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Invariant U2 snRNA nucleotides form a stem loop to recognize the intron early in splicing

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Abstract

U2 snRNA-intron branchpoint pairing is a critical step in pre-mRNA recognition by the splicing apparatus, but the mechanism by which these two RNAs engage each other is unknown. Here we identify a new U2 snRNA structure, the branchpoint interaction stem-loop (BSL), that presents the U2 nucleotides that will contact the intron. We provide evidence that the BSL forms prior to interaction with the intron, and is disrupted by the DExD/H protein Prp5p during engagement of the snRNA with the intron. In vitro splicing complex assembly in a BSL-destabilized mutant extract suggests that the BSL is required at a previously unrecognized step between commitment complex and prespliceosome formation. The extreme evolutionary conservation of the BSL suggests it represents an ancient structural solution to the problem of intron branchpoint recognition by dynamic RNA elements that must serve multiple functions at other times during splicing.

Introduction

The spliceosome is a highly dynamic ribonucleoprotein complex in which ordered rearrangement of the spliceosomal snRNAs accompanies the assembly of a functional splicing complex (Ares and Weiser, 1995) (Staley and Guthrie, 1998). The large number of spliceosomal RNA-RNA rearrangements greatly exceeds those known for any other RNA-protein complex, and their details remain poorly described. In particular many of the most conserved snRNA nucleotides have multiple functions, confounding standard analysis methods. Genetic and crosslinking studies show that U1 snRNA base pairs to the 5′ splice site and U2 pairs with the intron branchpoint sequence in an early complex in which the pre-mRNA reactive groups for the first step are identified and brought together. U1 then leaves, delivering the 5′ splice site to U6 snRNA. U4 snRNA, having entered the complex in association with U6 snRNA, also leaves, allowing U6 to pair with U2 (Ares and Weiser, 1995; Staley and Guthrie, 1998). As U4 departs, U6 forms an internal stem loop (ISL) that promotes catalysis in part through binding a divalent metal ion (Brow, 2002). This elaborate path of rearrangements demands that many snRNA nucleotides pair with more than one other nucleotide over time, and is distinctly different from the folding of the mechanistically similar group II introns (Toor et al., 2009; Toor et al., 2008). This additional complexity likely reflects the fact that the spliceosome acts in trans on a complex set of substrates in response to regulatory cues, whereas the typical group II intron removes itself constitutively from a single transcript.

Consistent with the requirement for RNA structural rearrangements, eight DExD/H box proteins play distinct and critical roles during the splicing process (Staley and Guthrie, 1998) (Brow, 2002). Two of these, Prp5p and Sub2p function early during ATP-dependent U2 snRNP

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recruitment to the pre-mRNA and stable prespliceosome formation (Kistler and Guthrie, 2001; Libri et al., 2001; O'Day et al., 1996; Ruby et al., 1993; Zhang and Green, 2001). One role for Prp5p is to mediate the U2 RNA structural transition from U2-stem IIc to U2-stem IIa, a reaction antagonized by the RNA binding protein Cus2p (Perriman and Ares, 2000; Perriman et al., 2003; Perriman and Ares, 2007). Stem IIa is required to form stable prespliceosomes (Yan et al., 1998; Zavanelli and Ares, 1991), and mutations in stem IIa are suppressed by alterations in Cus2p (Yan et al., 1998). Additional as yet unrecognized U2 RNA rearrangements might also be mediated by *PRP5* (Kosowski et al., 2009; Perriman et al., 2003; Xu and Query, 2007; Yan and Ares, 1996). Temperature sensitive *prp5* alleles render lethal several mutations in invariant U2 nucleotides that flank the U2 branchpoint interaction sequence (Yan and Ares, 1996). In addition, ATPase defective *prp5* alleles can suppress intron branchpoint mutants (Perriman and Ares, 2007; Xu and Query, 2007). Strikingly, the Prp5p ATP-binding function, but not Prp5p, is unnecessary when Cus2p is absent or if U2-stem loop IIa is hyperstabilized by mutation (Perriman et al., 2003). Thus *PRP5* might modulate additional rearrangements involving U2, but the nature of these have remained obscure.

Here we identify the branchpoint interacting stem loop (BSL), an evolutionarily conserved U2 RNA structural element that forms from pairing invariant sequences flanking the branchpoint interaction sequence. We show that this stem loop plays an important role with Prp5p in coordinating the fidelity and progression of splicing during the early steps of spliceosome assembly. Mutations that destabilize the BSL accumulate a unusual complex in vitro, whose snRNA requirements and kinetics suggest it is a product of commitment complex that is slow or unable to convert to stable prespliceosomes. Hyperstabilized BSL mutants relax the stringency of intron branchpoint selection, suggesting a role for the BSL in splicing fidelity. We propose the BSL presents U2 nucleotides to the intron branchpoint at a critical point in spliceosome assembly.

Results

Mutation of invariant U2 residues rescues a lethal truncation of Prp5p

To search for additional functions of Prp5p in association with U2 RNA we identified a C-terminal truncation of Prp5p we thought might be stable and bind ATP, but would not function as an ATP-dependent helicase. Crystal structures of individual domains from several DExD/H box proteins indicate that globular domains 1 and 2 form independent stable structures in vitro, and that the majority of ATP binding site contacts reside in domain 1 (Cheng et al., 2005; Fan et al., 2009; Rudolph et al., 2006). If a protein composed of the N-terminus and domain 1 of Prp5p is stable in vivo in the absence of domain 2 and the C-terminal residues, it might retain a partial Prp5p activity. We created *prp5-Δ494* by replacing amino acid 494 with a termination codon and removing domain 2 of the ATPase domain and the C terminus (see Fig 1A). This deletion is unable to complement the lethal phenotype of a *prp5* null mutation under a variety of growth conditions (Fig 1B, C; data not shown) and recombinant Prp5-Δ494p has no detectable ATPase activity as compared to the wild type protein in vitro (Supplementary Fig S1).

To determine whether a mutant U2 RNA could rescue *prp5-Δ494*, we screened a U2 mutant library (Yan and Ares, 1996). Because Cus2p is a negative regulator of Prp5p function (Perriman et al., 2003; Perriman and Ares, 2007) we performed the screen in a *cus2Δ* strain. We isolated a single U2 suppressor allele, U2-C46U, which was able to support growth of *prp5-Δ494* at 30°C (Figure 1B, C, D). To identify more suppressors, we screened a second pool of U2 snRNA alleles and identified another four mutations that rescue *prp5-Δ494:* U2-C29U, U2-U33A, U2-U42A and U2-C46U;U42A (Figure 1C, D). These mutants grow indistinguishably from wild type when wild type Prp5p is present, indicating that U2 function is not greatly compromised (Figure 1B, C, Yan & Ares 1996). Each suppressor mutation alters

a universally conserved residue near the branchpoint interaction region (Figure 1D). This region of U2 RNA contributes to U2 stem I (Sashital et al., 2007), U2–U6 helix Ia (Madhani and Guthrie, 1992), and U2–U6 helix III function (Sun and Manley, 1995). In addition, evidence from both yeast and mammalian systems indicates that conserved spliceosomal proteins interact with these nucleotides (Dybkov et al., 2006; Yan and Ares, 1996). Thus, these highly conserved U2 residues play numerous roles during splicing.

The distribution of suppressor mutations was inconsistent with disruption of any single known spliceosomal RNA structure, however they all fall within and disrupt a highly conserved 9 base pair imperfect inverted repeat (shaded in cyan in Figure 1D), not previously recognized as a U2 RNA structural element. A duplex formed by these sequences would present the U2 branchpoint interacting nucleotides (Parker et al., 1987; Zhuang and Weiner, 1989) in a terminal loop ("intron bp int", Figure 1D). Although aberrant formation of part of this structure was previously suggested to explain the growth defect of a U2-U44A allele (Yan and Ares, 1996), this stem loop has not been hypothesized to function in splicing. We have called this structure the branchpoint interacting stem loop, or BSL. The rescue of *prp5-Δ494* by U2 BSL destabilizing mutations is consistent with a role for Prp5p in disrupting the BSL during the normal process of splicing in wild type cells.

A model RNA comprising residues 23-49 of yeast U2 folds into a 9-bp stem loop

Of the 9 base appositions in the proposed BSL, one is a G–U pair, one is an A–C pair and the third is a U–U pair (Fig 2A). Elsewhere in the splicing machinery, non-Watson Crick pairings are observed, in particular where one RNA strand displaces another (Huppler et al., 2002) (Massenet et al., 1999). However because of the extreme evolutionary conservation of this sequence, phylogenetic covariation of residues that would support the existence of the stem is unavailable (see Fig 1D). To determine whether the BSL sequence forms a stem loop, we synthesized a 27 nt model BSL and tested its ability to fold using nuclease structure probing (Fig 2B). Nuclease V1, which cleaves bases in duplex or which are stacked (Auron et al., 1982), cleaves primarily in the stem of the model BSL (Fig 2B, lane 4, gray circles; Fig 2C, black arrows). In contrast to V1, nuclease T1, which is single stranded G-specific, cleaves primarily in the loop under native conditions (Fig 2B lane 6, white circles; Fig 2C, white triangles), but recognizes the stem G-residues under denaturing conditions (Fig 2B, lane 1). We conclude that the highly conserved 9 bp imperfect inverted repeat sequence of yeast U2 residues 25 to 47 is capable of adopting the predicted BSL structure. This experiment speaks only to the ability of the sequence to fold independently under physiological salt and temperatures, and does not incorporate adjacent spliceosomal snRNAs, proteins or modified nucleotides found in the native spliceosome. Other spliceosomal RNA elements have been studied at high resolution using similar model RNAs (Huppler et al., 2002;Sashital et al., 2004;Sashital et al., 2007;Stallings and Moore, 1997), and this finding (Fig 2) sets the stage for detailed structural studies of the BSL.

Suppression of the truncated *prp5* mutant requires disruption of the BSL

All five mutant U2 suppressors of *prp5-Δ494* destabilize potential Watson-Crick base pairing in the stem (Fig 1). To test the idea that suppression arises from BSL disruption, we asked whether mutations that restore potential Watson-Crick pairing to either the C46U or U42A suppressor alleles, (i. e. C46U; G26A or U42A; A30U) could abrogate suppression (Fig 3B). Suppression is lost when C46U is paired with G26A, a mutation that restores Watson-Crick pairing. The G26A mutant has a growth defect (Madhani and Guthrie, 1992), making evaluation of *prp5-Δ494* suppression problematic. Because the growth defect is suppressed when combined with C46U (see below), *prp5-Δ494* suppression can be evaluated in the double mutant, and it fails. Thus restoring the 26–46 base pair eliminates suppression of *prp5-Δ494* (Fig 3B). Similar results are obtained at the 30–42 base pair. The suppressor activity of U42A

is abrogated by A30U, which restores Watson-Crick pairing, but not by A30C, which does not (Fig 3B). Not all nucleotide substitutions that disrupt the BSL can suppress *prp5-Δ494* (Fig 3, and Table 1), probably because nucleotide identity at these positions is heavily constrained by other functions that employ these bases. Mutations that are predicted to hyperstabilize the BSL do not rescue *prp5-Δ494* (Table 1) and exhibit cold sensitive or lethal phenotypes in otherwise wild type strains (Yan and Ares, 1996); Table 1). This pattern of phenotypes is consistent with the interpretation that the BSL is a conserved dynamic structural element of U2 snRNA with a finely tuned stability, that must form and execute a function, and then be disrupted, most likely by the action of Prp5p, so that subsequent steps can occur.

BSL base pairs make multiple contributions to splicing

To this point our test of BSL function has involved rescue of a grossly defective Prp5 protein by disruption of the BSL. To determine whether the BSL is important for normal spliceosome function, we tested growth of U2 mutants in strains lacking Cus2p but expressing wild type Prp5p. Mutation of G26 causes severe growth defects, attributed to the disruption of other snRNA interactions during splicing (Madhani and Guthrie, 1992; Madhani and Guthrie, 1994b; McPheeters and Abelson, 1992). U2-G26 pairs with U2-C9 in U2 stem I (Fig 3A, top), and also with U6-C58 in U2–U6 helix Ia (Fig 3A, bottom). In the BSL, G26 is proposed to pair with C46 (Fig 3A, middle). To test whether G26 plays functional roles by pairing with C46 in the BSL, we asked whether base pairing between residues 26 and 46 contributes to function. C46U suppresses the G26A growth defect (Fig 3C). Similarly C46A suppresses the lethality of G26U, whereas non-Watson-Crick combinations do not function (Fig 3C and Table 1). We conclude that interaction between U2 bases 26 and 46 is important for splicing function, probably by forming the base of the BSL.

Since G26 participates in U2 stem I and U2–U6 helix Ia as well as in the BSL (Fig 3A), we tested the relative functional importance of each of the three interactions made by G26 by comparing the growth of cells lacking each of the interactions. A compensatory mutation that restores U2 stem I only (C9U; G26A), fails to rescue growth, arguing that disruption of stem I by G26A is not a major cause of the growth defect. In contrast, and consistent with previous reports (Madhani and Guthrie, 1992), the restoration of U2–U6 helix Ia by U6 C58U greatly improves growth compared with U2 G26A alone. Rescue via U2–U6 helix Ia restoration is slightly more robust than through restoration of the BSL (Fig 3C). We conclude that both the BSL and U2–U6 helix Ia have important functions, and that residues including G26 in the BSL exchange with the U2-U6 helix Ia during splicing.

Two non-standard base pairs are found in the BSL: U28–Ψ44 and A31–C41. Converting either of these base pairs to Watson-Crick pairs results in cold sensitive growth, and restoring non-Watson-Crick pairs at these locations restores function (Table 1). Cold sensitive phenotypes caused by hyperstabilization of RNA structures are observed elsewhere in the spliceosome where RNA structures must form and then disrupt for splicing to progress (Fortner et al., 1994;Hilliker et al., 2007;Li and Brow, 1996;Perriman and Ares, 2007;Staley and Guthrie, 1999;Zavanelli et al., 1994). Taken together, the data show that the BSL contributes to function, but must be disrupted, likely by the action of Prp5p, for the necessary program of snRNA rearrangements to proceed.

Destabilized BSL mutants accumulate an unusual splicing complex in vitro

Although the above genetic studies reveal important structural interactions between snRNAs at base pair resolution, they provide little information about the precise steps of splicing at which the BSL might function. To explore this, we made splicing extracts from yeast strains expressing a functional but destabilized BSL (U2-U42A; C46U), and analyzed spliceosome assembly using radiolabeled synthetic RP51A pre-mRNA as a substrate (Fig 4). U42A; C46U

extracts form prespliceosomes slowly, and accumulate an unusual complex (Fig 4A; asterisk) that migrates between commitment complex II (CC2) and the prespliceosome plus spliceosomes (PS/SP) bands on the native gel (Seraphin and Rosbash, 1989); Fig 4A; lanes 6-10). This complex is not apparent in wild type U2 splicing extracts (Fig 4A; lanes 1-5). In addition, the complex does not accumulate in hyperstabilized BSL mutant U2-U44A splicing extracts (data not shown).

To assess the kinetic relationship of the unusual complex with the known splicing complexes we measured the amount of RP51A RNA in each complex over time and plotted each as a percentage of total counts for each time point (Fig 4B). In comparison to wild type (left panel), the U42A; C46U extracts (right panel) show significantly slower turnover of commitment complex and production of prespliceosome and spliceosomes. The unusual complex peaks at 5 minutes and slowly disappears over time. As both the commitment complex and unusual complex disappear, there is a corresponding increase in prespliceosome and spliceosomes. We suggest the unusual complex represents a kinetic intermediate whose progress along the splicing path is retarded because of either delayed BSL formation or premature BSL destabilization. Alternatively the complex may represent a dead-end intermediate, the result of mis-timed pairing between U2 and the intron branchpoint. In either case, these results support the hypothesis the BSL helps engage U2 with the intron branchpoint during formation of prespliceosomes.

To support the conclusion that the unusual complex is related to standard splicing complexes, we determined the U snRNP, ATP (Fig 4C), and intron branchpoint (Fig 4D) requirements for its formation. We used snRNA-specific oligodeoxynucleotides and endogenous RNaseH activity in the extracts to digest the U1, U2 and U6 snRNAs (lanes 8-10). When these oligonucleotides are added to control wild type extracts, expected results (McPheeters et al., 1989;Perriman and Ares, 2000) are obtained, showing that U1 is first required for commitment complex formation, U2 is first required for prespliceosome formation, and U6 is not required until after prespliceosome formation (Fig 4C; lanes 3-5). In U42A; C46U splicing extracts, formation of the unusual complex requires U1 (lane 8) and U2 (lane 9), but not U6 (lanes 10) snRNAs, showing that it has similar snRNA requirements to authentic prespliceosomes. In contrast, depletion of ATP shows that the unusual complex can form in the absence of ATP (lane 7), suggesting that it shares some similarity with CC2, or perhaps has bypassed the ATP requirement, as observed for prespliceosome formation in extracts depleted of Cus2p (Perriman and Ares, 2000), note, these extracts contain Cus2p). A test of substrate requirements for formation of the unusual complex (Fig 4D) shows that like CC2 (Legrain et al., 1988) and prespliceosomes (Rymond and Rosbash, 1986), it requires a pre-mRNA branchpoint sequence (Fig 4D, compare lanes 1-6 with lanes 7-12). We conclude that disrupting the BSL sequence disrupts the normal course of prespliceosome formation, resulting in accumulation of an unusual stalled complex that is hung up in the transition between commitment complexes and prespliceosomes.

Hyperstabilizing the BSL relaxes branchpoint recognition

Given biochemical evidence that the BSL plays a role in stable association of U2 snRNP with the pre-mRNA and the provocative positioning of the intron contacting nucleotides in the loop of the BSL, we hypothesized that the BSL might play an important role in recognition of the intron branchpoint. To test this, we used *ACT-CUP1* reporter pre-mRNAs in which copper tolerance depends on splicing efficiency(Lesser and Guthrie, 1993). Reporters containing branchpoint region mutations (Fig 5A) were compared to wild type reporter in cells carrying different BSL mutations on copper medium (Fig 5B). Wild type *ACT-CUP* pre-mRNA containing a canonical branchpoint sequence shows a decrease in copper tolerance in cells expressing either the hyperstable or destabilized BSL mutants relative to wild type U2 (Fig

5C). This demonstrates that correct BSL stability is important for efficient splicing of wild type pre-mRNA. In contrast, branchpoint mutations A259G and C256A (UACUAAC to UACUAGC or UAAUAAC, Fig 5A) demonstrate increased copper tolerance in the hyperstabilized BSL mutant as compared to wild type U2 (Fig 5C). Hyperstabilization at position 44 is responsible for this, because disruption of the A44–U28 pairing in the U44A; U28C mutant eliminates both the diminished copper tolerance of wild type reporter, and the improved copper tolerance of the mutant reporters (Fig 4D). Thus hyperstabilizing the BSL (U44A) relaxes the stringency of branchpoint recognition, allowing mutant pre-mRNAs to be more efficiently spliced. Conversely destabilizing the BSL (U42A; C46U), does not allow improved splicing of mutant branchpoint introns and appears to cause a greater decrease in tolerance to copper when compared with wild type U2 (Fig 5C). The U2 BSL hyperstabilizing mutation does not suppress *ACT-CUP* pre-mRNAs mutated at the 5′ or 3′ splice sites or other mutations at the branchpoint interaction sequence (Supplementary Fig S2A, B), demonstrating the specificity of the suppression for the intron branchpoint sequence. In contrast, destabilizing the BSL causes a reduction in copper tolerance of the 5′ splice site mutation U2A, and more subtly for the 3′ splice site mutant A302U, as well as for the branchpoint mutants. Although functional interactions between U1, U2 and the 5′ exon are established in early in spliceosome assembly (Das et al., 2000; Donmez et al., 2007; Donmez et al., 2004; McGrail and O'Keefe, 2008) BSL destabilizing mutants may lead to a general reduction in splicing of pre-mRNA substrates of several types (Supplementary Fig S2).

BSL formation is mutually exclusive with the formation of other structures including U2–U6 helix Ia (see Fig 3A). To assess the possibility that a hyperstable BSL competes with U2–U6 helix Ia and that unstable helix Ia rescues branchpoint mutants, we tested strains carrying the U6-C58U mutation, which replaces a G–C pair in U2–U6 helix Ia with a G–U pair (see Fig 3B). For the most part, cells expressing U6-C58U demonstrate a similar pattern of copper tolerance for the tested pre-mRNAs as with wild type U6 (Fig 5C). The exception is that U6-C58U on its own detectably improves splicing of the C256 branchpoint mutant with wild type U2. Formally it is not possible to tell whether this weaker rescue of C256 is due to hyperstable BSL-mediated destabilization of helix Ia or destabilized helix Ia-mediated stabilization of the BSL. However U6-C58U may act by the independent mechanism of compromising proofreading by Prp16p, as previously suggested (Madhani and Guthrie, 1994a;Mefford and Staley, 2009). Since BSL hyperstabilization by U2-U44A is a more robust suppressor than U6-C58U (Fig 5C), we conclude that relaxed branchpoint usage is due primarily to hyperstabilizing the BSL, rather than by indirect effects of the BSL on U2–U6 helix Ia.

To resolve and measure the effects on splicing that lead to copper tolerance, we assayed splicing by primer extension (Fig 5E, F, and Supplementary Figure S2C). Consistent with the copper assays, splicing of C256A and A259G pre-mRNAs is improved by the BSL hyperstabilizing mutant U44A (Fig 5E, lanes 3 and 6), but is greatly reduced in the destabilizing U42A; C46U mutant (lanes 2 and 5). Quantification (Fig 5F) shows the first step of splicing of both C256A and A259G pre-mRNAs is significantly increased (reduced fidelity of branchpoint recognition) in strains expressing U44A (Fig 5F) but significantly decreased in strains expressing U42A; C46U. None of the three U2 snRNAs improve the very poor second step efficiency of the A259G pre-mRNA observed here and in previous studies (Burgess and Guthrie, 1993; Fouser and Friesen, 1986; Konarska et al., 2006). Although differences in copper tolerance are observed for wild type *ACT-CUP* pre-mRNA, splicing of the wild type reporter is robust (Fig 5E), and quantification shows only slight changes in the appropriate direction (Supplementary Fig S2B). Although the C256A substrate appears to show a decrease in the second step with the BSL destabilizing mutant (Fig 5F), the effect on the first step is so strong that the second step efficiency cannot accurately be estimated by this method. From these data we conclude that hyperstabilizing the BSL relaxes the stringency of branchpoint recognition and suppresses

branchpoint mutant pre-mRNAs at the first step of splicing, whereas destabilizing the BSL appears to cause a general loss of efficiency of branchpoint recognition and splicing.

Discussion

The binding of U2 snRNP to pre-mRNA is an early signature event in spliceosome assembly. U2 RNA and its partner U6 possess fluid structures that are dramatically influenced by a program of RNA-RNA interactions executed during spliceosome function. In this paper we describe a new dynamic U2 RNA structural element, the branchpoint-interacting stem loop or BSL, and place its function at the time of branchpoint recognition by the U2 snRNP (Fig 6). The identification of this structure greatly improves our understanding of the nearly invariant RNA sequences found at the core of the spliceosome. Its existence likely escaped detection because many of its nucleotides play other roles at other times in splicing. This underscores the fundamental difference between spliceosomal RNAs and other structural RNAs: extreme conservation is not a result of a highly constrained single function, but a grand compromise that optimizes the multiple functions imposed on the same sequence against each other.

We first recognized the BSL through its antagonism of a severely truncated Prp5 protein. Four single base changes in invariant U2 nucleotides relieve this, allowing the mutant protein to support growth (Fig 1). Without phylogenetic variation to predict a stem loop, we mapped the structure of a model RNA and found it forms a stem loop (Fig 2). Furthermore, compensatory mutations provide functional evidence that this stem is intrinsic to the splicing pathway. Its interaction with Prp5p likely reflects a role for Prp5p in unwinding the BSL, allowing subsequent functions of its nucleotides to be executed, for example as part of U2–U6 helix Ia (Fig 3). The provocative display of the U2 branchpoint interacting nucleotides in the loop of the BSL suggests a role in branchpoint recognition. Indeed the BSL is required during prespliceosome formation when the U2 snRNP recognizes the branchpoint (Fig 4), and its stablization allows greater flexibility in branchpoint usage (Fig 5). The stability of the BSL is tuned, as mutations that either destabilize or hyperstabilize it are deleterious (Table 1). Hyperstabilization creates cold sensitive growth but has a specific effect on the fidelity of branchpoint recognition, as hyperstable BSL mutants suppress mutations in the conserved branchpoint sequence of the intron (Fig 5).

New view of the RNA dynamics involved in prespliceosome assembly

The existence of the BSL now explains previously reported phenotypes of U2 RNA mutations (McPheeters and Abelson, 1992; Yan and Ares, 1996). Mutations that hyperstabilize BSL pairing are cold sensitive in vivo (Yan and Ares, 1996) and do not function in splicing reconstitution experiments in vitro (McPheeters and Abelson, 1992). In vivo structure probing of U2-U44A, the BSL hyperstablizing mutant we use in this study (Fig 5) shows increased reactivity of U2-C9 consistent with destabilization of competing U2 stem I (Yan and Ares, 1996), and BSL stabilization. Notably, U44A; U28C, which disrupts pairing between residues 44 and 28 (Fig 5), both suppresses the cold sensitivity of U44A and reverses the C9 increased reactivity (Yan and Ares, 1996). BSL residues are also important for U2 snRNP protein-RNA interactions. U2 mutations predicted to destabilize the BSL are synthetic lethal when combined with temperature sensitive alleles of *PRP5* and U2 snRNP SF3a protein complex components, *PRP9, PRP11* and *PRP21* (Yan and Ares, 1996). Consistent with this, SF3b protein component, SF3b14a (Snu17p/Ist3p in yeast; (Gottschalk et al., 2001) and SF3a60 (Prp9p), both crosslink to BSL sequences in purified mammalian 17S U2snRNPs (Dybkov et al., 2006). But whether and how these proteins might function with the BSL remains to be determined.

Pairing between U2 and the intron forms the “branchpoint helix” and is key to splicing progression. Splicing of pre-mRNAs with branchpoint mutations is improved by hyperstabilizing the BSL (Fig 5). This mechanism of suppression is likely to be distinct from

that observed by directly improving base pairing between U2 and the intron (McPheeters et al., 1989;Parker et al., 1987;Smith et al., 2009;Zhuang and Weiner, 1989), although stabilizing the BSL stem could allosterically stabilize base pairs between the BSL loop and the intron. BSL-mediated suppression promotes the first step of splicing (Fig 5), making it different from suppression by alleles of *PRP8* (Query and Konarska, 2004), or *prp16-1* (Burgess et al., 1990;Query and Konarska, 2004), both of which aid splicing progression after the first catalytic step. We suggest that BSL hyperstabilization reduces stringency of the step at which initial pairing of loop nucleotides is converted to an extended and more stable U2-branchpoint helix by prolonging or enabling an otherwise inappropriate interaction. In this view, a hyperstable BSL allows more time or repeated attempts to pass the initial pairing check between weakly complementary branchpoint sequences, allowing mutant branchpoints to be recognized. After initial intron recognition, a conformational change mediated by Prp5p leads to disruption of the BSL and extension of the U2-pre-mRNA pairing, forming prespliceosomes (see Fig 4A, Fig 6). In this model, Prp5p helps identify correct U2-branchpoint pairing and aids BSL stem opening allowing extended U2-intron interaction and stable prespliceosomes.

The formation and disruption of the BSL is consistent with the work of Xu and Query (2007) who found that splicing of intron branchpoint mutations could be suppressed by ATPase-compromised Prp5p alleles, and invoked an undefined conformational change associated with formation of stable U2-pre-mRNA complexes. We suggest BSL structure and dynamics embody this conformational change. In addition, the orthogonal yeast pre-mRNA splicing system developed by Smith et al., speaks to the extreme flexibility in U2-branchpoint helix sequence in the context of wild type BSL sequence (Smith et al., 2009). These observations suggest the BSL is able to present a variety of functional sequences.

Fine-tuning a structural feature of U2 snRNA

The striking invariance of BSL nucleotides strongly supports an ancient and integral role for this structure in the major spliceosome in all eukaryotes. Like the highly conserved internal stem loop of U6, the U2 BSL has few consecutive Watson-crick base pairs and includes unusual base appositions. This feature is likely important to the balanced stability of the helix with its competing structures. Another notable feature is an exceptional number of post-transcriptionally modified nucleotides. In mammalian U2 RNA, the BSL sequence contains ten modified nucleotides (Ares and Weiser, 1995) including three universally conserved pseudouridylations (Ψ; (Ma et al., 2005; Massenet et al., 1999). While the specific function of these is not fully understood, a role in stabilizing spliceosomal RNA elements has been demonstrated (Huppler et al., 2002; Lin and Kielkopf, 2008; Newby and Greenbaum, 2002; Sashital et al., 2007).

If BSL helix stability is affected by nucleotide modifications, the greater number of modified nucleotides in the mammalian BSL may resolve differences in branchpoint consensus conservation between the two systems. In yeast, intron branchpoint nucleotides are highly conserved but mammalian pre-mRNAs contain highly divergent branchpoint sequences (Brow, 2002). Presenting the U2 branchpoint interaction sequence as a loop at the end of a helix increases the chance to locate and pair with the intron branchpoint. Increasing BSL stability via nucleotide modifications could dramatically improve the chances of recognizing highly degenerate intron branchpoints (Fig 5), while still ensuring enough flexibility to unwind when required.

How do RNAs get together? Dynamics of snRNP binding to its targets via RNA

The predicted BSL function has parallels in the establishment of other important RNA-RNA interactions. The RNA primer required for ColEl plasmid replication is regulated by an antisense RNA via initial formation of a “kissing” complex intermediate that is converted to a

stable RNA-RNA interaction (Cesareni, 1982). Similarly, retroviruses package a diploid RNA genome. The pairing occurs at the dimerization initiation site (DIS) and requires passage from an unstable “kissing” loop intermediate through conversion to a stable duplex (Paillart et al., 1996). Further, proposed models for initiator tRNA/codon recognition suggest correct pairing triggers a conformational change that locks the complex into a stable state (Kolitz et al., 2009). In each case the presentation of key interacting nucleotides nucleates the interaction and is followed by disruption, and extended pairing with the target sequence in a process that mirrors our model for BSL function.

Since the RNA moieties within other snRNPs must bind and release a defined set of target RNAs during their passage through the pre-mRNA splicing cycle, some of these may be similarly structured to effectively engage their substrates. It seems likely that snRNP structure has evolved to enhance the presentation of important RNA strands. Interestingly, U12 snRNA, the minor spliceosome counterpart of U2 does not seem to form the BSL. This apparent divergence is consistent with established differences between the two splicing pathways in early steps of spliceosome assembly (Patel and Steitz, 2003). Presenting critical sequences in loops is an energetically favorable way of initiating and promoting specific RNA-RNA pairing events. In addition, as befits a complex cascade of RNA-RNA events, the establishment of the prior structure includes presentation of the next RNA segments that must be paired. In this regard we note that disruption of the BSL exposes the U2 nucleotides important for formation of U2-U6 helix I (Madhani and Guthrie, 1992) and helix III (Sun and Manley, 1995), which would be expected to occur in the phases of tri-snRNP addition and spliceosome activation.

Supplementary Material

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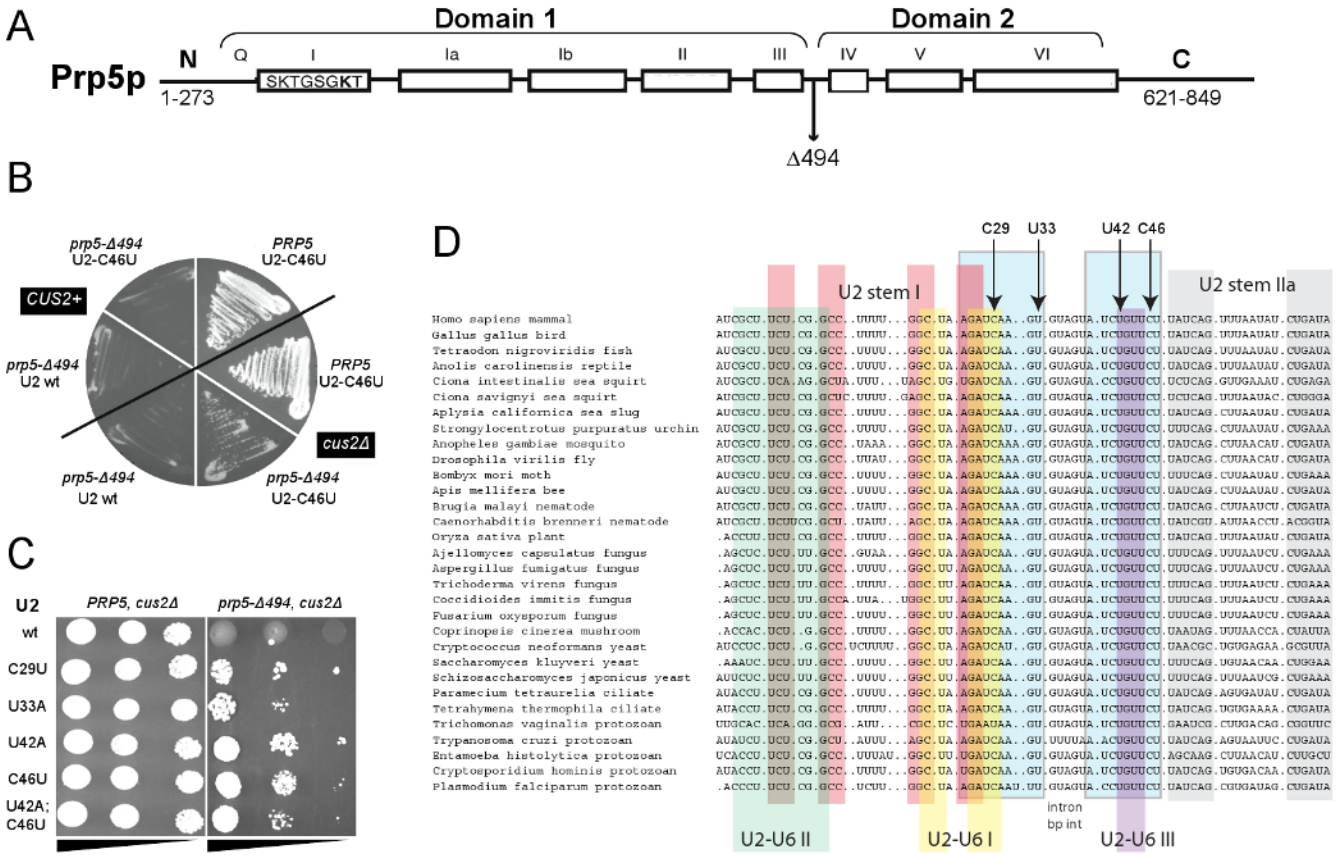

Figure 1. Point mutations in U2 snRNA rescue a large deletion of Prp5p

(A) Prp5p. The arrow shows position of truncation (residue 494), removing the C terminus and domain 2 of the conserved ATPase domain. (B) Rescue of *prp5-Δ494* by U2-C46U is *cus2Δ* dependent. Yeast disrupted for chromosomal *PRP5*, *U2* and *CUS2*, expressing *PRP5* or *prp5-Δ494*, Wt-U2 or U2-C46U, and *CUS2* or vector on 5FOA at 30°C for 5 days. (C) Other U2 mutations rescue *prp5-Δ494* when Cus2p is absent. Growth of yeast dilutions with *PRP5* (left) or *prp5-Δ494* (right) and the indicated U2 mutant. (D) Phylogenetic comparison of the 5′ end of U2 snRNA from 31 organisms demonstrates high conservation and universal conservation of suppressor residues (arrowed above). The 9 base inverted repeat (cyan) flanking the branchpoint interaction sequence (intron bp int) of the proposed BSL is indicated. Other RNA-RNA interactions involving overlapping portions of U2 RNA: U2-U6 helix II (green), U2 stem I (pink), U2-U6 helix I (yellow), U2-U6 helix III (purple).

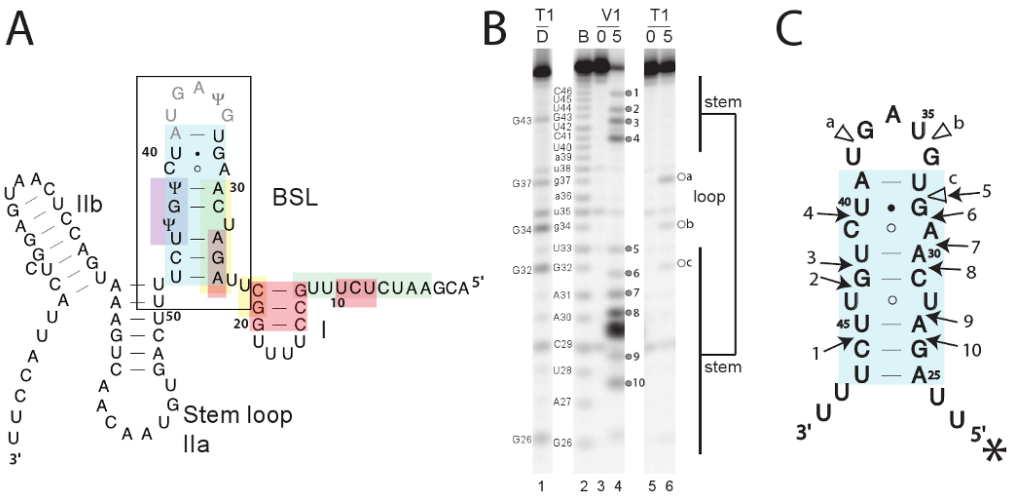

Figure 2. Structure mapping of a model BSL RNA

(A) U2 snRNA folded to contain the BSL. Nucleotides 1-92 of yeast U2 showing stem IIa, stem IIb, BSL and part of stem I. The branchpoint interaction sequence is in grey; Ψ: universally conserved pseudouridines. Nucleotides in other snRNA pairings are as in Fig 1D. (B) Nuclease T1 (lanes 1, 5, 6) and V1 (lanes 3-4) digestion of 5′ end labeled synthetic model BSL RNA. Lane 1: T1 digestion under denaturing conditions (“D”) with G residues indicated. Lane 2: alkaline ladder (“B”) with sequence indicated at left. Lane 3 and 4: V1 digestion for 0 or 5 minutes respectively with products indicated by grey circles. Lanes 5 and 6: T1 digestion for 0 or 5 minutes with products indicated by white circles. (C) V1 (arrows) and T1 (triangles) cleavages mapped to the model BSL sequence. Note that V1 cleavage products migrate more slowly than the corresponding T1 or alkaline hydrolysis products due to the absence of a 3′ phosphate, which reduces the negative charge to mass ratio of the RNA. The aberrant migration is exacerbated for lower molecule weight species where charge to mass ratio changes have a greater effect (Auron et al., 1982). Thus we consider assignment of the smaller V1 cleavage products to be tentative, but correct to within at least two residues.

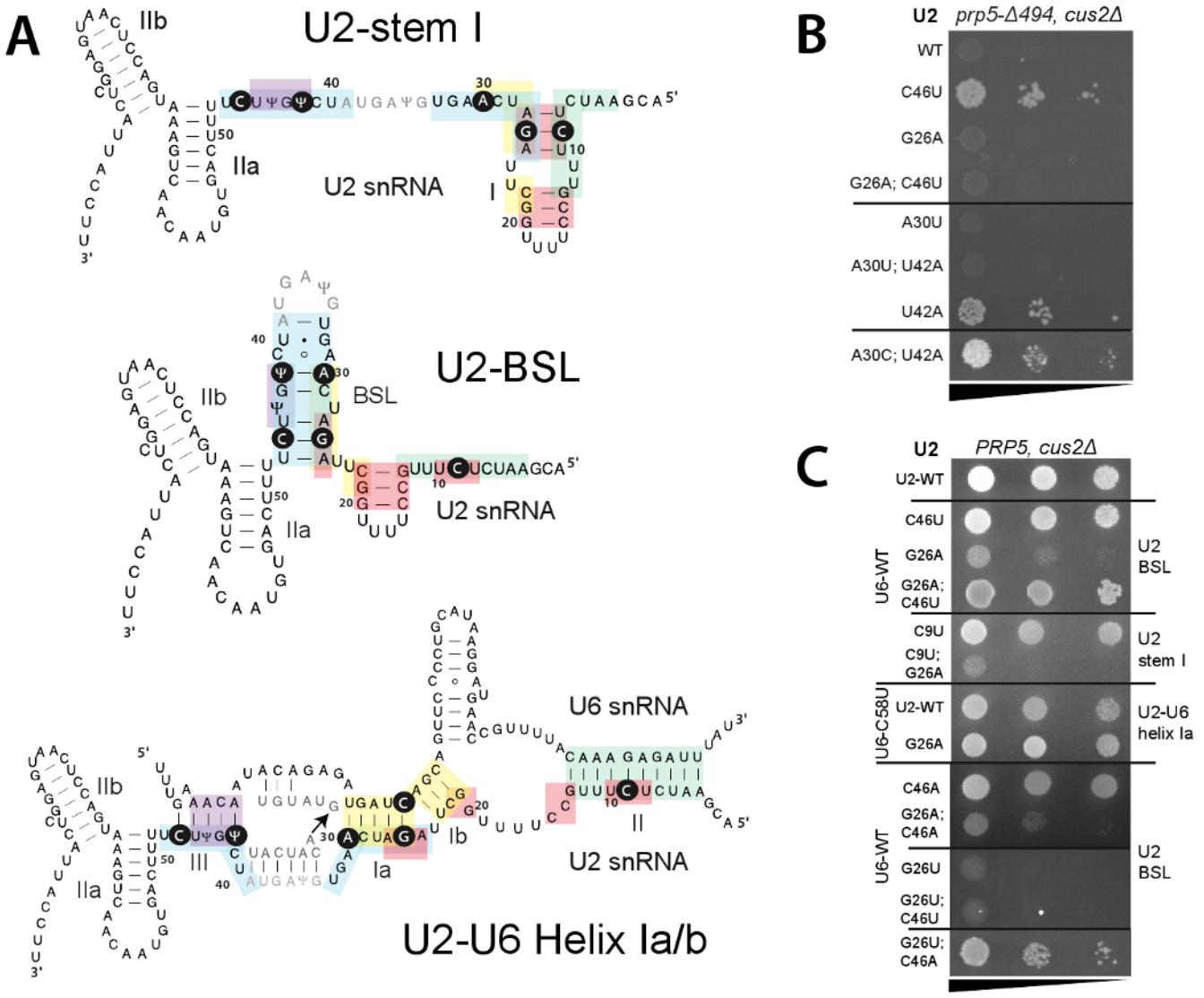

Figure 3. Genetic evidence for BSL function

(A) Structural rearrangements of U2 snRNA during splicing. U2 snRNA folded as U2 stem I form (top), the U2 BSL (middle) and U2-U6 helix Ia/b (bottom). The branchpoint interaction sequence is grey; U2 nucleotides C9, G26 and C46, A30 and U42, and U6 nucleotide C58 are highlighted in white on black circles. Colored sequences are as for 1D. (B) Watson-Crick pairing at the 26-46 or the 30-42 base pair abrogates *prp5-Δ494* suppression by non-Watson-Crick appositions. Growth of *cus2Δ* yeast expressing *prp5-Δ494* with indicated U2 genes on 5FOA, 4 days 30°C. (C) G26 interacts with both C46 in the BSL and U6-C58U in U2–U6 helix Ia. Growth of *cus2Δ* yeast expressing *PRP5* with indicated U2 and U6 genes on 5FOA, 4 days 30°C.

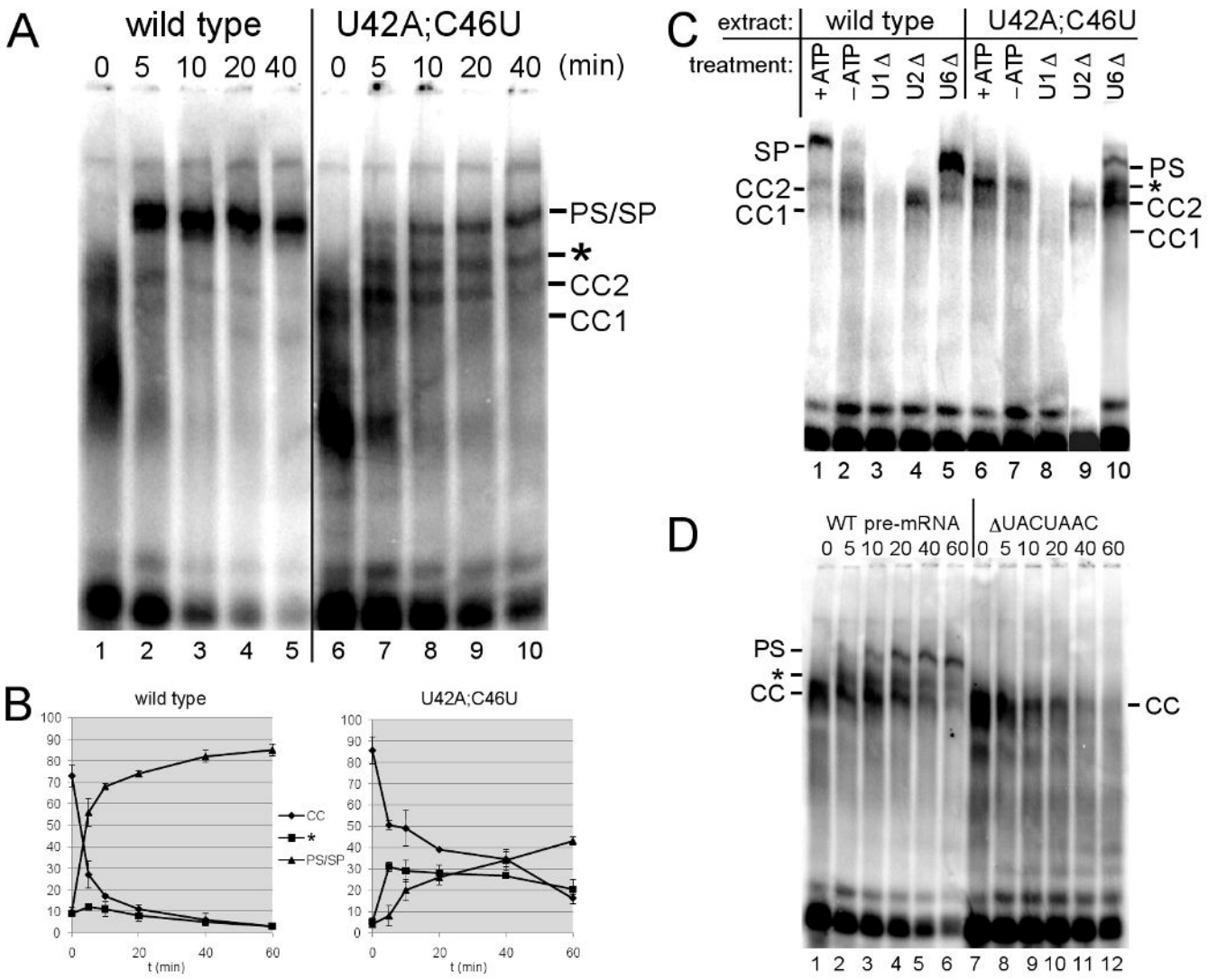

Figure 4. Splicing extracts with mutant U2 BSL accumulate an unusual complex

(A) Time course of splicing complex assembly at 17°C. RP51A pre-mRNA incubated in splicing extracts from U2 (lanes 1-5) or U42A; C46U (lanes 6-10) yeast. Reactions were stopped at 0, 5, 10, 20, 40 minutes after ATP + pre-mRNA addition, and run on native agarose-acrylamide gels. Prespliceosomes/spliceosomes (PS/SP) and commitment complexes 1 and 2 (CC1, CC2) are identified. The unusual complex is highlighted with an asterisk. (B) Quantification of splicing complex accumulation from three independent experiments: commitment complex (CC), the unusual complex (*) and prespliceosome/spliceosome (PS/SP) for U2 (left) or U42A; C46U (right) are plotted as time (X axis) verses % counts in each complex (Y axis). (C) Requirements for forming the unusual complex. U1 (lanes 3, 8), U2 (lanes 4, 9) or U6 (lanes 5, 10) oligonucleotide ablation of U2 (lanes 3-5) or U42A; C46U (lanes 8-10) extracts was performed followed by complex formation using RP51A pre-mRNA. ATP depletion of U2 (lane 2) or U42A; C46U (lane 7) extracts was performed followed by complex formation using RP51A pre-mRNA. Complexes are as in (A). (D) Substrate branchpoint requirement for forming the unusual complex. Time course from 0-60 minutes on WT (1-6) or ΔUACUAAC pre-RP51A in U2 and U42A; C46U extracts. Complexes are as in (A).

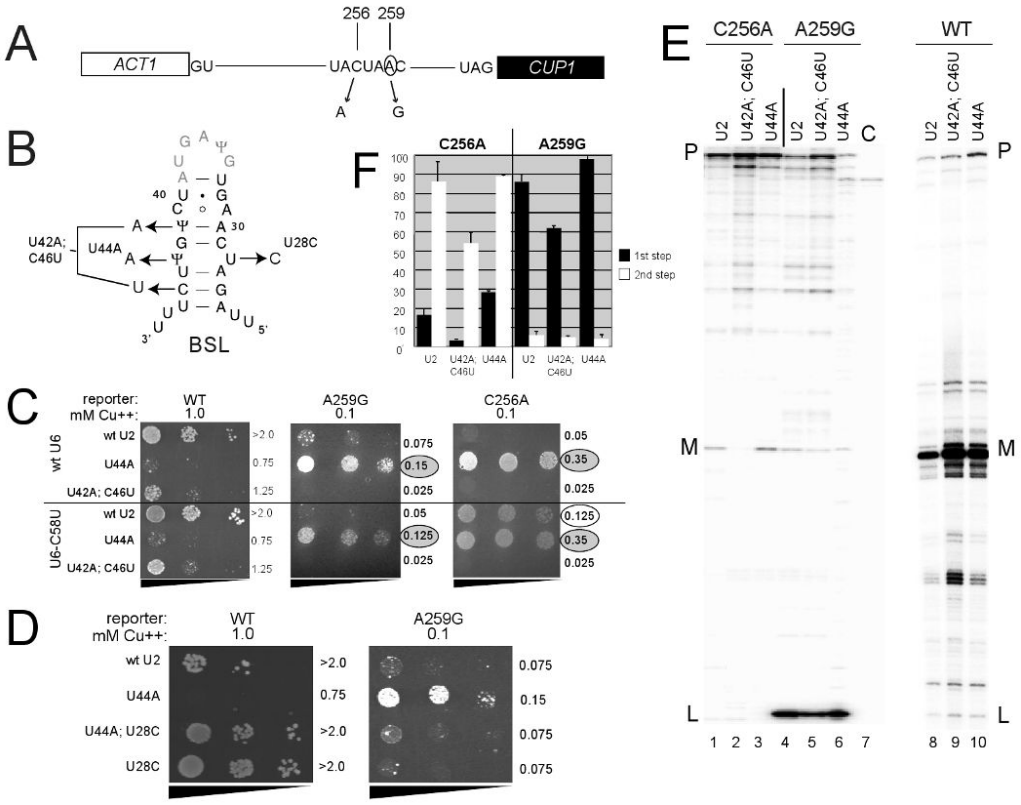

Figure 5. Hyperstable BSL suppresses branchpoint mutations

(A) The *ACT1-CUP1* reporter pre-mRNA. Point mutations C256A and A259G are shown. Circled A259 is the A residue that attacks the 5′ splice site for the first step of splicing to form the branch. (B) BSL secondary structure showing U44A, U42A; C46U, U28C mutations. (C) Copper sensitivity of *ACT-CUP* reporters in cells expressing wild type U6 (upper) or U6-C58U (lower) and wild type U2, U42A; C46U or U44A. Dilute cultures were spotted on 1.0 mM Cu ++ (WT) or 0.1 mM Cu++ (A259G and C256A). Maximum copper allowing growth is indicated (right of panel) with increased Cu++ tolerance circled. (D) Disrupting BSL hyperstabilization restores copper tolerance to wild type and abrogates branchpoint mutant suppression. Copper sensitivity assays of *ACT-CUP* (WT) or A259G when Watson-Crick pairing at positions 28–44 is disrupted (U44A; U28C). Dilutions and Cu++ are as for (C). (E) Spicing analysis of reporters. Primer extension products from strains expressing C256A (lanes 1-3), A259G (lanes 4-6) or wild type (lanes 8-10) *ACT-CUP* substrates. U2 alleles are indicated at top; Control “C” strain lacks any *ACT-CUP* plasmid. P, pre-mRNA, M, mature mRNA, L, lariat intermediate. (F) Quantification of step 1 and step 2 splicing efficiencies of C256A and A259G pre-mRNA with U2, U44A or U42A; C46U. First step (black bars) and second step (white bars) efficiencies are calculated as (M + L)/(P+M+L) and M/(M+L) respectively (Query and Konarska, 2004). Standard deviations are from three biological replicates.

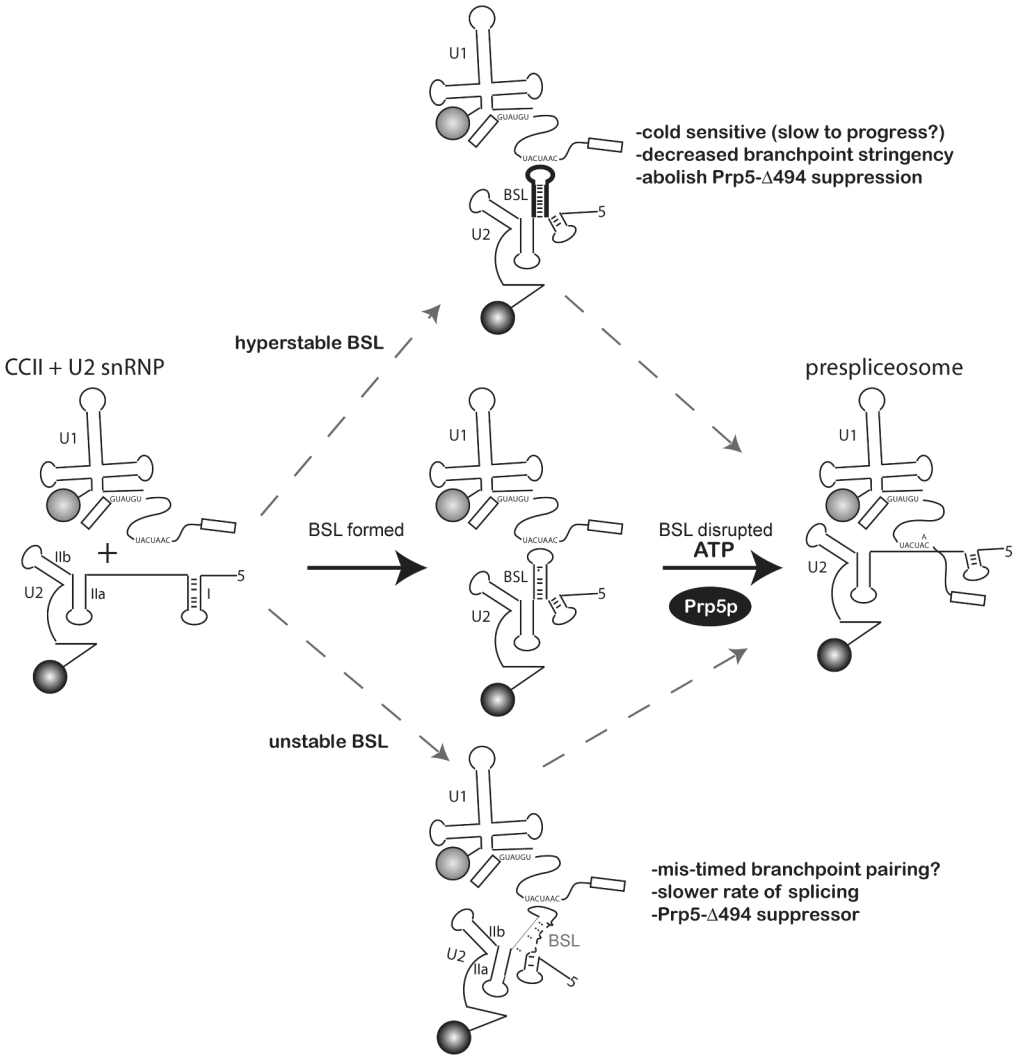

Figure 6. Model for BSL function during spliceosome assembly

In wild type, the BSL helps initiate the U2-branchpoint interaction (left to middle). The BSL is then disrupted, in a reaction involving Prp5p, allowing stable prespliceosomes to form leaving the BSL stem nucleotides free to basepair with U6 snRNA (middle to right). When BSL stability is altered, splicing efficiency becomes compromised. An unstable BSL (middle bottom) causes mis-timed branchpoint pairing, decreases the rate of splicing, but can suppress the *prp5-Δ494* lethal phenotype. Conversely, a hyperstable BSL (middle top) decreases the stringency of intron branchpoint selection, is cold sensitive, and abolishes *prp5-Δ494* suppression.

Table 1
Phenotypes of mutation in the base pairs of the BSL

BSL bases	mutation	base pair	growth	*prp5-Δ494* rescue
G26–C46	–	G–C	+	−
	C46U	G–U	+	+
	G26A	A–C	cs, ts	−
	C46A	G–A	+	−
	G26A; C46U	A–U	+	−*
	G26A; C46A	A–A	cs, ts	−
	G26U	U–U	-	−
	G26U; C46A	U–A	+	−
	G26U; C46U	U–U	-	−
U28–Ψ44	–	U–Ψ	+	−
	U28C	C–Ψ	+	
	Ψ44A	U–A	cs	−
	U28C; Ψ44A	C–A	+	
	U28C; Ψ44G	C–G	cs	
C29–G43	–	C–G	+	−
	C29U	U–G	+	+
	C29A	A–G	+	−
A30- Ψ42	–	A–Ψ	+	−
	Ψ42C	A–C	+	−
	Ψ42G	A–G	+	−
	Ψ42A	A–A	+	+
	A30C; Ψ42A	C–A	+	+
	A30U	U–Ψ	+	−
	A30U; Ψ42A	U–A	+	−*
A31–C41	–	A–C	+	−
	C41U	A–U	cs	−
U33–A39	-	U–A	+	−
	U33A	A–A	+	+
G26–C46	Ψ42A; C46U	G–U	ts	+
A30–Ψ42		A–A		

Ψ, pseudouracil replaces U in wild type; +, growth; −, no growth; cs, cold sensitive growth; ts, heat sensitive growth, *, abrogation of *prp5-Δ494* suppression by restoration of Watson-Crick pairing.