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trans-Splicing to Spliceosomal U2 snRNA Suggests Disruption of Branch Site-U2 Pairing during Pre-mRNA Splicing

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SUMMARY

Pairing between U2 snRNA and the branch site of spliceosomal introns is essential for spliceosome assembly and is thought to be required for the first catalytic step of splicing. We have identified an RNA comprising the 5′ end of U2 snRNA and the 3′ exon of the *ACT1-CUP1* reporter gene, resulting from a *trans*-splicing reaction in which a 5′ splice site-like sequence in the universally conserved branch site-binding region of U2 is used in *trans* as a 5′ splice site for both steps of splicing in vivo. Formation of this product occurs in functional spliceosomes assembled on reporter genes whose 5′ splice sites are predicted to bind poorly at the spliceosome catalytic center. Multiple spatially disparate splice sites in U2 can be used, calling into question both the fate of its pairing to the branch site and the details of its role in splicing catalysis.

INTRODUCTION

The removal of introns from pre-mRNA is catalyzed by the spliceosome—a large, conformationally and compositionally dynamic ribonucleoprotein complex comprising five small nuclear RNAs (snRNAs) and more than 100 proteins (Nilsen, 1998). The catalytic phase of splicing consists of two consecutive transesterification reactions: in the first step, the 2′ hydroxyl of the branch site adenosine (BS-A) attacks the phosphodiester bond at the 5′ splice site (5′SS), yielding a lariat intermediate and a free 5′ exon, which attacks the 3′SS in the second step to produce an excised lariat intron and spliced mRNA. Catalysis is preceded by an extended assembly phase: the 5′SS and branch site (BS) are bound by U1 and U2 snRNPs, respectively, and the 3′SS by protein factors; the [U4/U6•U5] tri-snRNP joins the complex, and a series of ATP-dependent conformational rearrangements results in the release of U1 and then U4 (Burge et al., 1999). The recruitment of the CDC5L complex (NTC in *S. cerevisiae*) completes the formation of the catalytically competent spliceosome (Makarov et al., 2002).

The pairing established between U2 snRNA and the BS during the early stages of spliceosome assembly (Parker et al., 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989) is thought to persist unaltered until after the first catalytic step of splicing, and possibly beyond. Experiments involving arabinosyl adenosine substitution at BS support the hypothesis that BS-A is activated for first-step catalysis by being bulged from the BS-U2 duplex (Query et al., 1994), and that this duplex therefore represents the catalytically relevant state of BS (Figure 1A). Further support for this model comes from NMR studies on model duplexes, which indicate that the conserved pseudouridine modification at U2 position 35 (*S. cerevisiae* numbering) selectively stabilizes the form of the duplex in which BS-A is bulged from, rather than intercalated into, the helix (Newby and Greenbaum, 2002).

U2 and U6 snRNAs appear to have intrinsic catalytic potential in combination with splicing substrates. In vitro-transcribed snRNAs, when incubated with a transcript containing a BS sequence, induce the formation of a covalent bond proposed to be a phosphotriester between the canonical BS-A and the guanosine in the universally conserved U6 AGC triad (Valadkhan and Manley, 2003). This reaction is stimulated by pseudouridylation of U2 position 35, suggesting that bulging BS-A from a duplex may be important for the observed reactivity. This reaction may have further biological relevance, as the site of reactivity in U6 is adjacent to the location of an intron in the *S. pombe* U6 snRNA gene (Tani and Ohshima, 1991), thought to have arisen due to an aberrant splicing event followed by retrotransposition of the resulting product into the genome.

U6 reactivity in a spliceosomal context has been observed directly (Yu et al., 1993). In an in vitro system designed to reconstitute nematode *cis*- and *trans*-splicing using *Ascaris lumbricoides* extracts, Nilsen and coworkers detected branched and linear RNA products indicating the use of U6 as both a branch acceptor and a 5′ exon in a 5′SS-independent splicing reaction. The reaction appears to represent an aberrant first step followed by a quasi-normal second step, as the canonical BS and 3′SS nucleotides are used, and U6 mutants that

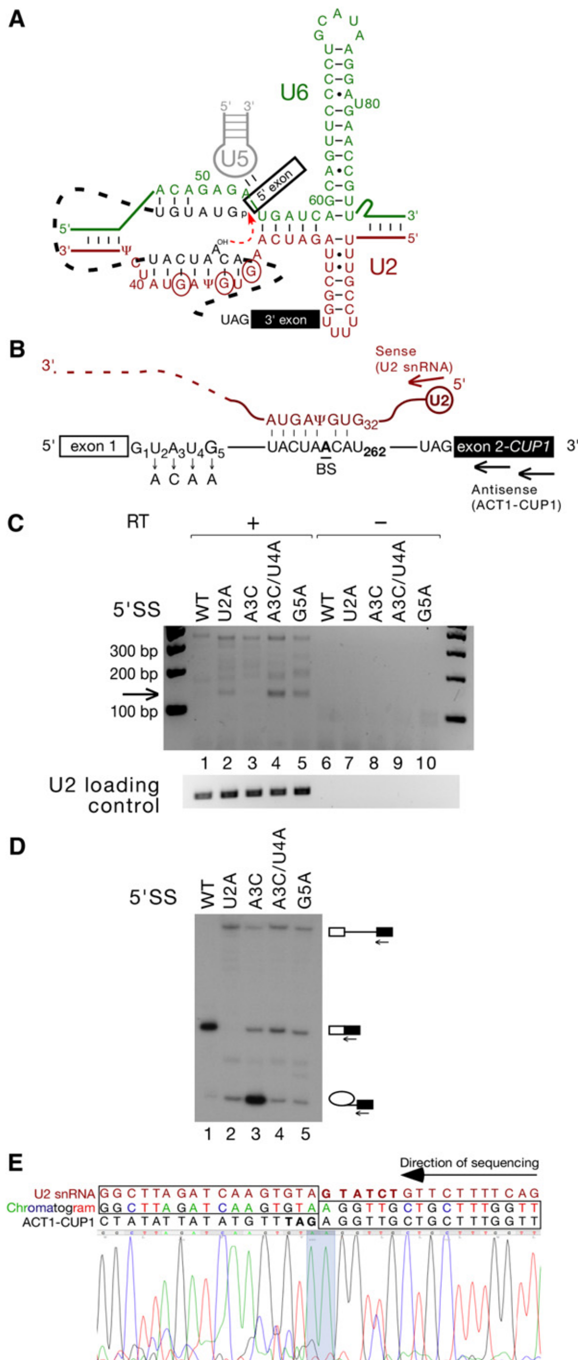

Figure 1. *trans*-Splicing in *S. cerevisiae* Can Generate an RNA Species Comprising the 5′ End of U2 snRNA and the 3′ Exon of the *ACT1-CUP1* Reporter

(A) Schematic of RNA-RNA interactions that contribute to the first step of splicing (modified from Konarska et al. [2006]) with the initial G of each of the three 5′SS-like sequences in the BS-binding region of U2 snRNA circled.

(B) Schematic of *ACT1-CUP1* reporter pre-mRNA and U2 snRNA, indicating the mutations used in (C) and (D), and the location of the RT and PCR primers (arrows) used in (C).

(C) RT-PCR using the primers indicated in (B) can amplify a product, of the size expected for a *trans*-splicing product generated using the BS-binding region of U2 as a 5′SS, from total RNA from *S. cerevisiae* Y04999 cells carrying reporters with 5′SS mutations as indicated.

(D) Primer extension analysis of RNA recovered from cells containing the *ACT1-CUP1* reporters as indicated. Primer complementary to the 3′ exon was used to reveal levels of pre-mRNA, mRNA, and lariat intermediate. Strain Y04999 (Δdbr1) was used in order to accurately monitor the efficiency of the first step.

(E) The 130 bp RT-PCR product corresponds to *trans*-spliced U2-ACT1-CUP1. Reverse-complemented sequencing trace obtained from the purified 130 bp product: U2 snRNA and *ACT1-CUP1* sequence, as well as the chromatogram read, are indicated above the trace; concordance between chromatogram and gene sequence is indicated by boxes, and the splice junction is highlighted on the chromatogram trace.

abolish the ability to reconstitute normal *cis*- and *trans*-splicing also render U6 *trans*-splicing undetectable.

Although U6 is the only snRNA that has been demonstrated to be reactive, the presence of multiple 5′SS-like sequences in the BS-binding region of U2 (Figure 1A), and the presence of a spliceosomal intron close to this region in *Rhodotorula hasegawae* (Takahashi et al., 1993), prompted us to search for the products of a *trans*-splicing reaction involving U2 in *S. cerevisiae*. Here we describe the formation of an RNA product comprising the highly conserved 5′ end of U2 and the 3′ exon of the *ACT1-CUP1* reporter gene in intact *S. cerevisiae* cells. The formation of this product requires full spliceosome assembly, and is only detectable when the reporter has a mutant 5′SS predicted to bind poorly at the spliceosome catalytic center. The predominant 5′SS used in U2 is in the middle of the predicted BS-U2 duplex, but multiple sites can be used; these data suggest that BS-U2 pairing is unlikely to be required for the second catalytic step of splicing, and that it may be at least partially disrupted prior to the first step.

RESULTS

The BS-Binding Region of U2 snRNA Can Be Used In *trans* as a 5′ Exon for Both Steps of Splicing

Attack of U2 snRNA by the BS nucleophile would be expected to generate a truncated U2 molecule covalently linked via its new 5′ end to the 2′ hydroxyl of BS, detectable as a strong stop by primer extension. An initial screen for such branched derivatives of U2 snRNA in *S. cerevisiae* total RNA was inconclusive due to unavoidable low levels of degradation (data not shown). We therefore reasoned that any "free 5′ exon" generated by such aberrant attack may be able to undergo the second step of splicing in *trans* to the 3′SS of a reporter gene, generating a novel linear RNA detectable by RT-PCR. We performed nested RT-PCR on DNase I-treated total RNA from strains carrying *ACT1-CUP1* reporter genes (Lesser and Guthrie, 1993) with various mutations, using antisense primers complementary to the CUP1 3′ exon and a sense primer corresponding to the 5′ end of U2 snRNA (Figure 1B). For multiple intron 5′SS mutants (G1C, G1U, U2A, A3G, [A3C + U4A], G5A, and G5C), we were able to detect

an ∼130 bp PCR product whose size corresponded to that expected for the *ACT1-CUP1* 3′ exon appended to ∼40 nucleotides (nt) of U2 (Figure 1C, lanes 2, 4, and 5 and data not shown). The 130 bp product was not generated from equivalent amounts of total RNA from strains carrying wild-type or 5′SS A3C *ACT1-CUP1* reporter, both of which show an efficient first step of canonical *cis*-splicing (Figures 1C and 1D, lanes 1 and 3).

Direct sequencing of the putative *trans*-splicing product indicated that it comprised the 5′-terminal 36 nt of U2 joined to the 3′ exon of *ACT1-CUP1* (Figure 1E). The homogeneity of the sequencing trace indicates a strong splice site preference, with the wild-type *ACT1-CUP1* 3′SS and the A/GUAUCΨ site in U2 (the best match in this region of U2 to G/GUAUGU, the *S. cerevisiae* consensus) apparently used substantially more efficiently than other sites.

The *trans*-splicing reaction appears to be very inefficient, with steady-state levels of the U2-ACT1-CUP1 product apparently lower than one molecule per cell (data not shown). We estimate that, in the case of the G5A mutant, *trans*-splicing represents around 1 per 100,000 two-step splicing events, comparable to the error rates of other macromolecular processes such as transcription.

The generation of the U2-ACT1-CUP1 RNA by an aberrant but otherwise mechanistically normal splicing reaction should also produce a second species—a branched RNA comprising the 5′ exon and intron of *ACT1-CUP1* joined via the 2′ hydroxyl of BS-A to the 3′ 1139 nt of U2, and an intermediate consisting of the "5′ exon" portion of U2 with a 3′ hydroxyl; although we attempted to detect these products using RT-PCR- and Klenow-based methods, respectively, we were unable to do so. This may in part be due to the rapid degradation of internally cleaved U2 snRNAs (McPheeters et al., 1989).

trans-Splicing to U2 snRNA Requires Poor Binding of the Canonical 5′SS at the Spliceosomal Catalytic Center

To investigate whether *trans*-splicing to U2 snRNA is simply a result of inhibition of the first step of the canonical splicing reaction, we analyzed the effects of non-5′SS reporter gene mutations on the efficiency of *trans*-splicing. Mutations at and around BS (C256G, U257A, U257C, BS-C, and BS-G—Figure 2A), which virtually abolish productive splicing of the reporter transcript (Figure 2C), did not promote *trans*-splicing (Figure 2B, lanes 1–7). When combined with a 5′SS G5A mutation, which alone showed easily detectable levels of *trans*-splicing, diverse mutations at and around BS inhibited the *trans*-splicing reaction to the extent that the product was no longer detectable (Figure 2B, lanes 15–19). Even mutations that show only mild inhibition of the first step of canonical *cis*-splicing of G5A mutants, such as an extension of the pairing interaction between BS and U2 snRNA, abolished *trans*-splicing (Figures 2B and 2C, lanes 15 to 16). 3′SS mutants exhibited behavior identical to that of BS mutants, failing to promote *trans*-splicing in an otherwise wild-type reporter and reducing it to undetectable levels in the context of a G1C mutation (data not shown). BS and 3′SS mutations exacerbate the inhibition of canonical mRNA splicing caused by the G5A mutation (Figure 2C, lanes 15–19 and data not shown), so their failure to further stimulate the aberrant *trans*-splicing reaction indicates that the requirements for this reaction extend beyond simple first-step inhibition.

Even among 5′SS mutants, the degree of first-step inhibition is not a good predictor of *trans*-splicing efficiency. For example, splicing is much more strongly inhibited by the 5′SS U2A mutation than by [A3C + U4A] (Figure 1D, lanes 2 and 4), yet the extent of *trans*-splicing to U2 snRNA is greater in the [A3C + U4A] mutant (Figure 1C, lanes 2 and 4). [A3C + U4A] has been shown to affect splicing predominantly by altering 5′SS binding at the catalytic center of the spliceosome (Konarska et al., 2006). Formation of the observed U2-ACT1-CUP1 RNA therefore appears to result from the aberrant attack by BS on U2 in the presence of a 5′SS that is poorly bound within the spliceosome catalytic center, rather than simply from an inefficient first step of splicing.

The Functional U2 snRNA Component of the Spliceosome Is the Substrate for *trans*-Splicing

To verify that the U2 snRNA portion of the *trans*-spliced product was derived from the U2 component of a functional spliceosome (rather than, for example, a second molecule of U2 displacing the reporter gene 5′SS), we took advantage of previously characterized compensatory mutations in U2 and U6 snRNAs. Mutation of U6 positions 56 and 57 from AU to UA is lethal except in the context of the compensatory (and likewise lethal in isolation) AU to UA mutations at positions 27 and 28 in U2, which are predicted to restore pairing in helix Ib (Madhani and Guthrie, 1992 and Figure 3A). *S. cerevisiae* U2/U6 double knockout strains were constructed in which plasmid-borne copies of both wild-type and mutant U2, and either wild-type or mutant U6, were present. In these strains, the identity of the U2 snRNA component of functional spliceosomes is expected to depend on the identity of the U6 snRNA gene carried, i.e., spliceosomes in the strain with wild-type U6 should contain wild-type U2, and those in the strain with mutant U6 should contain mutant U2 (Figures 3B and 3C). In both cases, the pool of "free U2," as well as early spliceosome assembly intermediates, should contain both wild-type and mutant U2. The "wild-type spliceosome" and "mutant spliceosome" strains were transfected with G5A *ACT1-CUP1* reporter, and the U2-ACT1-CUP1 *trans*-spliced product from each strain was amplified by RT-PCR. Sequencing indicated that the U2 snRNA portion of the *trans*-spliced product differed as predicted. The strain carrying a wild-type U6 snRNA gene generated a *trans*-spliced product in which U2 sequence was wild-type (Figure 3D), and that carrying the mutant U6 snRNA gene generated product with mutant U2 sequence (Figure 3E), indicating that the

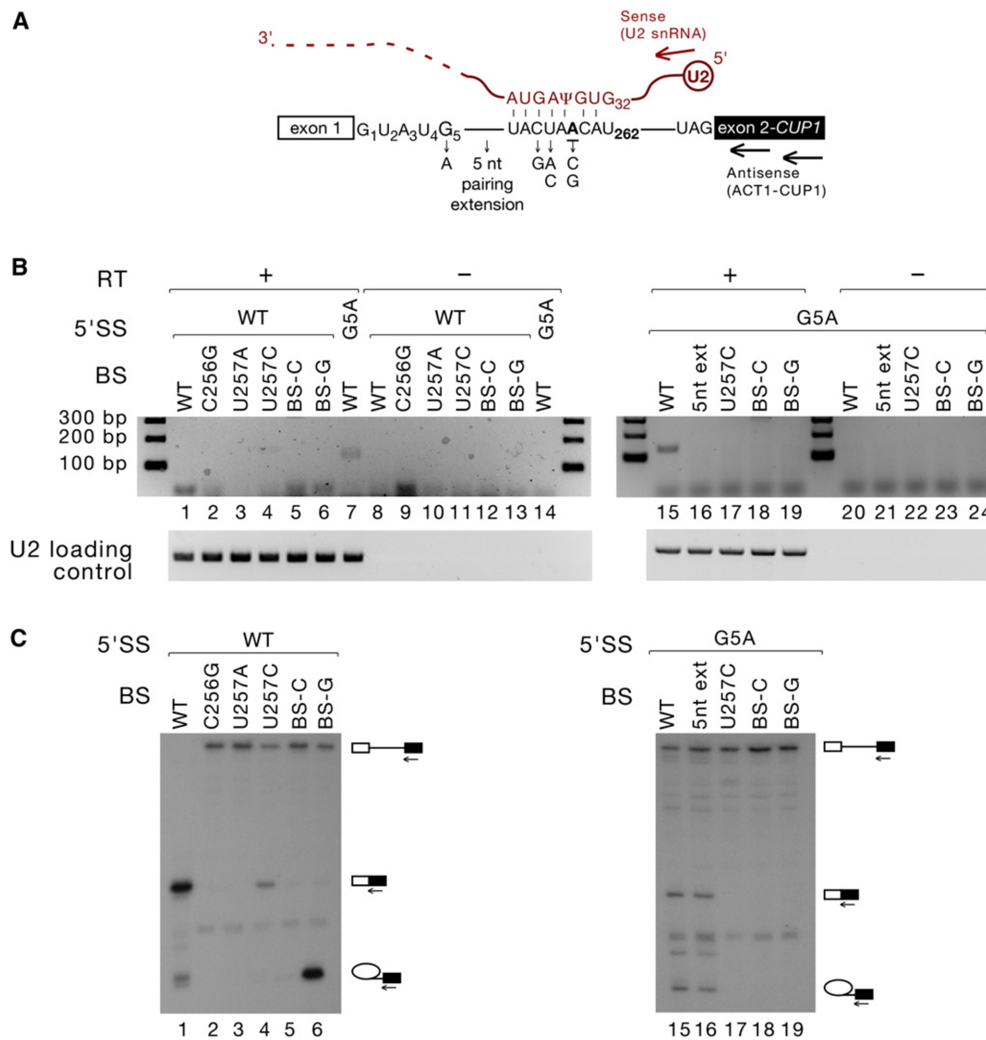

Figure 2. *trans*-Splicing Does Not Occur in Reporter Genes with Mutations outside the 5′SS

(A) Schematic of *ACT1-CUP1* reporter pre-mRNA and U2 snRNA, indicating the mutations used in (C) and (D), and the schematic location of RT and PCR primers (arrows) used in (B).

(B) Mutations at and around the BS do not stimulate *trans*-splicing in an otherwise wild-type context, and inhibit it in the context of an accompanying 5′SS mutation. RT-PCR analysis of RNA recovered from Y04999 cells containing the *ACT1-CUP1* reporters as indicated, as in Figure 1C.

(C) Primer extension analysis of RNA recovered from Y04999 cells containing the *ACT1-CUP1* reporters as indicated, as in Figure 1D.

trans-splicing reaction involves nucleophilic attack of the U2 snRNA component of a fully assembled, catalytically competent spliceosome, and therefore of the BS-binding region of U2 to which BS was bound.

Splicing Can Occur to All Three Predicted 5′SS in U2 snRNA

Although direct sequencing of the purified *trans*-splicing product had indicated the preferred use of CAAGUGΨA/GUAUCΨ as a 5′SS, and of the wild-type *ACT1-CUP1* 3′SS, we reasoned that the use of less-favored sites may be apparent if individual DNAs were analyzed. We therefore cloned the product into the pDrive sequencing vector (QIAGEN) and sequenced multiple clones. As expected, the majority of these clones (9/13) had used the preferred 5′SS in U2; however, one had used the GΨ/AG site 1 nt upstream, two had used the GU/GU site a further 2 nt upstream of this, and one had used the AA/GU a further 2 nt upstream (splice site usage in U2 snRNA is shown in Figure 4E). All clones had used the wild-type *ACT1-CUP1* 3′SS. Due to our inability to detect reaction intermediates, we do not know whether the levels of *trans*-spliced products reflecting the use of distinct 5′SS within U2 snRNA indicate differences in the efficiencies of the first step, the second step, or both, for each potential 5′SS. The 5′SS observed to be used span half a helical turn of RNA: the use of such spatially disparate 5′SS is inconsistent with nucleophilic attack by the

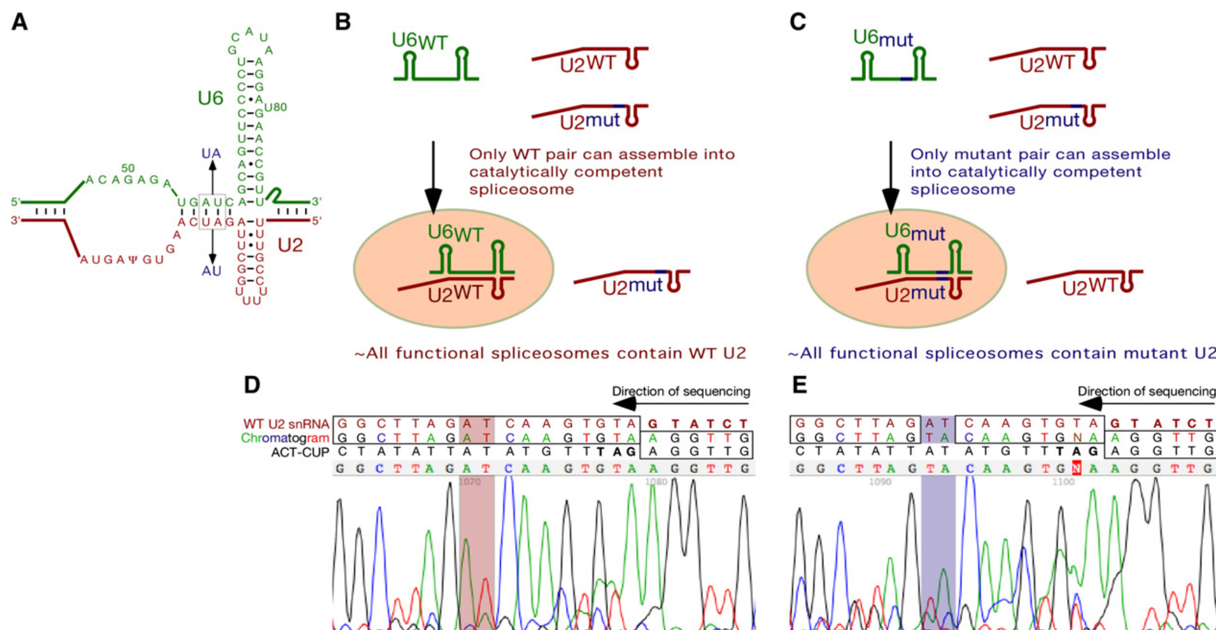

Figure 3. U2 snRNA in Functional Spliceosomes Is the Substrate for *trans*-Splicing

(A) Compensatory U2 and U6 snRNA mutations used in (C) and (D).

(B and C) Predicted behavior of wild-type and mutant U2 snRNAs in strains carrying a wild-type (B) or mutant (C) U6 snRNA gene.

(D and E) The *trans*-spliced product is generated by attack of U2 snRNA in functional spliceosomes. Reverse-complemented sequencing traces from purified *trans*-splicing product from yCQ62 cells carrying G5A *ACT1-CUP1* reporter, both wild-type and mutant U2 snRNA genes, and a wild-type (D) or mutant (E) U6 snRNA gene. Concordance between chromatogram and wild-type gene sequence is indicated by boxes, and the mutated/wild-type helix Ib U2 snRNA nucleotides are highlighted.

canonical BS-A within an intact, unaltered, and undisrupted BS-U2 duplex. Given that, even in the context of BS mutations that dramatically reduce splicing efficiency (BS-C, BS-U), *S. cerevisiae* introns have invariably been observed to branch from the pyrimidine nucleotide rather than the adjacent adenosine (Vijayraghavan et al., 1986 and D.J.S., C.C.Q., and M.M.K., unpublished data), at least some transient unwinding of the BS-U2 duplex seems more likely than aberrant attack by a noncanonical BS nucleotide.

The BS-Binding Region of U2, in the Context of the *ACT1-CUP1* Reporter Gene, Is Inefficiently Used In *cis* as a 5′SS and Shows a 5′SS Preference Distinct from that Observed in *trans*-Splicing

Given the tandem arrangement of three 5′SS-like sequences in the BS-binding region of U2 snRNA, and their use as 5′SS in the context of reporter gene mutations such as [A3C + U4A] and G5A that do not dramatically reduce the overall efficiency of *cis*-splicing, we investigated the efficiency with which U2 sequence could be used as a 5′SS in the context of the *ACT1-CUP1* reporter. Forty-eight nucleotides of reporter sequence around the 5′SS were replaced with sequence corresponding to the first 48 nt of *S. cerevisiae* U2 snRNA, such that the /GUAUCU 5′SS from U2 replaced the /GUAUGU 5′SS in the reporter (Figure 4A). Primer extension analysis of total RNA from strains expressing these *ACT1-U2-ACT1-CUP1* constructs revealed that this U2-substituted 5′SS was used inefficiently (Figure 4B). A small amount of spliced product could be detected by RT-PCR (Figure 4C); this RT-PCR product was cloned into pDrive (QIAGEN). Sequencing of eight clones revealed that 5′SS usage in U2 sequence in the context of the reporter gene (Figure 4D) was different from that in the context of U2 (Figure 4E). 5′SS usage in the U2-substituted reporter gene correlated relatively well with predicted U1 snRNA binding affinity (Seraphin and Rosbash, 1989), with the upstream-most 5′SS (AA/GUGUA) used in 4/8 clones and the downstream-most 5′SS (UA/GUAUCU) in only 1/8 (cf. 9/13 in the context of *trans*-splicing to U2). This suggests that, while this region of U2 snRNA sequence can be recognized by U1 snRNA and participate to a low level in a canonical mRNA splicing reaction when placed in the context of an otherwise wild-type reporter gene, it is a suboptimal substrate for splicing. This rationalizes the observation that U2 cannot compete effectively with a wild-type 5′SS. In addition, the different 5′SS preference observed between the *cis*- and *trans*-splicing reactions indicates that pairing between U1 and U2 snRNAs does not determine 5′SS usage in U2 snRNA in *trans*-splicing; the most likely candidate for such a determinant of 5′SS preference in *trans*-splicing is spatial proximity to the BS nucleophile. The use of multiple 5′SS in the *trans*-splicing

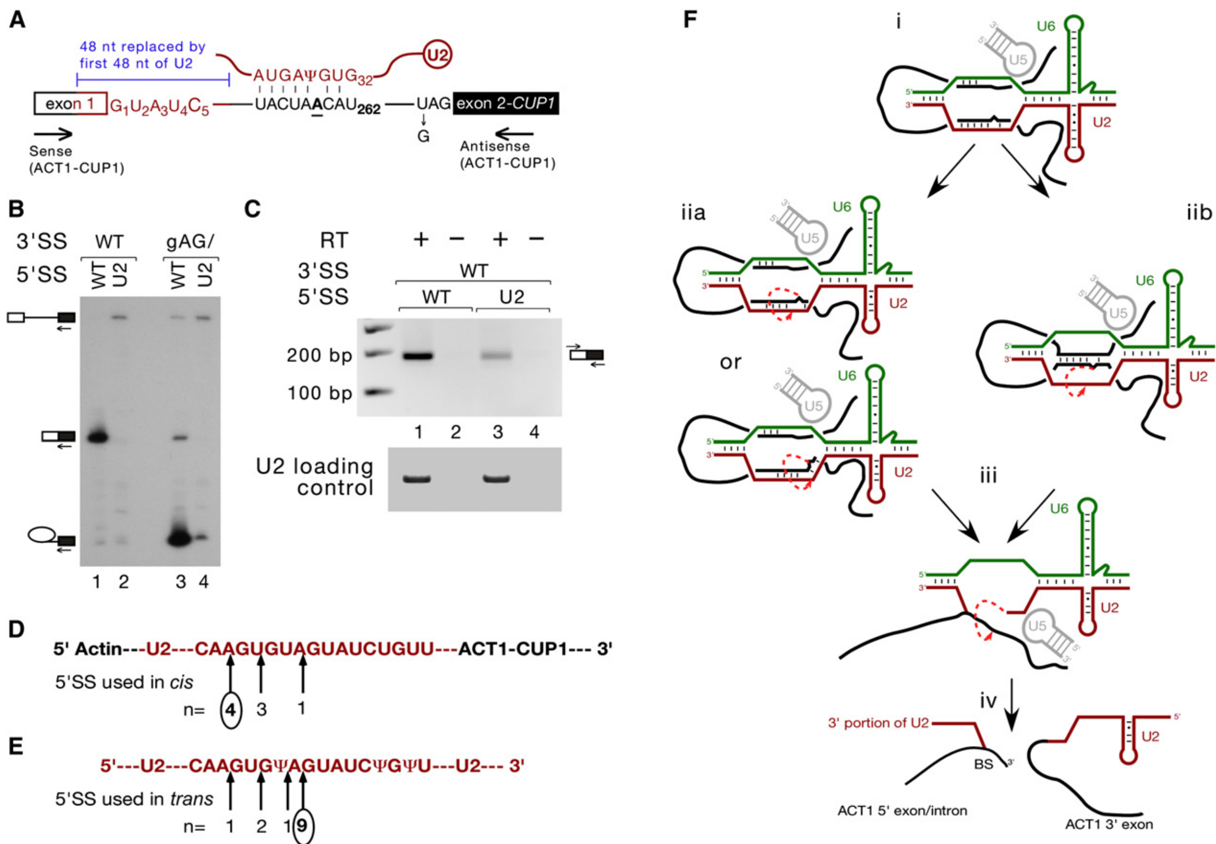

Figure 4. U2 snRNA Sequence Is a Suboptimal 5′SS in the Context of a Reporter Gene

(A) Schematic of *ACT1-CUP1* reporter pre-mRNA and U2 snRNA, indicating the U2 sequence substitution into *ACT1-CUP1* and 3′SS gAG/ mutation used in (B) and (C), and the RT-PCR primers used in (C).

(B) Primer extension analysis of RNA recovered from Y04999 cells containing the *ACT1-CUP1* reporters as indicated, as in Figure 1D.

(C) Spliced ACT1-U2-ACT1-CUP1 mRNA can be detected by RT-PCR (lane 3). Wild-type mRNA is shown for comparison (lane 1).

(D and E) Schematic indicating 5′SS usage in U2 sequence in *cis* in the context of *ACT1-U2-ACT1-CUP1* (D) and in *trans* in the context of U2 snRNA (E). n refers to the number of plasmids sequenced containing *trans*-spliced product that had used the 5′SS indicated by the arrow above, and circles indicate the predominant 5′SS detected in directly sequenced PCR products.

(F) Proposed mechanisms of *trans*-splicing: (i) RNA-RNA interactions in the fully assembled spliceosome, with BS paired to U2. (iia) BS and U2 unpair and re-pair potentially inaccurately, with BS nucleophilically attacking U2 from within the resulting helix, or (iib) BS and U2 unpair, with new pairing established between BS and the 5′SS; BS, bulged from this new helix, attacks U2. (iii) 5′SS-U6 and BS-U2 pairing both having been disrupted, the U2 5′ exon generated in (ii) attacks the reporter gene 3′SS, generating (iv) a branched product comprising the 3′ end of U2 appended to the 5′ exon and intron of the reporter gene, and a linear product comprising the 5′ end of U2 appended to the reporter 3′ exon.

reaction is therefore indicative of a high degree of flexibility in the spliceosomal catalytic center.

DISCUSSION

The Nature of the *trans*-Splicing Reaction

The data presented here demonstrate the formation of a *trans*-spliced RNA product comprising the 5′ end of U2 and the 3′ exon of an *ACT1-CUP1* reporter with a 5′SS predicted to bind poorly at the spliceosome catalytic center. The possibility of this product being generated by an RT artifact is ruled out by its strict dependence on 5′SS mutation. In strains expressing two distinct U2 snRNAs, the identity of the U2 portion of the *trans*-spliced product was observed to be dependent on the identity of the U6 expressed by the strain, with the potential for helix I formation between U2 and U6 an essential requirement for *trans*-splicing. Interactions between U2 and U6 are established during the late stages of spliceosome assembly, so the requirement for such interactions indicates that U2 snRNA within a functional spliceosome is nucleophilically attacked, presumably by the BS it recruited.

We were able to detect *trans*-splicing using reporter genes with wild-type 3′SS, but not with 3′SS mutants. In addition, all *trans*-splicing events observed involved the same 3′SS as is used in canonical *cis*-splicing of the reporter gene. These observations support the hypothesis that the *trans*-splicing reaction is essentially identical to

cis-splicing, but with an aberrant first catalytic step in which U2, rather than the 5′SS, is attacked; the exon generated by this aberrant first step proceeds through the second step in a quasi-normal fashion. As we were unable to detect reaction intermediates, the efficiency of this second step could not be investigated. Our inability to detect a branched intermediate also renders uncertain the identity of the first-step nucleophile, although the inhibition of *trans*-splicing by mutations at and around BS supports a 2′-5′ branching reaction. A hydrolytic first step analogous to that observed in group II introns (Podar et al., 1998) cannot be ruled out by our data, but we feel that the existence of such a first step is unlikely and would not substantially affect our interpretation.

Flexibility in the Spliceosome Catalytic Center, and the Fate of the BS-U2 Duplex for the First Catalytic Step of Pre-mRNA Splicing

Splicing is a multistep reaction that necessarily involves conformational rearrangements as well as complex assembly and disassembly phases; global conformational flexibility is clearly required for such a reaction. In addition to this necessary flexibility, however, splicing is characterized by the need for nucleotide precision; this is especially true in *S. cerevisiae*, an organism with strong splice site consensuses in which functionally important alternative splicing has not been demonstrated. Therefore, while both global flexibility throughout the assembly-reaction-disassembly pathway and local flexibility at the catalytic center between the two chemical steps are inevitable, tight substrate binding between rearrangements is expected. As noted by Nilsen and coworkers (Yu et al., 1993), snRNA reactivity in spliceosomes is indicative of catalytic center flexibility. The reactivity of an snRNA region intimately involved in substrate binding during assembly, and possibly also catalysis, extends our view of this flexibility to include pairing interactions important for splicing fidelity. The nucleophilic attack of four sites in U2 is inconsistent with reactivity within a BS-U2 duplex that has remained intact from early spliceosome assembly until the catalytic phase, even in the unlikely scenario of a hydrolytic first step. To rationalize the reactivity of multiple sites in U2, it is therefore necessary to invoke at least transient disruption of the interaction between BS and U2. Following this unpairing, three possibilities exist between which we cannot currently distinguish: the BS, the presumed nucleophile for the first step of the *trans*-splicing reaction, could attack U2 while in a single-stranded state; this seems unlikely, as such circumvention of the need for nucleophile activation would be highly surprising in a reaction as important and precise as splicing. A second possibility is that BS may simply re-pair somewhat inaccurately with U2, and attack from within the resulting reformed duplex (Figure 4F, iia). The third possibility is that BS may pair to another RNA strand and attack U2 from within this duplex (Figure 4F, iib). Given the similarity between the sequences of the yeast consensus 5′SS and the BS-binding region of U2, the 5′SS represents an obvious candidate for such a partner. While a complete strand exchange reaction, in which U2 and the 5′SS are able to swap positions and roles after assembly, does not appear consistent with the wide range of 5′SS mutations that facilitate *trans*-splicing, partial strand exchange is not ruled out. We are currently investigating the possibility of partial displacement of U2 from the BS-U2 duplex by the 5′SS prior to the first catalytic step of canonical pre-mRNA splicing.

The Fate of the BS-U2 Duplex for the Second Catalytic Step of Pre-mRNA Splicing

The persistence after the first step of splicing of the entire U2-BS helix, established during the early stages of spliceosome assembly, is not inconsistent with any previously published data. Our data, however, clearly suggest that the persistence of this helix is at least not required for the second step of splicing. As indicated in Figures 1A and 4E, the predominant 5′SS used in U2 lies in the middle of this helix, which must therefore be at least partially disrupted in order for the "U2 5′ exon" to be free to attack the 3′SS during the second step. Given that the structural consequences of the absence of a helix normally present in the second-step conformation of the spliceosome would likely cause a severe, if not terminal, second-step block, and that the second step of the *trans*-splicing reaction appears to be analogous to the second step of canonical *cis*-splicing, we hypothesize that the U2-BS helix does not normally persist during the second step and that the removal of the lariat intermediate, generated in the first step, from the catalytic center prior to the second step may be linked to the unpairing of its BS portion from U2 snRNA as well as the disruption of the 5′SS-U6 snRNA interaction (Konarska et al., 2006). Although these conclusions are based on an infrequent *trans*-splicing reaction, the capacity of the spliceosome to support such a reaction has important implications for physiologically relevant pre-mRNA splicing.

EXPERIMENTAL PROCEDURES

Strains and Reporter Plasmids

S. cerevisiae strains used in this study were Y04999 [*MATa*, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YKL149c::kanMX4*] (derived from BY4741), 46ΔCUP [*MATa*, *ade2 cup1Δ::ura3 his3 leu2 lys2 trp1 ura3, GAL+*] (Lesser and Guthrie, 1993), and yCQ62 [*MATa*, *ade2 cup1*Δ::*ura3 his3 leu2 lys2 trp1 ura3 snr6*Δ::*loxP snr20Δ*::*loxP*, pU6 + U2 (*SNR6 SNR20 URA3* CEN ARS)]. *ACT1-CUP1* reporter plasmids (Lesser and Guthrie, 1993) were as described (Query and Konarska, 2004) or prepared by overlapping PCR and in vivo gap repair cloning.

RT-PCR

Yeast total RNA was treated with DNase I (Sigma) according to the manufacturer's instructions. Reverse transcription was carried out using primer YAC94 5′-CGTCGCTGTTACACCC-3′. Reverse-transcribed DNA was amplified by PCR using primers YAC94 and yU2-22 5′-ACGAATCTCTTTGCCTTTTGGC-3′, diluted 1:1000, and amplified using primers yU2-22 and YAC6 5′-GGCACTCATGACCTTC-3′. Products were separated in 2% agarose gels. RT-PCRs for loading controls were performed with yU2-138-154 5′-AAAGTCTCTT

CCCGTCC-3′ and yU2-22 as antisense and sense primers, respectively. PCR products were purified for sequencing and cloning using Qiaquick gel extraction kits (QIAGEN).

Primer Extension

Primer extensions were carried out as described (Siatecka et al., 1999), using primer YAC6, complementary to exon 2 of *ACT1-CUP1*. Extension products were separated in 7% polyacrylamide/8 M urea gels and visualized by autoradiography.

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