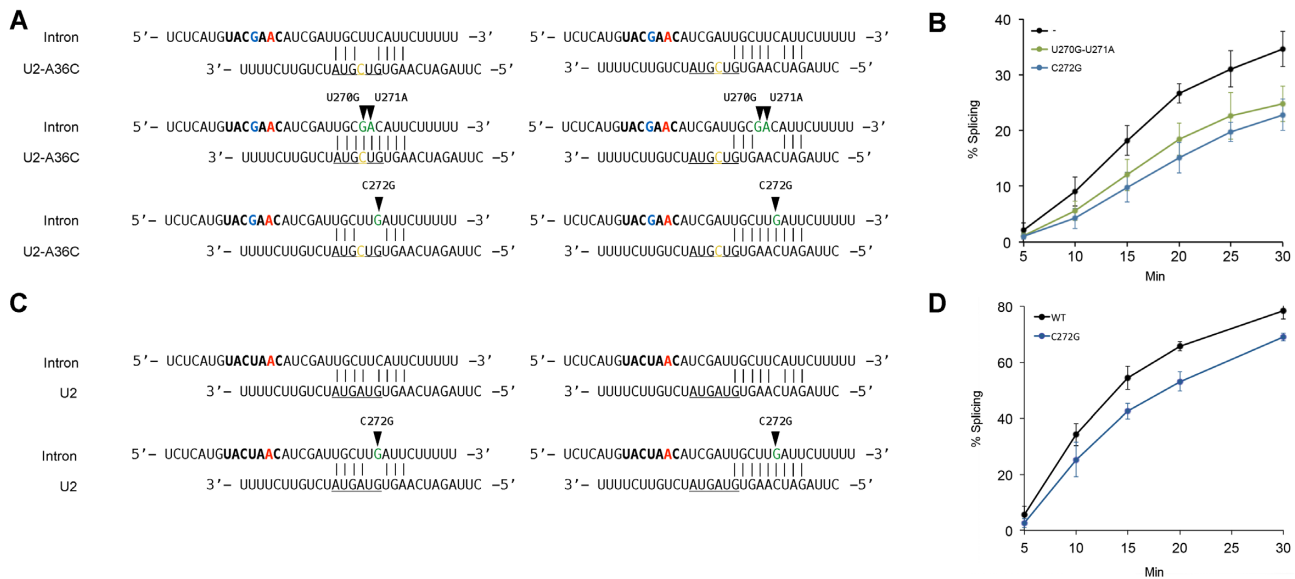
Figure 6. Enhanced base pairing between U2 and BS downstream region retards splicing. (**A** and **C**) Two proposed base pairing modes between U2 and the BS downstream region of U257G (A) or wild-type (C) pre-mRNA. The BS sequence is in bold, the branchpoint is in red, nucleotide 257G is in blue, and the nucleotides changed on the pre-mRNA are in green. The BSL loop of U2 is underlined, and U2-A36C is in yellow. (**B** and **D**) Time course of the splicing reactions performed at 25°C with U257G or U257G-U271A pre-mRNA in U2-depleted extracts supplemented with U2-A36C (B) or at 15°C with wild-type and C272G pre-mRNAs in wild-type extracts (D). RNA was quantified using a PhosphorImager, and the molar ratios of the sum of lariat intermediate and mRNA to that of total RNA were calculated as a percentage of splicing. Data were average numbers from three experiments with standard deviations indicated.

sociated with the pre-mRNA. Furthermore, splicing could not be rescued by suppressor U2. This scenario indicates that the binding of Msl5 to the CBS is important for stabilizing the association of U2 with the pre-mRNA and for its targeting to the authentic BS. It also shows that the sites for Msl5 binding and branchpoint usage are separable. Msl5 recognizes the BS sequence, and it might bridge the 5′ splice site and the BS by interacting with the U1 component Prp40 (29,30). Although U2 snRNP also interacts with U1 snRNP, its simultaneous interaction with Msl5-Mud2 might be important for stable association of U2 snRNP with the pre-mRNA.

Interactions of Msl5-Mud2 with U2 components have been well documented. Human U2AF has been shown to interact with the U2 component SF3b1 (54), and yeast Mud2 interacts with the SF3a2 orthologue Prp11 (35). Yeast two-hybrid assays identified interactions of the human Msl5 orthologue SF1 with SF3a1 and CHERP, which is more loosely associated with the U2 snRNP and functions in alternative splicing (50,55,56). More recently, a human G-patch-containing protein, SUGP1, was demonstrated to interact with SF1, U2AF2 and SF3b1 in bridging U2 and the BS. SF3b1 mutations that cause myelodysplastic syndromes (MDS) and many cancers disrupt the interaction of SF3b1 with SUGP1, and consequently with SF1, leading to aberrant usage of an upstream branchpoint and cryptic 3′ splice site (36). These findings further imply that SF1 plays a role in guiding branchpoint selection.

Branchpoint mapping for lariat intron-exon 2 generated from low levels of splicing of U257 mutant pre-mRNA revealed that the branching reaction occurred primarily at the mutated BS, using A_{-1} (the base upstream of the correct A residue) as the branchpoint, and much less at the CBS

where Msl5 binds. Since U2 can potentially form extensive base pairings with the pre-mRNA at both sites (Supplementary Figure S6), branchpoint selection might involve additional factors. Cryo-EM structures of the spliceosome have revealed extended U2/BS helix within the spliceosome (57–62). Whether such extended helix is functionally important to account for preferential selection of the branchpoint at the mutated BS is not clear. It is also not known whether U2 could form extended helix with the CBS at different sites. Functional significance of the U2/BS extended helix awaits further study.

How U2 is recruited to the BS to displace Msl5-Mud2 was not clear. U2 becomes stably associated with the splicing complex upon direct binding to the pre-mRNA near the BS. Given that Msl5-Mud2 remain associated with FIC, neither displacement of Msl5-Mud2 nor U2-BS base pairing is necessary for stable association of U2 with the complex. Our crosslinking analysis revealed that several U2 components that interact strongly with the pre-mRNA near the BS on the prespliceosome showed no or only weak interactions with the pre-mRNA on FIC, suggesting that U2 was not located at a correct position on the pre-mRNA. Consistently, our psoralen crosslinking showed crosslinking of $U2-U_{40}$ to the +3 and +8 positions of the U257G pre-mRNA downstream of the BS, as opposed to the -7 position of the wild-type pre-mRNA on the prespliceosome. This result indicates that U2 might move more dynamically along the pre-mRNA in the BS downstream region when it is unable to anchor on the BS. The crosslinked sites likely represent regions that more stably interact with U2, possibly with extensive base pairing. We have proposed two possible modes of U2-BS base pairing, and we have demonstrated that further enhancement of the base pairing in these
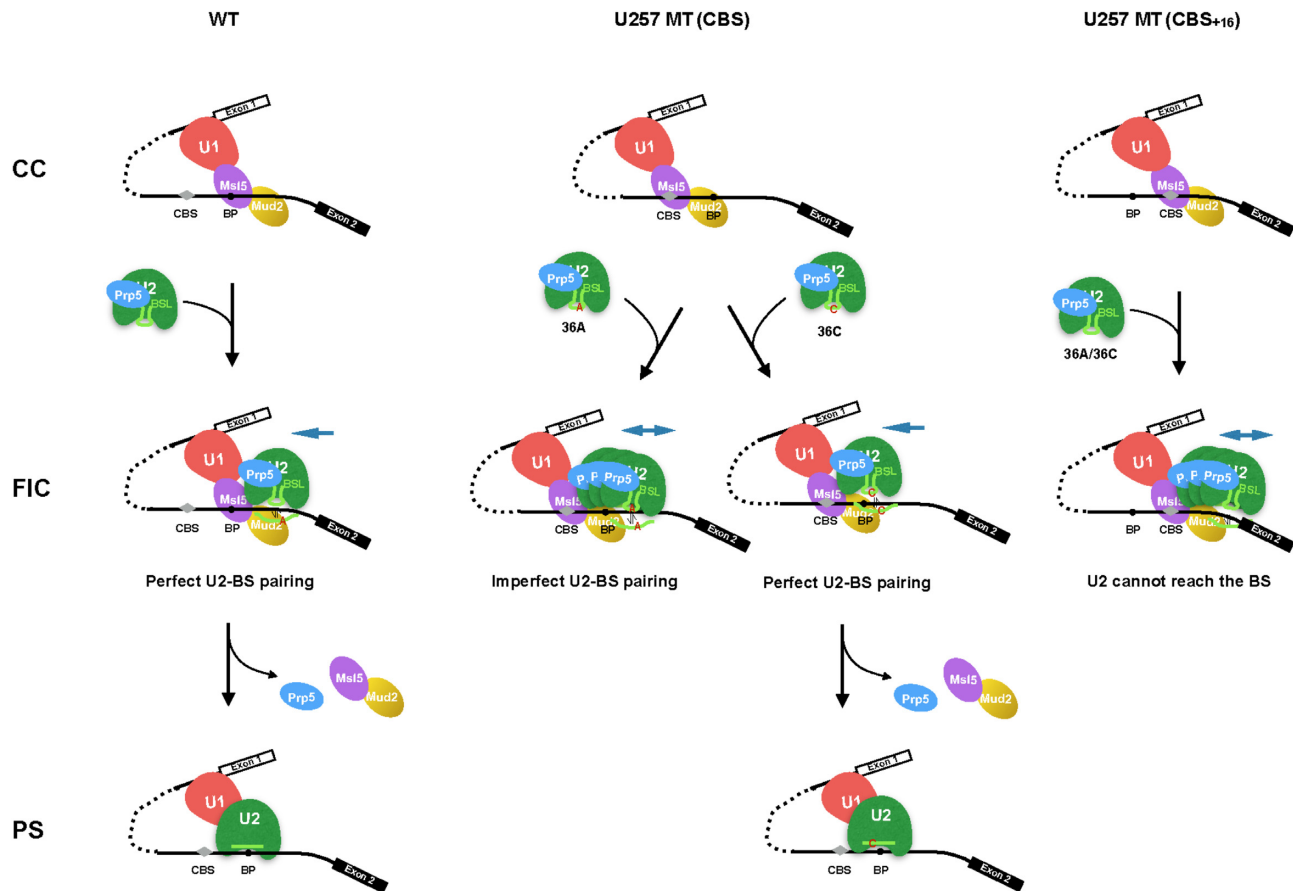
Figure 7. A model showing how U2 interacts with the intron for wild-type and U257 mutant pre-mRNA to enable prespliceosome formation. On the commitment complex, U1 binds to the 5′ splice site and the Msl5-Mud2 heterodimer binds to the BS. Through the interaction of Msl5 with the U1 component, the 5′ splice site and the BS are brought close to each other. Prp5 is required for U2 activation and remains associated with U2 to bridge U1 and U2 for recruitment of U2. U2 is then deposited onto the pre-mRNA downstream of the BS to form FIC. On wild-type pre-mRNA, U2 either moves in a 3′ to 5′ direction, or docks and undocks from the pre-mRNA to probe for the BS. Upon base pairing with the BS, Prp5 and Msl5-Mud2 are released to form the prespliceosome (PS). On U257 mutant pre-mRNA, Msl5 does not bind the BS, but binds the CBS instead. When the CBS is upstream of the BS, U2 can reach the branch site but cannot form proper base pairing. When the CBS is downstream of the BS, U2 cannot pass Msl5-Mud2 to reach the BS. In both cases, U2 continues moving on the BS downstream region of the pre-mRNA, and the spliceosome stalls at the FIC stage. BP, branchpoint, indicated as a black circle; CBS, cryptic branch site, indicated as a grey diamond; blue arrows indicate the directions of U2 motion. The BSL is shown to switch dynamically between the stem-loop and the flat forms upon interacting with the pre-mRNA.

regions elicited slower kinetics for the splicing reactions that proceed through the authentic branchpoint. Thus, U2 may be loaded onto the pre-mRNA downstream of the BS, and then it moves along the pre-mRNA to search for the BS. Alternatively, U2 could dynamically dock and undock from the pre-mRNA, being restricted within a region downstream of the BS due to structural constraint, until it locates the BS. The fact that the same U_{40} residue of U2 crosslinked to different sites of the intron suggests a common mode of U2 binding to the pre-mRNA during its action. Notably, our proposed modes for U2 base pairing with the BS downstream sequence require disruption of the BSL. The cryo-EM structure of the human 17S U2 snRNP reveals that the BSL is surrounded by U2 components, among which TAT-SF1 (human orthologue of Cus2) is positioned adjacent to the loop (24). Cus2 is known to inhibit U2 function in yeast, and its removal to activate U2 requires Prp5-catalyzed ATP hydrolysis (21). It is possible that the BSL may have been destabilized by displacement of Cus2 before

U2 is loaded on to the pre-mRNA, or the conformation of the BSL may fluctuate dynamically upon binding of U2 to the pre-mRNA, to allow interactions of U2 with the pre-mRNA to search for the branch site. Consistent with this scenario, interactions of spliceosomal components with the BS appear to be more dynamic at early stages of the spliceosome assembly pathway as revealed by lower resolution in the BS than the 5′SS region of the cryo-EM structures of both the commitment complex (or E-complex) and the prespliceosome (or A complex) (30,63).

DEAD-box protein Sub2 has been implicated in displacing Msl5-Mud2 from the BS (20), but the mechanism of its action is totally unknown. Replacement of Msl5 with U2 to form the prespliceosome does not require prior binding of Msl5 on the BS, given that Msl5 does not bind the U257 mutant BS. Nevertheless, Msl5 binding on the CBS is necessary for targeting U2 to the BS as deletion of the CBS inhibits splicing. Deletion of the CBS also prevents stable association of U2 with the pre-mRNA, suggesting that U2 may be-

come stably associated with the pre-mRNA only when U1 can interact with the BS by interacting with Msl5. Alone, interaction of U2 with U1 is not sufficient for stable association of U2 with the pre-mRNA after U1 binds to the 5′ splice site. The interactions of U1 and U2 with Msl5-Mud2 would likely place a constraint for loading of U2 onto the pre-mRNA only downstream of the Msl5-binding site. Only when Msl5 binds on or upstream of the branch site can U2 access the branch site by moving in a 3′ to 5′ direction or by dynamically probing along the pre-mRNA. When the CBS is moved downstream of the BS, U2 can be recruited to and is stably associated with the pre-mRNA. The fact that the U2-A36C mutant fails to rescue splicing of U257G mutant pre-mRNA suggests that U2 cannot access the BS, possibly due to steric hindrance by Msl5. Stable interaction of U2 with the BS may involve not only proper U2-BS base pairing but also interactions of U2 components with the BS sequence. Formation of a stable interaction between U2 and the BS may trigger a conformational change in U2 snRNP to release Prp5, and a subsequent structural change of U1 snRNP, resulting in destabilization of Msl5-Mud2 from the splicing complex.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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