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Cotranscriptional Spliceosome Assembly Dynamics and the Role of U1 snRNA:5′ss Base Pairing in Yeast

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Summary

To investigate the mechanism of spliceosome assembly in vivo, we performed chromatin immunoprecipitation (ChIP) analysis of U1, U2, and U5 small nuclear ribonucleoprotein particles (snRNPs) to intron-containing yeast (*S. cerevisiae*) genes. The snRNPs display patterns that indicate a cotranscriptional assembly model: U1 first, then U2, and the U4/U6•U5 tri-snRNP followed by U1 destabilization. *cis*-splicing mutations also support a role of U2 and/or the tri-snRNP in U1 destabilization. Moreover, they indicate that splicing efficiency has a major impact on cotranscriptional snRNP recruitment and suggest that cotranscriptional recruitment of U2 or the tri-snRNP is required to commit the pre-mRNA to splicing. Branchpoint (BP) mutations had a major effect on the U1 pattern, whereas 5′ splice site (5′ss) mutations had a stronger effect on the U2 pattern. A 5′ss-U1 snRNA complementation experiment suggests that pairing between U1 and the 5′ss occurs after U1 recruitment and contributes to a specific U1:substrate conformation required for efficient U2 and tri-snRNP recruitment.

Introduction

Eukaryotic pre-mRNA is covalently processed in three important ways prior to export from the nucleus. Transcripts are capped at their 5′ end with a methylated guanosine nucleotide, introns are removed by splicing, and 3′ ends are cleaved and extended with a polyA tail. Whereas capping and 3′ end formation have been shown to be intimately connected to transcription in all studied eukaryotes, direct evidence for cotranscriptional splicing derives mainly from studies in higher eukaryotes with obvious implications for alternative splicing (Bauren and Wieslander, 1994; Beyer and Osheim, 1988; Jensen et al., 2003; Kiseleva et al., 1994; Kornblihtt et al., 2004; LeMaire and Thummel, 1990; Wuarin and Schibler, 1994).

Splicing occurs within the spliceosome, a large complex including five small ribonucleoprotein particles (U snRNPs). Two decades of in vitro studies in yeast, invertebrates, and vertebrate systems support an ordered and dynamic pathway for spliceosome assembly on a pre-mRNA. U1 snRNP associates first, committing the transcript to the splicing pathway. U2 snRNP is then added in the first ATP-dependent assembly step to form the prespliceosome, which is then recognized by a preassembled tri-snRNP consisting of U4, U5, and U6 snRNPs. Several rearrangements take place, ultimately leading to dissociation of U1 and U4 snRNAs and formation of a catalytically active splicing complex (Burge et al., 1999; Jurica and Moore, 2003; Villa et al., 2002).

This assembly pathway is orchestrated mainly by a set of consensus sequences that demarcate the substrate intron. These sequences, 5′ss, BP, and 3′ splice site (3′ss), are remarkably conserved among yeast introns such that 75% conform exactly to the 5′ss consensus GUAUGU, 92% to the BP consensus UACU AAC, and 100% to the 3′ss AG (Spingola et al., 1999). During spliceosome assembly, the 5′ss region associates with U1 snRNP through RNA-RNA interactions with the 5′ arm of U1 snRNA as well as RNA-protein interactions with U1 snRNP proteins (Du and Rosbash, 2002; Michaud and Reed, 1991; Seraphin and Rosbash, 1989; Zhang et al., 2001; Zhang and Rosbash, 1999). The BP sequence base pairs with U2 snRNA, which is thought to displace two BP-splicing factors, BBP and Mud2p (Abovich et al., 1994; Abovich and Rosbash, 1997; Berglund et al., 1997; Parker et al., 1987; Rutz and Seraphin, 1999). The U5 snRNP component Prp8p has been shown to interact directly with the 5′ss, the BP, and the 3′ss, and U5 snRNA itself contributes to 5′ss selection through base-pairing interactions. U6 snRNA base pairing ultimately replaces U1 at the 5′ss, and rearrangements that establish base pairing between U2 and U6 snRNAs bring the ends of the intron into close proximity (Kandels-Lewis and Séraphin, 1993; MacMillan et al., 1994; Madhani and Guthrie, 1992; Newman and Norman, 1991; Newman and Norman, 1992; Reyes et al., 1996; Teigelkamp et al., 1995; Umen and Guthrie, 1995; Wyatt et al., 1992). These events ultimately result in the first chemical step of splicing: nucleophilic attack immediately upstream of the 5′ss by an adenosine in the BP sequence (Query et al., 1994).

It is unclear whether this in vitro spliceosome assembly pathway governs in vivo pre-mRNA splicing. In contrast to in vitro-synthesized substrates, intronic sequence elements appear at distinct times during transcription (Neugebauer, 2002). In addition, the physical coupling of spliceosome assembly with the transcription machinery might substantially alter the nature and order of events (Kornblihtt et al., 2004).

Recent evidence suggests that yeast introns are also recognized during transcription. U1 snRNP proteins have been shown to crosslink preferentially to DNA from intronic regions (Abruzzi et al., 2004; Kotovic et al., 2003), and intron recognition influences the cotranscriptional association of mRNA export factors (Abruzzi et al., 2004; Lei and Silver, 2002). Moreover, nuclear run-on assays indicate that an intron can increase polymerase density in yeast as well as in mammals (Furger et al., 2002), suggesting that introns can have a positive influence on transcription. This is an especially attractive notion for yeast, because intron-containing genes account for ~50% of the steady-state RNA population

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despite comprising only ~5% of the genes (Ares et al., 1999).

Despite the evidence that yeast introns are recognized by some of the splicing machinery during transcription, it is unclear to what extent in vivo spliceosome assembly resembles the in vitro pathway described above. Indeed, recent studies have challenged the traditional step-wise model and reinvigorated the discourse on in vivo spliceosome assembly. Complexes purified from yeast extracts under splicing-permissive low-salt conditions contain all five U snRNAs and almost all known snRNP components (Stevens et al., 2002). The authors proposed a preassembled penta-snRNP model in which all five splicing snRNPs associate prior to substrate binding. An additional challenge comes from a *trans*-splicing study in which the U5 snRNP component Prp8p could be crosslinked to the 5′ss without U1 or U2 snRNP association in human cells and nematodes (Maroney et al., 2000). These data are difficult to explain within the confines of the traditional assembly model: U1, then U2 followed by U4/U6•U5.

As a consequence, we decided to address yeast in vivo spliceosome assembly by monitoring the cotranscriptional recruitment of multiple snRNPs using ChIP. U2 and U5 snRNPs, as well as U1 snRNP (Abruzzi et al., 2004; Kotovic et al., 2003), are specifically recruited to nascent transcripts of an endogenous intron-containing gene. U2 and U5 show spatial/temporal patterns that clearly separate them from U1, and analysis of a well-studied intron-containing reporter gene showed remarkably similar patterns. This enabled us to exploit previously characterized *cis*-splicing mutations for their effects on in vivo snRNP assembly. The extent of apparent U2 and U5 recruitment roughly paralleled splicing efficiency. Surprisingly, 5′ss mutations had bigger effects on U2 and U5 signals than on U1, whereas BP mutations influenced U1 signals even more strongly than the 5′ss mutations. Indeed, results with a complementary U1 snRNA mutation, which improves base pairing with a 5′ss mutant, suggest that U1:substrate base pairing is less important for U1 snRNP recruitment than for the transition to a U2-containing complex. Based on these observations and those in the accompanying manuscript, in vivo spliceosome assembly is discussed.

Results

U1, U2, and U5 snRNPs Are Recruited Similarly to *ACT1* and an *RP51A:lacZ* Fusion Reporter Plasmid

To address cotranscriptional spliceosome assembly, we performed ChIP with representative proteins from U1, U2, and U5 snRNPs. TAP-tagged U1C, Lea1p, and Prp8p, for U1, U2, and U5, respectively (Anderson et al., 1989; Caspary and Seraphin, 1998; Tang et al., 1997), were immunoprecipitated with IgG Sepharose beads from formaldehyde-crosslinked chromatin, and the DNA was analyzed by quantitative real-time PCR (see Experimental Procedures). We first characterized snRNP recruitment to the endogenous *ACT1* gene. *ACT1* has a single 5′ proximal intron of 307 base pairs (bp) with a consensus 5′ss (GUAUGU) and BP (UACUAAC; Figure 1A). We also assayed a well-studied 2 μm plasmid-encoded chimeric reporter gene, including the 5′ end of *RP51A* with the complete intron and the *lacZ* open reading frame (ORF). Like the chromosomal *RP51A* gene, the canonical 397 bp *RP51A* intron begins shortly after the initiation codon, and pre-mRNA splicing is efficient (pHZ18; Figure 1A; Jacquier et al., 1985; Jacquier and Rosbash, 1986; Pikielny and Rosbash, 1985; Seraphin et al., 1988; Teem and Rosbash, 1983).

Quantitative real-time PCR using five sets of primer pairs directed to different regions of the *ACT1* locus

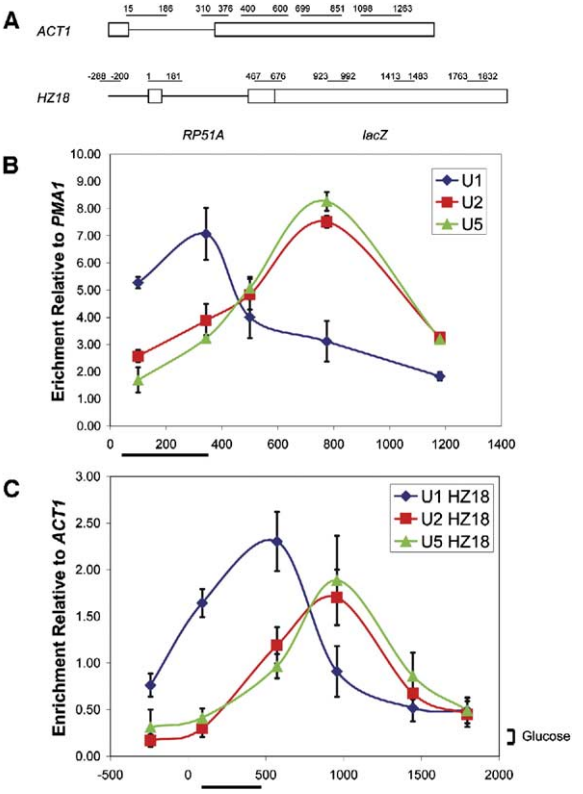

Figure 1. U1, U2, and U5 snRNPs Show Similar Recruitment to the Endogenous *ACT1* Gene and to a *lacZ* Fusion Reporter Gene Harboring the *RP51A* Intron

(A) The schematic shows positions of the primers used for *ACT1* and *HZ18* ChIP analysis (see Experimental Procedures). The *HZ18* reporter consists of a fusion between the *GAL4* UAS and the *CYC1* promoter directing galactose-dependent expression of a gene containing the entire *RP51A* intron plus 63 codons of exon two fused to the *lacZ* ORF (Teem and Rosbash, 1983).

(B) ChIPs against U1 snRNP (U1C-TAP, blue), U2 snRNP (Lea1-TAP, red), and U5 snRNP (Prp8-TAP, green) were performed as described (see Experimental Procedures). The x axis represents the distance in base pairs from the ATG start codon. The black bar underneath shows the position of the *ACT1* intron. U2 and U5 show recruitment patterns that are clearly distinct from U1.

(C) The strains described above were transformed with pHZ18 ([A] and Figure 2A) and subjected to ChIP. The data are remarkably similar to the patterns seen on *ACT1,* where U2 and U5 are separated from U1. ChIP signals from the same three strains grown in glucose where reporter transcription is repressed range from 0.1 to 0.3 and are denoted by the bracket to the right.

Error bars in (B) and (C) represent the average deviation from at least two independent experiments.

(Figure 1A) showed that all three proteins significantly enriched *ACT1* DNA relative to a highly transcribed region in the middle of the intronless gene *PMA1* (see Experimental Procedures). U1 snRNP recruitment peaks directly after the intron, decreases substantially 200 bp into exon 2, and continues to decrease 500 and 900 bp into exon 2 (Figure 1B). Importantly, the pattern of *ACT1* from U1C IPs is essentially identical to that for a different tagged U1 snRNP protein, Prp42p (data not shown; Abruzzi et al., 2004). In contrast, U2 and U5 snRNPs show a different pattern: levels are low early, gradually increase to maximal around 500 bp past the intron, and then decrease 900 bp past the intron (Figure 1B).

For the *HZ18* reporter gene, PCR signals were normalized to the *ACT1* fourth primer pair. This served as a better strategy for a plasmid-derived intron-containing gene and especially for comparisons of *cis* mutations. This is because the actin gene gives strong and reproducible signals for all three tagged proteins in the same wild-type (wt) strain background and can therefore accommodate differences in IP efficiency between replicates (Figure 1C; see Experimental Procedures).

The *HZ18* patterns for all three snRNPs are similar to those of *ACT1* (compare Figures 1C and 1B), indicating that spliceosome assembly on plasmid-derived nascent pre-mRNAs is similar if not identical to that on chromosomal pre-mRNAs. The U1 snRNP pattern peaks just past the intron and precedes the U2 and U5 snRNP patterns, which are essentially identical and peak ~500 bp after the intron. All three signals decline by ~1000 bp past the intron, and the U2 and U5 signals decline further by ~1400 bp. snRNP signals on *HZ18* are reduced to background levels when the tagged strains are grown in glucose (Figure 1C), because *HZ18* is under *GAL4* UAS transcriptional control (Figure 2A; Teem and Rosbash, 1983).

The snRNP patterns on *HZ18* and *ACT1* suggest that a uniform spliceosome assembly-recruitment strategy is taking place on canonical yeast intron-containing nascent transcripts. U2 and U4/U6•U5 tri-snRNP recruitment are similar: they follow U1 snRNP and appear coupled to U1 snRNP destabilization. This is based on the assumption that U5 snRNP recruitment reflects tri-snRNP recruitment, terms that we use interchangeably in the rest of the text.

Strong BP and 5′ss Mutations Show Altered Assembly Patterns

To verify and extend these spliceosome assembly results, we analyzed two *HZ18* mutations that strongly inhibit in vivo splicing (Figure 2B; Jacquier and Rosbash, 1986; Pikielny and Rosbash, 1985). The 3′III mutation is an A to C transversion at the BP adenosine responsible for nucleophilic attack during the first step of splicing (UACUAAC→UACUAcC). The 5′0 mutation is a 67 bp deletion within the intron, resulting in a mutated 5′ss (GUAUGU→GUAUac) as well as loss of some relevant nonconserved adjacent sequences (Pikielny and Rosbash, 1985; Puig et al., 1999). Both mutations efficiently block splicing, as there is no detectable *HZ18* mRNA or β-galactosidase activity (Figure 2B; Jacquier and Rosbash, 1986; Pikielny and Rosbash, 1985).

U1 snRNP shows a delayed pattern to 3′III compared to the wt reporter gene: the peak is shifted from the third to the fourth primer pair 500 bp past the intron, and higher signals extend further downstream than on the wt gene (Figure 3A). This indicates that the BP influences some aspect of stable U1 snRNP recruitment. Not surprisingly, the U2 snRNP signal is strongly decreased by the 3′III mutation (Figure 3B), consistent with abundant evidence linking the BP to U2 snRNP recruitment in yeast (Abovich et al., 1994; Berglund et al., 2001; Libri et al., 2001; Parker et al., 1987; Pascolo and Séraphin, 1997; Ruby et al., 1993; Zhang and Green, 2001). The U5 signal is even more severely reduced, with levels comparable to those observed when transcription is shut off by growth in glucose (Figure 3B).

The U1 pattern on the 5′0 mutant reporter gene is similar to the delayed pattern observed for 3′III. However, signals are further increased at 500 and 1000 bp past the intron compared to the 3′III pattern (compare Figures 3A and 3C), and these delayed signals are even increased relative to the maximal values of the wt *HZ18* recruitment pattern (Figure 3C). The 3′III and 5′0 patterns suggest that optimal U1 snRNP recruitment requires a 5′ss as well as a BP sequence and is normally followed by a robust decrease in stable association with the nascent transcript.

In contrast, U2 and U5 levels on 5′0 are barely detectable with signals close to those when transcription is repressed. They may be even lower than those of 3′III (Figures 3B and 3D). These results suggest that U1 snRNP recruitment is insufficient for stable U2 and tri-snRNP association with a nascent intron and that the failure to recruit these snRNPs is relevant to the splicing deficiency of these mutant introns. Moreover, the enhanced U1 snRNP signal at the third, fourth, and fifth primer pairs might be due to reduced U2 and/or U5 recruitment (see Discussion).

U1 snRNP Recruitment without a 5′ss or a BP

The 3′III and 5′0 results inspired complete deletions of the *HZ18* 5′ss and BP regions (see Experimental Procedures). Deletion of the GUAUGU leaves no sequence within the first 1900 transcribed nucleotides that resembles a 5′ss (best = three GUAUs at +275, +383, and +425). Likewise, deletion of the UACUAAC leaves no sequence that resembles a BP (Figure 2C; see Discussion). Deletion of the BP shifted the U1 peak from 200 bp past the intron to 500 bp past the intron with a sustained and substantial signal at 1350 bp past the intron (Figure 4A). The pattern is reminiscent of the 3′III mutant but with even higher and more sustained levels (compare Figures 3A and 4A). Surprisingly, the 5′ss deletion had the highest U1 levels observed in this study, 2-fold higher than those of the wt intron (Figure 4C). The results suggest that the RNA substrate requires either a 5′ss or a BP to recruit substantial U1 snRNP but that both are needed for successful U2/U5 recruitment (Figures 4B and 4D). The lack of splicing, as well as U2/U5 recruitment, suggests that nascent pre-mRNA is accumulating in a dead-end U1 snRNP-containing complex. Finally, the persistent inverse relationship between U1 and U2/U5 levels continues to implicate the latter in U1 destabilization.

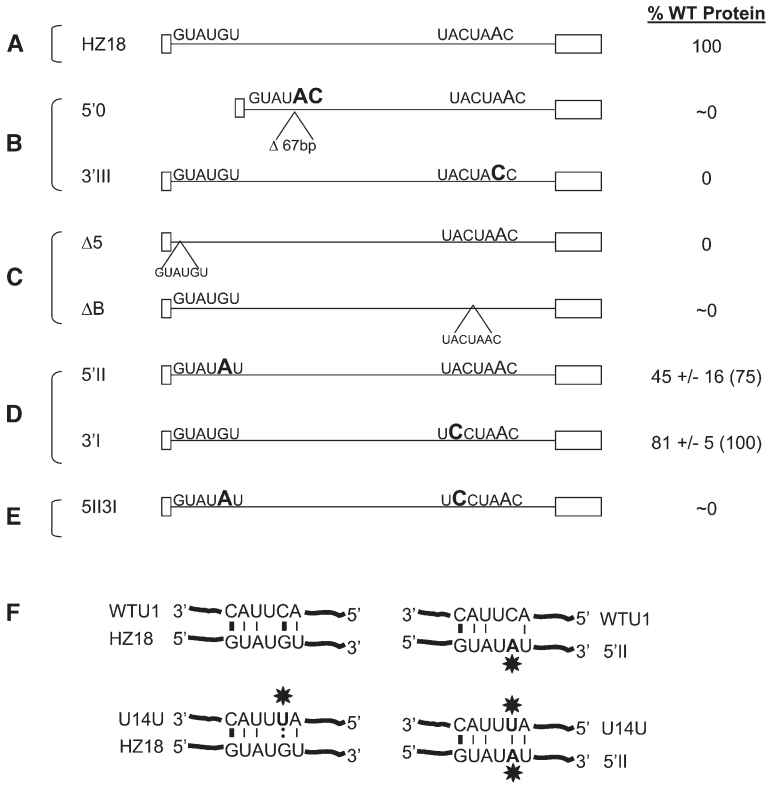

Figure 2. Schematic Diagrams of the *HZ18* Intron and All of Its Derivatives Used in This Study

In each case, the *cis* mutations are shown in bold type, and the relative protein levels are shown to the right from the current study and from previous studies in parentheses (Jacquier et al., 1985; Pikielny and Rosbash, 1985).

(A) Wt *HZ18* intron with its 5′ss and BP sequences (Teem and Rosbash, 1983). The BP adenosine responsible for the first step of splicing is augmented.

(B) The 5′0 and 3′III mutant introns. Both constructs display undetectable levels of mRNA.

(C) Full 5′ss and BP deletions leaving no recognizable splicing signals and which give less than 1% wt protein levels.

(D) The weaker 5′ss 5′II mutation and the weaker BP 3′I mutation.

(E) 5II3I is a combination of the 5′II and 3′I mutations.

(F) Base-pairing match ups between wt *HZ18* and wt U1 snRNA (wtU1); 5′II *HZ18* and wt, wt *HZ18*, and U14U snRNA (U14U); and 5′II *HZ18* and U14U. Thick bars between bases represent strong wt G-C pairings, whereas thinner bars represent the weaker wt A-U pairing. The dotted connection represents the weaker wobble G-U pairing, and the mutated nucleotides are marked with stars.

Effects of Weaker BP and 5′ss Mutations and Their Interactions

We also analyzed three pHZ18 reporters containing weaker *cis* mutations. The 5′II and 3′I mutations result in pre-mRNA accumulation as well as 40%–75% and 80%–100% mRNA levels, respectively (Figure 2D; Jacquier et al., 1985). The 3′I mutation is an A to C transversion at position 2 of the BP and results in a ~5 fold increase in pre-mRNA accumulation, whereas the 5′II construct is a G to A transition at position 5 of the 5′ss and results in a larger ~15 fold increase in pre-mRNA accumulation (Figure 2D; Jacquier et al., 1985).

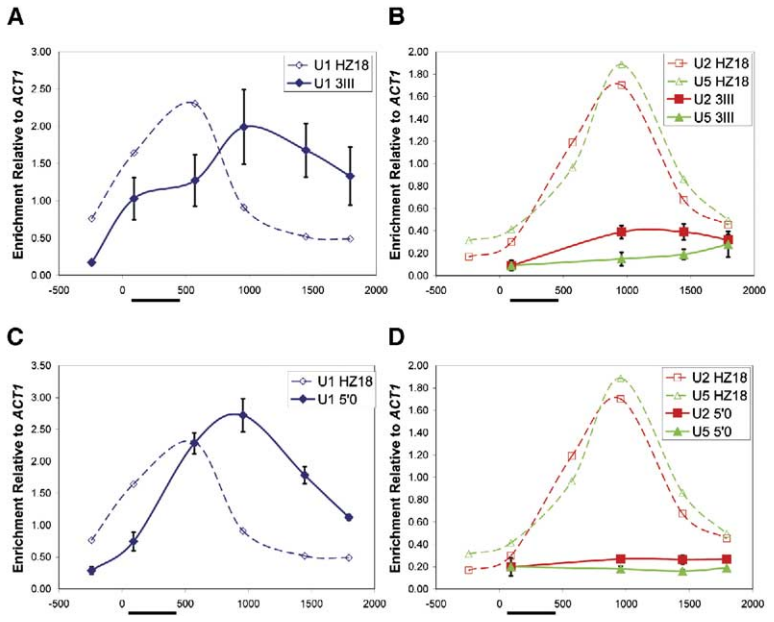

Figure 3. 5′ss and BP Mutations that Eliminate Splicing Alter snRNP Recruitment

ChIPs were performed on the indicated strains and analyzed by real-time PCR using the *HZ18* primers. In all cases, recruitment to the *cis* mutants are shown in solid lines compared to the wt *HZ18* (dashed lines), and the black bars under each graph show the position of the intron. The x axis represents the distance in base pairs from the ATG start codon. Error bars represent the average deviation from at least two independent experiments.

(A) U1 snRNP (U1C-TAP, blue) shows a delayed recruitment pattern to the 3′III BP mutant construct (Figure 2B; solid line) compared to the wt intron (dashed line).

(B) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP, green) recruitment is severely reduced by the BP mutation (solid lines) compared to the wt intron (dashed lines).

(C) U1 snRNP (U1C-TAP, blue) recruitment to the 5′0 mutant (Figure 2B). This construct displays high levels of U1 in a delayed type of pattern.

(D) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP; green) are reduced significantly to the strong 5′0 mutant construct.

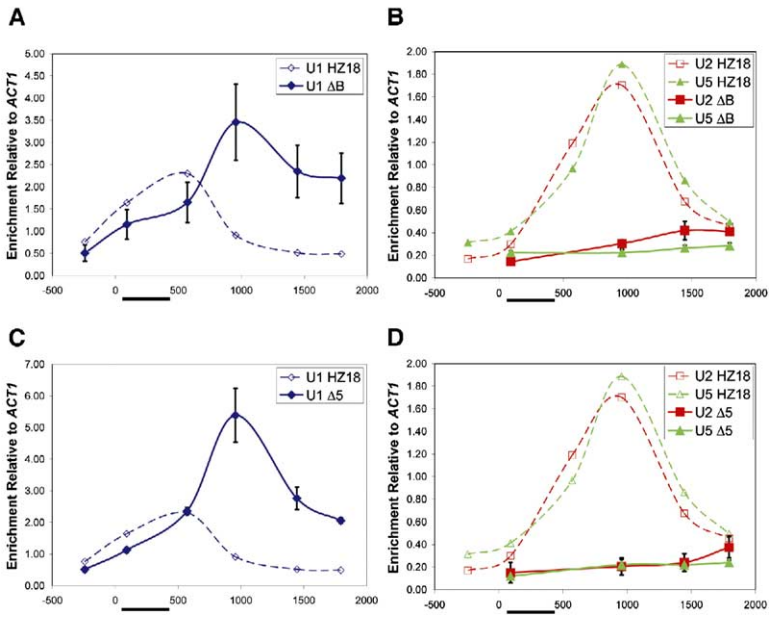

Figure 4. Complete Deletion of Either the BP or the 5′ss Results in Elevated Levels of U1

ChIPs were performed on the indicated strains and analyzed by real-time PCR using the *HZ18* primers. In all cases, recruitment to the *cis* mutants are shown in solid lines compared to the wt *HZ18* (dashed lines), and the black bars under each graph show the position of the intron. The x axis represents the distance in base pairs from the ATG start codon. Error bars represent the average deviation from at least two independent experiments.

(A) U1 snRNP (U1C-TAP, blue) shows a delayed recruitment pattern to the ΔB BP deletion construct (Figure 2C; solid line) compared to the wt intron (dashed line).

(B) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP, green) recruitment is severely reduced by the BP deletion (solid lines) compared to the wt intron (dashed lines).

(C) U1 snRNP (U1C-TAP, blue) recruitment to the Δ5 mutant (Figure 2C). This construct displays the highest levels of U1 in a delayed type of pattern.

(D) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP, green) are reduced significantly to the strong Δ5 mutant construct.

The U1 pattern is slightly delayed by the 3′I mutation (Figure 5A), and the U2 and U5 patterns show only a modest decrease in peak levels (Figure 5B). The 5′II patterns are more severely affected, with a larger U1 delay and a more prominent decrease in U2/U5 levels (Figures 5C and 5D). However, even the effects of the 5′II mutation are more modest than those of the stronger 5′0 and Δ5 mutations (compare Figures 5C and 5D with 3C and 3D and 4C and 4D). The effects of all these mutants on U2/U5 levels correlate roughly with their splicing efficiencies, whereas the delayed and increased U1 levels correlate negatively with splicing. This suggests that efficient U2 and tri-snRNP recruitment is accompanied by U1 dissociation from nascent transcription complexes.

We also examined a construct containing both the 5′II and 3′I mutations within the same gene. This combination eliminates mRNA production and accumulates high levels of pre-mRNA (Figure 2E; Jacquier et al., 1985). Even U1 snRNP levels are very low (Figure 5E); a stronger phenotype than from any single mutation assayed in this study and indicative of a dual 5′ss-BP influence on U1 recruitment/retention. U2/U5 levels are also low, comparable to glucose conditions (Figure 5F).

Effects of a Compensating U1 snRNA Mutation

The effects of the 5′II mutation on splicing were previously shown to be partially rescued by a compensating mutation at position 4 within the 5′ arm of U1 snRNA (Seraphin et al., 1988). U1 snRNA position 4 contains a C, which normally base pairs with the G at position 5 of a consensus 5′ss (G-C). To restore base pairing with the 5′II mutation A, the wt U14C was mutated to U14U, to replace the A-C mismatch with an A-U pair (Figure 2F; Seraphin et al., 1988). To assay the U1 and U2 patterns on the 5′II gene in the presence of the U14U mutation, strains were constructed containing U1C-TAP or Lea1-TAP and a knock out of the endogenous U1 snRNA covered by a plasmid expressing U14U or wt U1 (see Table S1 available in the Supplemental Data with this article online). These strains were then transformed with pHZ18 or with its 5′II mutant version.

Surprisingly, there was no substantial rescue of the 5′II-delayed U1 snRNP recruitment pattern by the U14U snRNA gene. Moreover, U1 recruitment to the wt *HZ18* gene was even delayed in the U14U background (compare Figures 6B and 6A), suggesting that the G-U pair of this 5′ss-U1 combination alters some feature of U1 recruitment relative to the wt-wt G-C pairing—despite no previously observed effect on wt splicing efficiency with this combination (Seraphin et al., 1988).

In contrast, the U14U snRNA gene noticeably rescued the 5′II U2 snRNP pattern; it has the same shape and a maximum at the same primer pair as the *HZ18* U2 snRNP pattern, which is similar in a U14U and in a wt U1 background (Figures 6D and 6C). Taken together with earlier results (Figures 3, 4, and 5; Seraphin et al., 1988), the U14U experiments suggest that U1 snRNA:5′ss base pairing is less important for initial U1 snRNP recruitment than for transitioning to a U1 snRNP-pre-mRNA conformation that promotes stable U2 snRNP recruitment, mature spliceosome formation, and splicing (see Discussion).

Discussion

Although it is not yet known whether splicing occurs cotranscriptionally in yeast, ChIP has been used to show that U1 snRNP recruitment occurs on nascent transcripts (Abruzzi et al., 2004; Kotovic et al., 2003). In this study, the same assay indicates that U2 snRNP and U5 snRNP, the latter almost certainly indicative of the U4/U6•U5 tri-snRNP, are also recruited cotranscription-

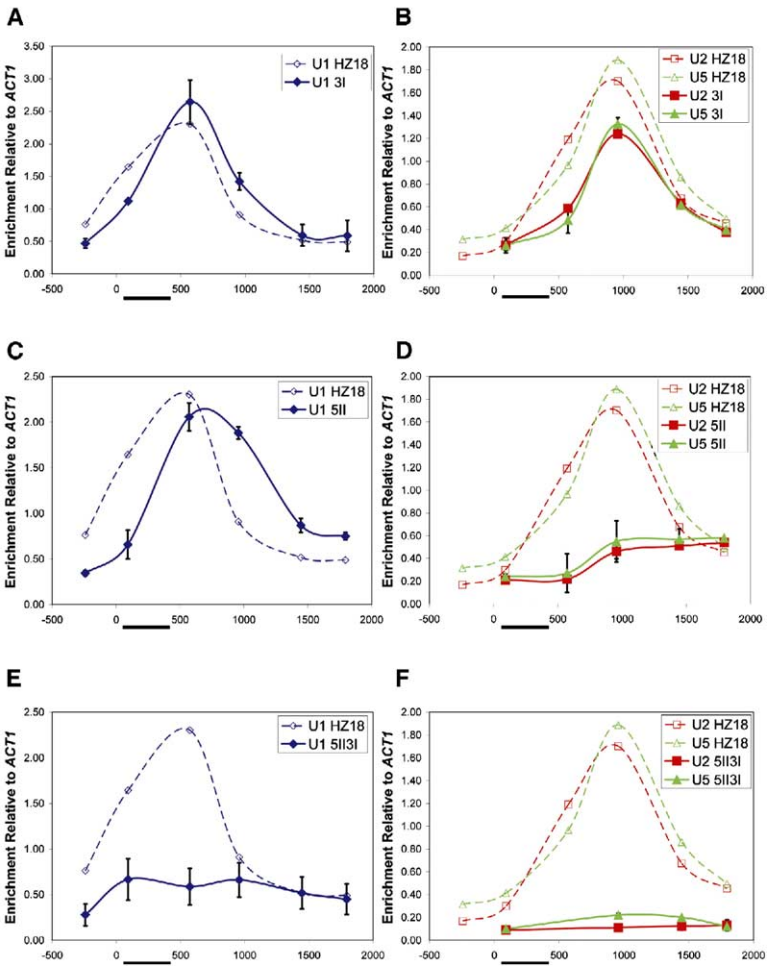

Figure 5. Weak 5′ss and BP Mutations Alter snRNP Recruitment and Combine to Severely Diminish Nascent snRNP Association

ChIPs were performed on the indicated strains and analyzed by real-time PCR using the *HZ18* primers. In all cases, recruitment to the *cis* mutants are shown in solid lines compared to the wt *HZ18* (dashed lines), and the black bars under each graph show the position of the intron. The x axis represents the distance in base pairs from the ATG start codon. Error bars represent the average deviation from at least two independent experiments.

(A) U1 snRNP (U1C-TAP, blue) recruitment to the 3′I mutation (Figure 2D). The U1 pattern to this weak mutant is slightly delayed.

(B) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP, green) show a modest drop in recruitment levels to the 3′I mutant reporter.

(C) U1 snRNP (U1C-TAP, blue) shows a delayed recruitment pattern to the weak 5′II mutant (Figure 2D).

(D) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP, green) respond similarly to the 5′II mutation, showing a reduced and flattened pattern.

(E) U1 snRNP (U1C-TAP, blue) recruitment to the 5II3I double mutation (Figure 2E) is the most severely affected of all constructs assayed.

(F) U2 snRNP (Lea1-TAP, red) and U5 snRNP (Prp8-TAP, green) are also poorly recruited to the 5II3I construct, suggesting that little to no cotranscriptional assembly is taking place.

ally to chromosomal as well as plasmid-derived nascent intron-containing RNAs. Canonical introns from the *ACT1* and *RP51A* genes show almost identical snRNP patterns. For both genes, U1 signals peak well before those for U2 and U5, which are almost indistinguishable. U1 signals are surprisingly robust with either a BP or a 5′ss, whereas both elements are needed for subsequent recruitment/assembly steps. The effects of weak as well as strong *cis*-splicing mutations are suggestive of a multistep in vivo spliceosome assembly pathway: U1 snRNP recruitment, then recruitment of U2 and the tri-snRNP followed by U1 dissociation. Cotranscriptional U2 and/or tri-snRNP levels correlate with splicing efficiency. Finally, our data suggest that base pairing between the 5′ss and U1 snRNA is more important for transitioning to the U2/tri-snRNP complexes than for U1 snRNP recruitment itself.

snRNP recruitment as assayed by ChIP is predominantly, if not completely, dependent upon intron RNA sequences. (1) *HZ18* transcription requires galactose, and there is only a trace signal when this strain is grown in glucose (Figure 1C). (2) *LGSD5*, which encodes the cDNA version of *HZ18* (Guarente et al., 1982; Teem and Rosbash, 1983), shows no recruitment, i.e., U1 signals are comparable to those of pHZ18 grown in glucose (data not shown). (3) With a few possible exceptions, U1 recruitment appears limited to intron-containing chromosomal loci (Kotovic et al., 2003). (4) U1 signals correlate well with intron position, even when the intron is relatively far from the promoter (Abruzzi et al., 2004; Kotovic et al., 2003). These considerations indicate that U1 snRNP is not detectably recruited by promoter elements, consistent with the fact that the galactose promoter of the *HZ18* gene is not derived from an intron-containing locus yet recruits spliceosome components as well as the chromosomal *ACT1* gene (Figures 1B and 1C).

An important caveat is that the ChIP assay requires direct or indirect crosslinking of the tagged protein to DNA. In this regard, snRNPs could be efficiently prerecruited by promoters (even of nonintron-containing genes) but without sufficient proximity to DNA for a robust ChIP signal. Transfer of the snRNPs from the transcription machinery to nascent RNA could be accompanied by a dramatic increase in nucleic acid proximity and therefore "crosslinkability," due, for example, to snRNA-pre-mRNA base pairing. The same or additional conformational rearrangements could also result in increased epitope accessibility.

Given these possibilities, it is remarkable that Lea1p and Prp8p have almost identical recruitment patterns and even respond similarly to the *HZ18* splicing mu-

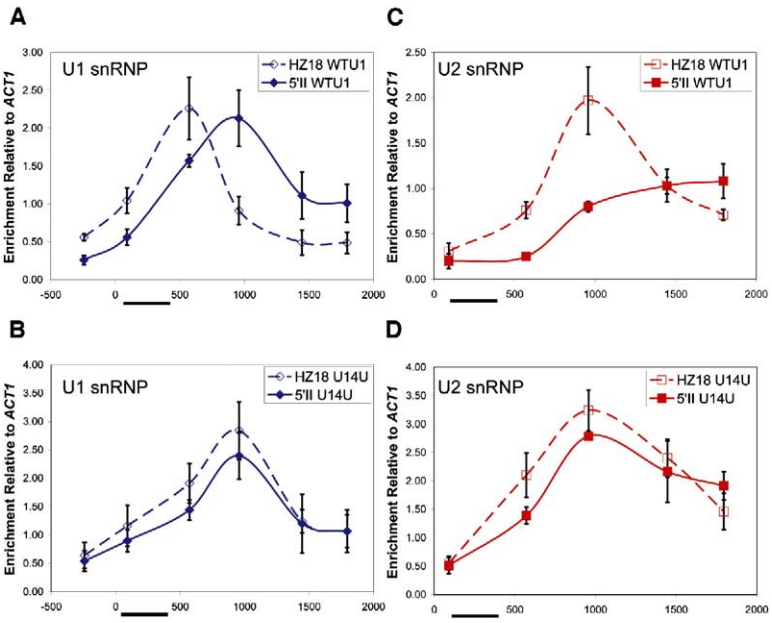

Figure 6. U1 snRNA:5′ss Base Pairing Is Less Important for Initial U1 Recruitment Than for Transitioning to a U2-Containing Complex

A knockout of the essential *SNR19*-encoding U1 snRNA was introduced into the U1C- and Lea1-TAP strains and covered by a CEN plasmid expressing either a wt U1 snRNA (wtU1; dashed line) or a U1 snRNA harboring a C to U transition at position 4 (U14U; solid line). Each strain was then transformed with wt pHZ18 or the 5′II derivative of pHZ18, and ChIPs were performed. The x axis represents the distance in base pairs from the ATG start codon. Error bars represent the average deviation from at least two independent experiments.

(A) The U1 snRNP patterns to the wt and 5′II mutant introns repeat with a plasmid-born wt U1 snRNA (U1C-TAP).

(B) U1 snRNP (U1C-TAP) patterns on the wt intron are negatively affected in the U14U despite little or no change in mRNA levels (Seraphin et al., 1988), and the U1 pattern is not rescued to the 5′II mutant.

(C) U2 snRNP recruitment to the wt and 5′II mutant introns repeat in the plasmid wt U1 snRNA background.

(D) U2 snRNP recruitment to the 5′II mutant is rescued in the U14U background. U2 snRNP levels in the U14U background are increased; an observation mirrored in the U2/U1 ratio on *ACT1* in the U14U background (data not shown). These changes do not affect the patterns on the reporter genes, which we emphasize as the central point of the experiment.

tants. Moreover, U1C and Prp42p have very similar ChIP patterns at the *ACT1* locus (data not shown; Abruzzi et al., 2004), suggesting that changes in epitope availability do not make a major contribution to the U1 snRNP patterns. We therefore interpret most of the strong increases and decreases in ChIP signal at different gene positions as predominantly indicating changes in snRNP recruitment or snRNP dissociation at preferred positions during transcription as the nascent spliceosome assembly/disassembly process proceeds. We also interpret position as "time," i.e., more 5′ recruitment = earlier recruitment, and the splicing mutants we study here show no systematic effect on polymerase density by ChIP (Figure S1).

The striking pattern difference between the U1 and U2/U5 patterns suggests a step-wise recruitment pattern, consistent with the conclusion of the accompanying paper (Görnemann et al., 2005) and inconsistent with the penta-snRNP proposal of Abelson and colleagues (Stevens et al., 2002). The similar U2 and U5 snRNP patterns might reflect a two-step assembly pathway, first U1 snRNP and then a U2/4/5/6 tetra-snRNP (Figure 1B; Stevens et al., 2002). Although none of the *cis* mutations dramatically resolved the U2 and U5 patterns, our two introns might be suboptimal for this purpose. Indeed, data in the accompanying paper indicate separate U2 and tri-snRNP recruitment: there is a delay in the U5 pattern relative to the U2 pattern as well as a preferential effect of CBC deletions on U5 recruitment (Görnemann et al., 2005). These results suggest an optimistic interpretation of the small difference between the U2 and U5 patterns in the 3′III mutant (Figure 3B). Alternatively, and as discussed above, these spatial/temporal differences might reflect conformational changes that alter "crosslinkability," e.g., different orientations between U5 snRNA and the 5′ss (Newman, 1997).

Although the same caveat applies to the advanced U1 pattern, differences between the U1 and the U2/U5 patterns are dramatic and present in all genes and constructs. U1 snRNP recruitment is thought to be predominantly determined by the pre-mRNA 5′ss sequence and its pairing interaction with the 5′ arm of U1 snRNA, as yeast U1 snRNP associates efficiently in vitro with a pre-mRNA substrate containing only a 5′ss (Seraphin and Rosbash, 1989; Seraphin and Rosbash, 1991). However, nuclear retention of a yeast pre-mRNA requires a BP as well as a 5′ss, suggesting that successful recruitment of U1 snRNP requires both of these sequence elements (Galy et al., 2004; Legrain and Rosbash, 1989; Rutz and Seraphin, 2000). Indeed, all three BP mutants used in this study decrease U1 signals at the second primer pair, i.e., at or near the intron and well before any substantial U2 or U5 signal (3′III, Figures 3A and 3B; ΔB, Figures 4A and 4B; and 3′I, Figures 5A and 5B). Moreover, the U1 snRNP signal on the 5′II mutant gene is dramatically reduced when combined with the 3′I mutation (Figure 5E). This collaborative view is consistent with the accompanying paper, which also shows higher U1 snRNP levels after the BP region rather than before (Görnemann et al., 2005). We attribute the modest differences in other snRNP patterns between the two papers to the different genes examined, different primer positions, possible effects of the different tags, and/or minor technical differences.

Not surprisingly, all three 5′ss mutants also decreased U1 signals at the second primer pair (5′0, Figure 3C; Δ5, Figure 4C; and 5′II, Figure 5C). A decrease

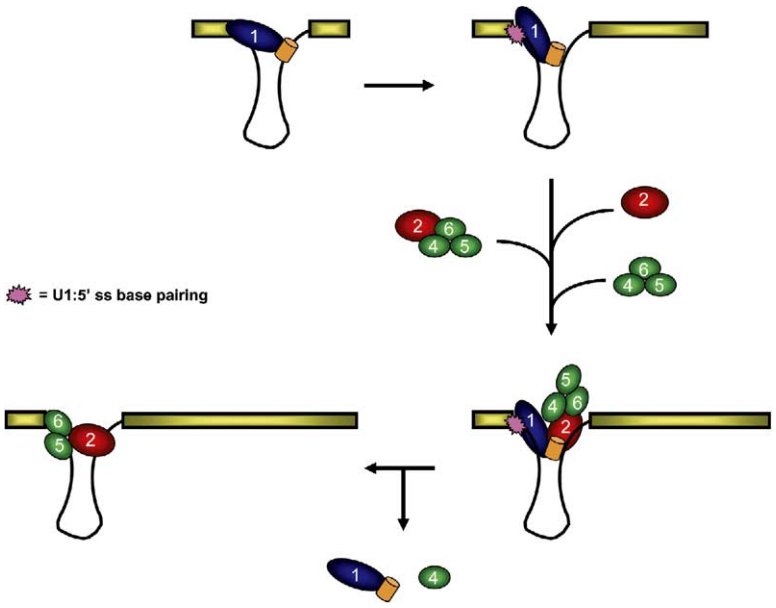

Figure 7. A Model for Cotranscriptional Spliceosome Assembly

U1 snRNP (blue) recruitment to a nascent RNA is influenced by interactions at the 5′ss as well as the BP, presumably through the BBP (orange). Once recruited, base pairing between U1 snRNA and the 5′ss (pink) switches the U1:substrate complex to a conformation that promotes U2 snRNP (red) and tri-snRNP (green) recruitment either separately or together as a tetra-snRNP. Rearrangements then take place as suggested by previously proposed in vitro models (see Discussion).

is even apparent with a wt 5′ss and the U14U snRNP (Figures 6A and 6B), perhaps reflecting the less stable G-U base pair compared to the normal G-C base pair. However, the base-pairing complementation experiment was unable to restore normal U1 recruitment (Figure 6). Because other aspects of this complementation were successful (see below), we take the negative result seriously and suggest that the 5′ arm of U1 snRNA contributes in a more indirect way to initial recognition of pre-mRNA by U1 snRNP (Du et al., 2004). Many U1 snRNP proteins as well as the cap binding complex (CBC) interact with the pre-mRNA 5′ss region (Colot et al., 1996; Lewis et al., 1996; Puig et al., 1999; Zhang and Rosbash, 1999), and U1C may interact directly with the 5′ss GUAUGU (Du and Rosbash, 2002). In any case, the data indicate that the 5′ss region, the BP region, and the 5′ arm of U1 snRNA are all important for optimal U1 snRNP recruitment (Figure 7).

In contrast, the altered U2 snRNP pattern on the 5′II construct was rescued by the U14U gene. All of these considerations indicate that the U1 snRNA:5′ss pairing interaction is more important for transitioning to a U2 snRNP-containing complex than for initial U1 snRNP recruitment. Although the low U2 snRNP levels on 3′III can be interpreted as a direct effect of the mutant BP sequence on U2 snRNA:BP interactions, it is also possible that the 3′III mutant BP creates a U1 snRNP:pre-mRNA configuration unfavorable for U2 snRNP recruitment. Moreover, the relative effects of the two BP mutations on U2 recruitment correlate with their reported effects on interactions with the branchpoint binding protein (BBP or SF1), a component of the in vitro U1 snRNP:pre-mRNA commitment complex (Abovich and Rosbash, 1997; Berglund et al., 1997). We therefore favor the notion that the nascent U1 snRNP:pre-mRNA complex, including U1 snRNA:5′ss base pairing and proteins associated with the BP region, constitute a single binding platform for U2 snRNP or tetra-snRNP recruitment (Figure 7).

There is an inverse correlation between U2/U5 levels and U1 levels. Although other interpretations are possible, we suggest that successful U2 and/or U5 snRNP recruitment then results in U1 snRNP dissociation. There is abundant evidence implicating the tri-snRNP in the in vitro dissociation of U1 from the 5′ss (Kandels-Lewis and Séraphin, 1993; Konforti et al., 1993; Lesser and Guthrie, 1993; Sawa and Abelson, 1992; Staley and Guthrie, 1999; Wassarman and Steitz, 1992), and data in the accompanying manuscript suggest an in vivo role for the CBC in promoting a switch between U1 and tri-snRNP at the 5′ss (Görnemann et al., 2005). This would explain why some of the severe mutants show higher U1 signals than wt, i.e., the highest wt levels are already experiencing dissociation. We cannot exclude other possibilities, e.g., the mutant dead-end complexes recruit unusually high levels of U1 snRNP or that they have increased formaldehyde-sensitive contacts with nascent RNA or DNA. We note that there is no in vitro precedent for the recruitment of U1 snRNP without a 5′ss. We also note that several of the mutants show some decrease in U1 levels even without an apparent U2 or U5 signal. This could reflect some cryptic U2 or tri-snRNP activity and/or might be due in part to the modest pol II signal decrease in the more 3′ regions of the *HZ18* genes (see Figure S1). In any case, most of the mutant decreases in U1 signal are more modest than that from wt *HZ18*.

The ability of the U14U snRNP to promote some in vitro U2 snRNP:pre-mRNA association without ATP might also reflect this interplay between U2/tri-snRNP and U1 snRNP dissociation (Liao et al., 1992). Indeed, the U14U and U1C L13 mutations act as bypass suppressors for a deletion of the gene encoding Prp28p. This DEAD box protein is required for splicing, has ATPase activity, and is implicated in disrupting U1:5′ss base pairing as well as promoting U6:5′ss base pairing (Chen et al., 2001; Staley and Guthrie, 1999). All of these observations suggest that decreasing U1:sub-

strate complex stability can increase the efficiency of U2 and tri-snRNP recruitment to the point of decreasing or perhaps even alleviating the ATP requirement. Taken together, they reinforce the suggestion that U2/tri-snRNP or perhaps tetra-snRNP recruitment to nascent splicing complexes ultimately results in U1 snRNP dissociation.

There is a positive correlation between the U2/U5 levels detected on the different genes and their relative splicing efficiencies as measured by mRNA levels or β-galactosidase activity (Jacquier et al., 1985; Jacquier and Rosbash, 1986; Pikielny and Rosbash, 1985). This is especially apparent in the U14U experiment in which only the U2 rescue parallels the increased splicing of the 5′II mutant (Figure 6; Seraphin et al., 1988). The observations suggest that cotranscriptional U2/U5 recruitment or stabililization, rather than U1, commits the pre-mRNA substrate to splicing. A presupposition is that the U14U U1 patterns reflect delayed recruitment and not a delayed postrecruitment conformational change that masks a prior rescued U1 snRNP recruitment. Perhaps cotranscriptional snRNP recruitment is in competition with the cotranscriptional deposition of hnRNP proteins on splicing signals, which then prevents the posttranscriptional recruitment of functional snRNPs. Assuming cotranscriptional splicing, these considerations suggest that genes with different mRNA levels due to differences in splicing efficiency do not differ in when/where cotranscriptional splicing occurs along the gene but rather in the extent to which they cotranscriptionally recruit U2/U5. This may not apply to more subtle splicing efficiency decreases resulting only in pre-mRNA level increases. To verify these predictions, it will be essential to develop a high-resolution assay that can measure where/when splicing actually takes place along these different intron-containing lacZ genes.

Experimental Procedures

Strains

All strains used in this study are listed in Table S1. We used standard methods for yeast manipulations (Guthrie and Fink, 1991). For galactose induction of the reporter plasmids, strains were grown in 3% galactose and 1% raffinose.

Plasmid Construction

Primers DT147 (5′-ACGTGCGCGGCCGCACGTTGTATCATTGCTG-3′) and DT150 (5′-GCACGTCTCGAGTGAGTGGCAAAGCTCACA TTCTC-3′) were used to amplify U1 snRNA from wt genomic DNA. PCR products were cut with NotI and XhoI and cloned into pRS314 cut with NotI and XhoI to make pDT1. pHZΔ5 and pHZΔB were made by performing QuikChange Site-Directed Mutatgenesis (Stratagene catalog number 200518) on pHZ18 with the following primers: SALO203 (5′-CTCGAGACTAGCAATAACAAAATGTAATATGGA CTAAAGGAGGCTTTT-3′) and SALO204 (5′-AAAAGCCTCCTTT AGTCCATATTACATTTTGTTATTGCTAGTCTCGAG-3′) for Δ5 and SALO205 (5′-ATTATAATGTGTTTTTGATATCAGTAAAGTTGAATTGC ATTTACAAAC-3′) and SALO206 (5′-GTTTGTAAATGCAATTCAA CTTTACTGATATCAAAAACACATTATAAT-3) for ΔB.

ChIPs

ChIPs were performed essentially as described previously (Abruzzi et al., 2004), with the following exceptions. ChIPs for the TAP tag were performed with IgG Sepharose beads (Amersham Biosciences), whereas ChIPs for pol II were performed as described (Abruzzi et al., 2004). After decrosslinking, DNA from input and IP samples was purified by using the QIAquick PCR Purification Kit (Qiagen). PCR analysis was performed on a Rotorgene 3000 PCR machine (Corbett Research). Differences in primer pair efficiencies were accounted for by forming standard curves from serial dilutions of appropriate genomic DNA preparations during each real-time run. All samples in a single PCR run were assayed in triplicate. All data represent the average of at least two independent experiments with the error bars displaying the average deviation.

For *ACT1* analysis, inputs and IP signals were normalized to a primer pair amplifying a region in the center of the highly transcribed intronless gene *PMA1* (Abruzzi et al., 2004). *PMA1* levels are ~2-fold above a nontranscribed intergenic region for all three factors (data not shown). For analysis of *HZ18* and its derivatives, inputs and IPs were normalized to *ACT1* primer pair KA223 and KA235 to account for differences in IP efficiency between replicates. The *ACT1* primers used in this study were KA227, KA237, KA223, KA235, KA225, and KA236, whose sequences were published previously (Abruzzi et al., 2004), in addition to primer pair two SALO258 (5′- ATGTTTAGAGGTTGCTGCTTTGGTTATTGATAACGGT-3′) and DT206 (5′-GCAAAACCGGCTTTACACAT-3′) and primer pair three SALO259 (5′- CCCATCTATCGTCGGTAGACCAAGACACCA-3′) and SALO261 (5′- GGGCAACTCTCAATTCGTTGTAGAAGGTAT-3′). The primers for *HZ18* and its derivatives are as follows: primer pair one, SALO254 (5′- TGAAAGTTCCAAAGAGAAGGTTTTTTTAG GCTA-3′) and SALO255 (5′-AGTTGCCTGGCCATCCACGCTATATA CACGCCT-3′); primer pair two, SALO83 (5′-ATGACCGGATCCC AAG-3′) and SALO84 (5′-CGGTATCGCAGTTCCATATCGTCTGAAA ATATCGTC-3′); primer pair three, DT44 (5′-GTCAAGCGTGCTTCT AAGGC-3′) and SALO257 (5′-TCTTTTCACCAGTGAGACGGGCA ACAGCCA-3′); primer pair 4, SALO96 (5′-ATCTTCCTGAGGCCGA TACTGTCGTCGTCC-3′) and SALO97 (5′-TAGATGGGCGCATCGTA ACCGTGCATCTGC-3′); primer pair five, SALO110 (5′-GGAGGCT GAAGTTCAGATGTGCGGCGAGTT-3′) and SALO111 (5′-ACCCT GCCATAAAGAAACTGTTACCCGTAG-3′); and primer pair six, SALO120 (5′-ACGAGCATCATCCTCTGCATGGTCAGGTCA-3′) and SALO121 (5′-TTCATCAGCAGGATATCCTGCACCATCGTC-3′).

β-Galactosidase Activity Assays

β-galactosidase assays were performed as previously described (Jacquier et al., 1985).

Supplemental Data

Supplemental Data include one figure and one table and are available with this article online at http://www.molecule.org/cgi/content/full/19/1/65/DC1/.

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