Mutations in the SF1-U2AF<sup>59</sup>-U2AF<sup>23</sup> Complex Cause Exon Skipping in Schizosaccharomyces pombe<sup>*</sup>

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To identify genes involved in the mechanism to ensure ordered 5′ to 3′ exon joining in constitutively spliced pre-mRNAs, we screened for mutants that cause exon skipping in the fission yeast *Schizosaccharomyces pombe* using a reporter plasmid, which contains the *ura4<sup>+</sup>* gene with the *nda3* intron 1-exon 2-intron 2 sequence. The reporter plasmid was designed to produce the functional *ura4<sup>+</sup>* mRNA, when the central *nda3* exon is skipped during the splicing reaction. We mutagenized cells harboring the plasmid by UV irradiation and isolated 34 *ura<sup>+</sup>* mutants that grew on minimal medium. Of those, eight mutants were found to be temperature sensitive (*ts*) for growth. Complementation analyses revealed that the *ts* mutants belong to three distinct complementation groups named *ods* (ordered splicing) 1, 2, and 3. RT-PCR analyses showed that products of exon skipping were actually generated in the *ods* mutants. We cloned the genes responsible for the *ods* mutations, and found that *ods1<sup>+</sup>*, *ods2<sup>+</sup>* and *ods3<sup>+</sup>* encode splicing factors Prp2p/U2AF<sup>59</sup>, U2AF<sup>23</sup>, and SF1, respectively, which form a SF1-U2AF<sup>59</sup>-U2AF<sup>23</sup> complex involved in recognition of the branch-point and 3′ splice site sequences in a pre-mRNA. We also showed that mutations in the SF1-U2AF<sup>59</sup>-U2AF<sup>23</sup> binding sequences in the reporter plasmid result in exon skipping in wild-type *S. pombe* cells. In addition, drugs that decrease the rate of transcription elongation were found to suppress the exon skipping in the *ods* mutants. These results suggest that co-transcriptional recognition of a nascent pre-mRNA by the SF1-U2AF<sup>59</sup>-U2AF<sup>23</sup> complex is essential for ordered exon joining in constitutive splicing in *S. pombe*.

The coding regions of typical metazoan genes are interrupted by multiple noncoding introns. Removal of introns from a pre-mRNA (pre-mRNA splicing) is thus essential for expression of eukaryotic genes. The splicing reaction takes place in a dynamic complex termed a spliceosome (for a review, see Ref. 1). A set of five small nuclear ribonucleoprotein particles (snRNPs)<sup>2</sup> and more than 50 accessory proteins are assembled onto each intron to form the spliceosome. Experiments using the *in vitro* splicing system revealed that, at the initial step for the spliceosome formation, the U1 snRNP binds to the 5′ splice site through base pairing between the splice site and the U1 snRNA. The branchpoint, which is required for formation of a lariat intermediate, and the polypyrimidine tract are bound by SF1 (also called BBP in *Saccharomyces cerevisiae*) and the large subunit of U2AF (U2AF<sup>65</sup>), respectively (for a review, see Ref. 2). In addition, the small subunit of U2AF (U2AF<sup>35</sup>) binds to AG at the 3′ splice site. The earliest complex containing the U1 snRNP and U2AF in spliceosome assembly is called the E (early) or commitment complex. The U2 snRNP, the U4/U5/U6 tri-snRNP and other splicing factors are then assembled sequentially to form the final spliceosome, in which the splicing reaction is carried out.

In a multi-intron pre-mRNA, alterations in splice site choice can produce diversity in protein synthesis. Such alternative pre-mRNA splicing is now known to be as high as 60% for human genes (3). In general, alternative splicing occurs in specific sets of introns and exons, and is carried out in a highly regulated manner (3). Most other exons, in contrast, are joined in an orderly manner and are included in the final mature mRNA that directs the synthesis of a functional protein (constitutive splicing). Although binding of regulatory proteins to pre-mRNA is shown to activate or repress the use of splice sites in alternative splicing (4, 5), little is known about the mechanisms that ensure ordered exon joining in constitutive splicing.

In the fission yeast *Schizosaccharomyces pombe*, more than 43% of the protein-coding genes contain introns, and individual genes can contain up to 15 introns (6), some of which have been reported to be alternatively spliced (6–8). These facts indicate that the *S. pombe* splicing system is closer to that of mammals than is the system of *S. cerevisiae*, in which only a small portion of the genes (about 4% of the total genes) contains an intron and most of them have only one intron per gene (9). From that point of view, we have thought that *S. pombe* is a good model organism to analyze the splicing mechanisms including that for constitutive splicing.

In this report, we isolated three classes of mutations that cause exon skipping in *S. pombe* and revealed that the genes carrying these mutations encode subunits of the SF1-U2AF<sup>59</sup>-U2AF<sup>23</sup> complex. We also showed that exon skipping in these mutants was repressed when cells are treated with inhibitors of transcription elongation. These results suggest that the initial...
co-transcriptional recognition of pre-mRNA by the SF1-U2AF59, U2AF23 complex is important to ensure ordered exon joining in constitutive splicing.

**EXPERIMENTAL PROCEDURES**

*S. pombe Strains and General Methods—* *S. pombe* strains used in this study are listed in Table 1. The complete media YPD or YE (10, 11) and the minimal medium MM (10) were used for standard culture of *S. pombe* strains. Appropriate growth supplements (adenine, leucine, and uracil, 80 mg/liter each) were added to MM. SPA medium was used for induction of mating and sporulation of *S. pombe* (10). Medium containing 2% agar was used for plating. Standard genetic methods used for *S. pombe* were as described (12).

Construction of a Reporter Plasmid to Detect Exon Skipping—To construct a reporter plasmid to detect exon skipping in *S. pombe*, we synthesized the intron 1-exon 2-intron 2 sequence and the 5'-tubulin sequence and the H9252, S. Urushiyama strains used in this study (Table 1) and tetrad analysis was performed. Analysis of 10 tetrads for each mutant showed that the ts and wild-type phenotypes segregated 2:2 in all cases. Each mutant was backcrossed at least three times with UR470. Co-segregation of the exon skipping phenotype (ura4+) with the ts phenotype was confirmed by re-transformation of backcrossed mutants with pURA46. To determine whether the ts mutations in isolated mutants are dominant or recessive, heterozygous diploids were obtained by crossing with strain RS1 (Table 1). All diploids grew at the restrictive temperature of 37 °C, demonstrating that the mutations were recessive.

Preparation of RNA and RT-PCR—Cells containing pURA4β were grown in 10 ml of appropriate medium (MMU for wild-type cells and MM for ods mutants) to mid-log phase at the permissive temperature (26 or 30 °C), and then shifted to the restrictive temperature (37 °C) for 2 h. In the case of snh29, cells were cultured to the mid-log phase at 26 °C and shifted to the restrictive temperatures of 30 or 36 °C for 2 h. Cells were washed twice with sterile water, and total RNA was prepared by glass beads disruption as described by Urushiyama *et al.* (15). The yield of total RNA was usually 40–200 μg.

RNA samples were treated with RQ1 RNase free DNase (Promega) in 5 mM MgCl2 and 50 mM Tris–HCl (pH 8.0) at 37 °C for 1 h to remove contaminating genomic DNA, followed by extraction with phenol/chloroform/isooamyl alcohol and ethanol precipitation. Reverse transcription was then performed using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences), 3.33 μM gene-specific RT primer (tub-4: 5'-CTTTGGAAGACATTTAGC-3') or oligo dT (50 mer), and 2 μg of total RNA according to the manufacturer’s protocol. PCR was done in a solution containing Ex Taq polymerase (TaKaRa), 0.2 mM dNTP mix, 3.33 μM 5'-forward primer (tub-3: 5'-ATATGCATCTGGTGTAC-3'), 3.33 μM tub-4 primer, and 2 μl of reverse-transcribed cDNAs. The tub-3 and tub-4 primers are complementary to the 3'-end of the *ura4*+ first exon and the 5'-end of the *ura4*+ second exon, respectively. The amplified products were separated on a 5% polyacrylamide gel and stained with ethidium bromide solution (1 μg/ml).

Southern Blot Analysis—After electrophoresis on a 5% polyacrylamide gel, the amplified products were transferred onto a Gene Screen Plus filter (PerkinElmer Life Sciences) by capillary blotting and detected by hybridization with an oligonucleotide probe complementary to the sequence in the *ura4*+ cDNA produced by the exon skipping spliced product (5'-TCTTTAGGCCCTTGTATA-3'), which was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (16). Hybridization was performed in a solution containing 6× SSC, 50 mM sodium phosphate, 5× Denhardt’s solution, 0.1% SDS, 100 μg/ml boiled salmon sperm DNA and the 32P-labeled probe at 42 °C. After hybridization, filters were exposed to a Fuji imaging plate and analyzed using a Bioimaging Analyzer BAS-1500 (Fuji Photo Film Co., Ltd.).

Site-directed Mutagenesis of pURA4β—The various mutations of pURA4β were generated by site directed mutagenesis of single-stranded DNA using QuikChange II site-directed mutagenesis kit (Stratagene) with oligonucleotides listed in Table 2.
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**TABLE 2**
List of oligonucleotides

| Names | Sequences |
|-------|-----------|
| **Primers for RT-PCR analysis** | |
| tub-3 | 5'-atatgcatctggtttgac-3' |
| tub-4 | 5'-ctttgagaggctgta-3' |
| **Probe for exon-skipped product** | |
| ura4 probe | 5'-tctttgagaggctgta-3' |
| **Oligonucleotides for site directed mutagenesis** | |
| nda3In2-5ss.mut' | 5'-gaagttagttataaatctttcatctttcttataaagttgccccttc-3' |
| nda3In2-5ss.mut | 5'-tgattttcagctgtatccttatccttataaagttgccccttc-3' |
| ln2 polyY-y | 5'-tctttgagaggctgta-3' |
| ln2 polyY-y | 5'-tctttgagaggctgta-3' |
| ln2 5'ss-as | 5'-tctttgagaggctgta-3' |
| ln2 5'ss-as | 5'-tctttgagaggctgta-3' |
| ln2 5'ss-as | 5'-tctttgagaggctgta-3' |

**Analyses of Exon Skipping using Drugs That Slow the Transcription Elongation**—Strain UR471 (Table 1) or ods1-1 cells harboring pURA4β were cultured in MM containing uracil at 26 °C to A600 of 0.5. After washing once with sterilized water, cells were resuspended in MM containing 6-azauracil (6-AU, 0.08 mg/ml) or mycophenolic acid (MPA, 0.1 mg/ml) and then incubated with shaking for 4 h at 26 °C. After cultivation, total RNAs were isolated and subjected to RT-PCR analyses with the tub-3 and tub-4 primers, as described above, to detect the exon skipping.

**RESULTS**

Isolation of S. pombe Mutants That Cause Exon Skipping—To isolate S. pombe mutants that induce exon skipping, we constructed a reporter plasmid (pURA4β) carrying a chimeric *ura4*+ gene, in which the intron 1-exon 2-intron 2 sequence of the *nda3*+ gene was inserted at a site previously shown to tolerate the insertion of an artificial intron (17) (Fig. 1A). In *ura*− cells harboring pURA4β, if skipping of the inserted β-tubulin exon occurs, then functional *ura4*+ mRNA is regenerated, and the cells are expected to become prototrophic for uracil, resulting in growth on minimal medium (MM) (Fig. 1B). We mutagenized about 15 million cells containing pURA4β by UV irradiation and isolated 34 *ura*− mutants that grew on MM plates. Of those, eight mutants (shn4, 19, 25, 28, 29, 32, 33, and 34) were temperature-sensitive for growth (Fig. 2A) and chosen for further analyses.

To exclude a possibility that the *ura*+ phenotype of the isolated mutants resulted from mutations in the intron-containing *ura4*+ gene of the reporter plasmid, we dropped the plasmid from the mutants and then re-introduced pURA4β. As expected, the mutant cells without pURA4β could not grow on MM plates; whereas, mutant cells re-transformed with pURA4β recovered the ability to grow on MM plates (Fig. 2B). These results indicated that the expression of the *ura*+ phenotype of the isolated mutants depended on pURA4β and probably resulted from exon skipping in the splicing of pre-mRNAs from the intron-containing *ura4*+ gene.

The mutants were backcrossed three times with a wild-type strain to remove extra mutations. Tetrad analysis showed 2:2 segregation of the ts− and ts+ phenotypes in all cases, suggesting that the ts− phenotype is caused by a single mutation. The ability to grow on MM co-segregated with the ts− phenotype of the isolated mutants resulted from mutations in the intron-containing *ura4*+ gene. The ability to grow on MM co-segregated with the ts− phenotype in all the mutants, indicating that in each mutant a single mutation is responsible for both phenotypes. All mutations are recessive, as the heterozygous diploids with the wild-type alleles grew well at the restrictive temperature.

After backcrossing, each mutant was subjected to complementation analysis in all combinations. As shown in Fig. 2C, the isolated mutants fell into three different complementation groups named *ods* (ordered splicing) 1 to 3. We categorized shn4, 25, 28, 29, and 32 in *ods*1, shn19 in *ods*2, and shn33 and 34 in *ods*3, respectively.

Detection of Exon Skipping Products in the ods Mutants—To demonstrate that exon skipping is induced in *ods* mutants, we isolated total RNAs from wild type (UR471) and ods cells carrying pURA4β and subjected them to RT-PCR analyses using primers for amplification of the intron-containing region of the...
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Figure 2. A, temperature sensitive growth of the isolated mutants. Each mutant and the wild type strain (UR471) was streaked on YEALU plates and incubated at indicated temperatures for 5 days. B, growth of the isolated mutants on MM plates depends on the pURA4 (UR471) was streaked on YEALU plates and incubated at indicated temperatures for 5 days.

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Figure 3. RT-PCR analyses of the transcripts from pURA4β in the wild-type strain (UR471) and ods mutants. The amplified products were electrophoresed on a 5% acrylamide gel, stained with ethidium bromide (Fig. 3, upper panel). Southern blot analysis (Fig. 3, lower panel) and the sequence analysis (not shown) of the products revealed that those are actually derived from the exon-skipped transcripts. In contrast, bands for pre-mRNA with two introns and exon-skipped products were barely detected in the wild-type cells. From these results, we concluded that skipping of the central exon in the chimeric ura4+ pre-mRNA was induced in the isolated mutants, although the amount of the exon skipping products is low compared with those for constitutively spliced mRNAs or unspliced products.

Cloning of the ods1+, ods2+, and ods3+ Genes—To clone the ods1+, ods2+, and ods3+ genes, we transformed each ods mutant with an S. pombe genomic library, and isolated clones that were able to complement the ts− growth defects. In the case of the ods1-1 (snh4) mutation, we obtained two cosmid clones that rescued the ts− phenotype: the complementing activity localized to a 9.5-kb XhoI/SacI fragment in a region shared by the two clones (Fig. 4A). That fragment contains the prp2+ gene encoding the large subunit (U2AF59) of the U2 snRNP auxiliary factor (U2AF), which binds to a polypyrimidine tract between the branchpoint sequence and the 3’ splice site (18, 19). As only the subcloned fragment that included the complete prp2+ gene could rescue the ts− phenotype, we presumed that the ods1 mutation residues in the prp2+ gene. Sequence analysis of the prp2 gene in ods1-1 revealed a single nucleotide change (G to A) at position 919 of the protein-coding region, which results in replacement of aspartic acid at amino acid 307 of the Prp2 protein with asparagine. The prp2 alleles in the snh25, snh28, and snh32 mutants were found to have the identical mutation. Thus, we categorized these mutant strains as ods1-1. We named snh29 as ods1-2 as it has a C to A change at nucleotide position 1148 of the protein-coding region, which results in replacement of alanine with glutamine at amino acid 383 in the Prp2 protein. Identification of mutations in the prp2 gene in the ods1 mutants establishes that the ods1+ gene is identical with prp2+.
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Mutations in the SF1-U2AF59-U2AF23 Binding Sequence in the First Intron Cause Exon Skipping—We found that the genes responsible for the ods1, ods2, and ods3 mutations encode components of the SF1-U2AF59-U2AF23 complex, suggesting that the complex plays an important role to ensure ordered exon joining during the splicing reaction. To examine further requirement of the SF1-U2AF59-U2AF23 complex in ordered splicing, we mutagenized the sequence of the binding site region for the SF1-U2AF59-U2AF23 complex (21), that is, the region spanning from the branch site to the 3' splice site, in the chimeric ura4+ gene, as shown in Fig. 5A. The putative branch site sequence GCCTAA, which matches the S. pombe branch site consensus sequence PuCT-PuAPy (Pu, purine; Py, pyrimidine) (22), was changed to GTGATT and two pyrimidines between the branch site and the 3' splice site were changed to A. The conserved 3' splice site AG was also mutagenized to CT. In addition to those mutations, we generated a mutation in the 5' splice site of the second intron. The mutations were introduced into the ura4 reporter gene plasmid in all combinations. The efficiency of exon-skipping was then evaluated by growth of transformants on MM plates and South-
ern blot analyses of the RT-PCR products amplified from total RNAs prepared from the transformants.

Fig. 5B shows the results of the growth test on MM plates. The cells transformed with the plasmid containing the mutation at the 5′ splice site could not grow on MM, suggesting that, as with the unmutated ura4Δ construct in wild-type cells, exon skipping did not occur, which was confirmed by Southern blot analysis (Fig. 5C, lanes 1 and 2). In contrast, the transformants with the plasmid carrying the branch site mutation grew well on MM (Fig. 5B), and the product of exon skipping was detected by Southern blot analysis (Fig. 5C, lanes 3, 4, 6, and 7). Interestingly, the mutation of the branch site in the first intron resulted in accumulation of pre-mRNA containing not only the first intron, but also the second intron, suggesting that the splicing of the second intron was also impaired to some extent by the mutation in the first intron (Fig. 5C, lanes 3 and 4). In the case of the transformant with the 3′ splice site mutation (Fig. 5A, mutant 5), growth on MM was slow (Fig. 5B), and the amount of the exon-skipped product was low (Fig. 5C, lane 5). Sequence analysis of the RT-PCR product revealed that a cryptic 3′ splice site localized 13 nucleotides downstream of the authentic 3′ splice site in the first intron was used for the splicing of this mutant pre-mRNA. In construct 4, combining the 3′ splice site and branch point mutations appear to decrease the efficiency of exon skipping compared with construct 3, which has only the branch site mutation (Fig. 5C, lane 4).

Addition of the 5′ splice site mutation in the second intron to the branch site mutation in the first intron did not enhance the exon skipping above that produces by the branch point mutation alone (Fig. 5C, compare lanes 3 and 6). As expected, addition of both the 3′ splice site mutation in the first intron and the 5′ splice site mutation in the second intron to the branch point mutation in the first intron abolished splicing of both the first and second introns separately and promoted exon skipping exclusively (Fig. 5C, lane 7). The plasmid containing mutations of both the 3′ and 5′ splice sites also caused exon skipping, but the efficiency was lower than that of the gene with the branch site mutation (Fig. 5C, lane 8). These results, which demonstrate that cis-acting mutations in the region recognized by the SF1-U2AF59-U2AF23 complex cause the same type of exon skipping as trans-acting mutations in the subunits, support a role for the SF1-U2AF59-U2AF23 complex in the maintenance of orderly, constitutive splicing in multi-exon pre-mRNAs.

Treatment of the ods Mutants and Drugs That Slow the Transcription Elongation Represses Exon Skipping—Recently, Howe et al. (23) found that exon skipping in S. cerevisiae is suppressed when transcription is slowed by treatment of cells with 6-azauracil (6-AU) or mycophenolic acid (MPA), inhibitors of nucleotide biosynthesis that deplete cellular nucleotide pools required for efficient transcription by RNA polymerase (23). Based on this phenomenon, Howe et al. (23) speculated that a drug-induced reduction in the rate of transcript elongation relative to splicing represses exon skipping by allowing more time for branchpoint recognition in the first intron to occur before the second intron is transcribed.

Using our pURA4Δ reporter system, we examined how the rate of transcriptional elongation affects the fidelity of ordered exon joining in the ods mutants. We found that both 6-AU (0.08 mg/ml) and MPA (0.1 mg/ml) induced impaired growth of ods mutant cells on MM at 26 °C, suggesting suppression of exon skipping (data not shown), which was confirmed by the reduction of the exon-skipped product detected by RT-PCR followed by Southern blot analysis for the probe specific for the exon-skipped product (Fig. 6). As the amounts of the constitutively spliced chimeric ura4Δ product (Fig. 6, upper left panel) and actin transcript (lower left panel) were not changed, decrease of the exon-skipped product seems not to be the outcome from the general inhibition of the transcription. Rather, it appears that a shift in the choice of splicing pathway from exon skipping to exon inclusion has occurred as a result of the slowed elongation of the nascent transcript.

DISCUSSION

To understand the molecular mechanisms that ensure ordered exon joining in constitutive splicing, we screened for mutations that cause exon-skipping during splicing of the pre-mRNA transcribed from a reporter gene and found that all of the mutations fell within the three genes that encode the subunits of the SF1-U2AF59-U2AF23 complex, which is essential for the recognition of the branchpoint sequence and the 3′ splice site (2). Cis-acting mutations that reproduced the exon skipping phenotype of the mutations in the SF1-U2AF59-U2AF23 complex confirmed the importance of branchpoint recognition in the maintenance of ordered exon joining.

In the isolated ods mutants, the amounts of the exon-skipped products detected were low, although those were sufficient to support growth of the plasmid-bearing mutants on MM. Actually, we detected a small amount of exon 2-skipped transcripts from the endogenous nda3Δ gene in the ods1-1 mutant (data not shown). As severe splicing perturbations result in cell mortality, ods alleles that cause strong induction of exon skipping are expected to be difficult to recover in our screening.

We showed that mutating the 5′ splice site of the second intron produced exclusive retention of the mutated intron during splicing, in contrast to the case of vertebrates that causes exon skipping (24). This result indicates that splicing in fission yeast is conducted via intron definition, which is consistent with the data reported by Romfo et al. (25).
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In addition to induction of exon skipping, branchpoint mutations that block splicing of the first intron decreases the splicing efficiency of the second intron, leading to accumulation of the unspliced pre-mRNA (Fig. 5C, lanes 3 and 4). Contrary, the 5′ splice site mutation in the second intron neither significantly reduces the efficiency of the first intron splicing, nor induces exon skipping (Fig. 5C, lane 2). There might be a mechanism that represses splicing of the downstream intron when splicing of the preceding intron is inhibited, besides a mechanism that induces exon skipping.

In *S. pombe*, SF1 was shown to bind U2AF^{59} and U2AF^{23} and form a stable complex required for pre-spliceosome complex A formation (21). Genetic and biochemical experiments in *S. cerevisiae* have shown that BBP (a homologue of SF1) interacts directly with both Mud2p (a homologue of U2AF^{65}) and the U1 snRNP protein Prp40p (26). As BBP and Prp40p are involved in recognition of the branchpoint sequence and the 5′ splice site, respectively, the interaction is thought to define bridging between the two ends of an intron (26). In mammals, SF1 interacts with U2AF^{65} and with U1C through FBP21, a Prp40p like protein, indicating that the interaction between SF1 and Prp40p is conserved among organisms (26). Interestingly, *S. cerevisiae* Prp40p was shown to bind the phosphorylated C-terminal domain (CTD) of elongating RNA polymerase II, suggesting co-transcriptional formation of a commitment complex for splicing (27).

In *S. cerevisiae*, mutations in the DYN2 gene that reduce recognition of the branchpoint sequence in the first intron cause exon skipping, indicating that efficient recognition of the first intron is important for exon inclusion (23). In addition, treatment of cells with inhibitors for nucleotide biosynthesis that reduce the rate of transcription elongation was found to inhibit exon skipping in vivo. Those observations are consistent with the predictions of the previously proposed “first come, first served” model, which suggest the co-transcriptional recognition of splice sites (28).

In this report, we provided another evidence supporting the “first come, first served” model in *S. pombe*. We found that mutations in the SF1-U2AF^{59}.U2AF^{23} complex or the reporter gene that affect branchpoint recognition enhances exon skipping in *S. pombe*. We also showed that drugs that slow transcription elongation suppress exon skipping in the ods mutants, suggesting that co-transcriptional recognition of a nascent pre-mRNA by the SF1-U2AF^{59}.U2AF^{23} complex is essential for ordered 5′ to 3′ exon joining. Taken together with the previously reported results mentioned above, we propose the ordered splicing model shown in Fig. 7. In this model, the U1 snRNP bound to the CTD of elongating RNA polymerase II through Prp40p recognizes the 5′ splice site of a nascent transcript first, and then the SF1-U2AF^{59}.U2AF^{23} complex associated with U1 snRNP through Prp40p recognizes the branchpoint sequence and the 3′ splice site, which appears in the transcript as transcription proceeds (Fig. 7A). In this model, the association between U1 snRNP and the SF1-U2AF^{59}.U2AF^{23} complex via Prp40p assures co-transcriptional ordered exon joining. In the ods mutants, reduced fidelity of the branchpoint recognition in the first intron by mutations in the SF1-U2AF^{59}.U2AF^{23} complex might induce exon skipping (Fig. 7B). If U1 snRNP is stalled at the 5′ splice site of the first intron, as shown in Fig. 7B, then recognition of the 5′ splice site of the second intron by U1 snRNP would be hampered, which may explain a mechanism for repression of the second intron splicing by mutations in the first intron (Fig. 5C).

The vast majority of metazoan pre-mRNAs have multiple introns, which typically range in size from 10^3 to 10^4 nucleotides. Exons in these pre-mRNAs are relatively short, averaging less than 200 nucleotides. It has been thought that initial splice site recognition in metazoan pre-mRNAs is established across a short exon (exon definition), rather than across an intron with long length (29). In *S. pombe*, in contrast, introns are short (the average size is 10^7 nucleotides) relative to exons that appear not to be constrained in size (30). Thus, there is less of a need for a system that recognizes exons in this organism. In *S. pombe*, a system with a strong dependence on recognition of the branchpoint-3′ splice site sequence by the SF1-U2AF^{59}.U2AF^{23} complex might have evolved to make sure that a spliceosome is not assembled until both the 5′ and 3′ splice sites in an intron are recognized.
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