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Exon Definition Complexes Contain the Tri-snRNP and Can Be Directly Converted into B-like Precatalytic Splicing Complexes

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SUMMARY

The first step in splicing of pre-mRNAs with long introns is exon definition, where U1 and U2 snRNPs bind at opposite ends of an exon. After exon definition, these snRNPs must form a complex across the upstream intron to allow splicing catalysis. Exon definition and conversion of cross-exon to cross-intron spliceosomal complexes are poorly understood. Here we demonstrate that, in addition to U1 and U2 snRNPs, cross-exon complexes contain U4, U5, and U6 (which form the tri-snRNP). Tri-snRNP docking involves the formation of U2/U6 helix II. This interaction is stabilized by a 5′ splice site (SS)-containing oligonucleotide, which can bind the tri-snRNP and convert the cross-exon complex into a cross-intron, B-like complex. Our data suggest that the switch from cross-exon to cross-intron complexes can occur directly when an exon-bound tri-snRNP interacts with an upstream 5′SS, without prior formation of a cross-intron A complex, revealing an alternative spliceosome assembly pathway.

INTRODUCTION

Pre-mRNA splicing is catalyzed by the spliceosome, which catalyzes intron removal, and exon ligation to form mature mRNA. The spliceosome consists of the U1, U2, U4/U6, and U5 snRNPs and a multitude of other splicing factors (Will and Lührmann, 2006). When introns are relatively short (less than ~200 nt), spliceosomes assemble first across the intron (Fox-Walsh et al., 2005). Thus, U1 base pairs with the 5′ splice site (SS) of the pre-mRNA to form the E complex, and U2 then interacts stably with the branchpoint sequence (BPS) to form the A complex. Subsequent recruitment of the U4/U6.U5 tri-snRNP generates the B complex. Major conformational and compositional rearrangements follow. At the 5′SS, U1 base pairing is replaced by interaction of U6 via its ACAGAG box. Subsequently, U4 dissociates from U6, and the latter base pairs with U2 (Nilsen, 1998). After release of U1 and U4, the activated B* complex is formed. It catalyzes step 1 of splicing, during which the 5′SS is cleaved and the intron's 5′ end is ligated to the branch adenosine. This generates the C complex, which catalyzes step II of splicing, during which the intron is excised and the 5′ and 3′ exons are joined.

Most mammalian introns span more than 200 nt (Deutsch and Long, 1999), whereas exons have a rather fixed length of only ~120 nt on average (Ast, 2004). When a pre-mRNA contains a long intron, initial splicing complex formation occurs across an exon, a process called exon definition (Berget, 1995). During the latter, U2 binds the BPS upstream of the exon and U1 the 5′SS downstream of it. Splicing enhancer sequences within the exon (ESEs) recruit SR proteins that establish a network of protein-protein interactions across the exon, thereby bridging U2 and U1 and stabilizing the exon-defined complex (Hoffman and Grabowski, 1992; Reed, 2000). As the chemical steps of splicing occur across an intron, subsequent to exon definition the 3′SS must be paired with an upstream 5′SS across the adjacent intron. Thus, it is thought that cross-exon interactions are disrupted and the cross-exon complex is converted into a cross-intron A complex, where a molecular bridge now forms between U2 and U1 bound to an upstream 5′SS (Reed, 2000). This step is decisive in determining which 5′ and 3′ exon will ultimately be spliced together; recent data indicate that regulation of exon inclusion or skipping occurs during the switch from a cross-exon to cross-intron complex (Bonnal et al., 2008; House and Lynch, 2006; Sharma et al., 2008). Although splice site pairing was shown to occur during A complex formation (Lim and Hertel, 2004), it is not known how long the cross-exon molecular interactions persist and whether they must be disrupted to allow cross-intron complex formation. In current models, the tri-snRNP first binds after exon definition, interacting with the cross-intron A complex, thereby generating a B complex.

Dissection of the swap between cross-exon and cross-intron interactions has proven difficult, due to the inability to form solely a cross-exon complex on single or multiple intron pre-mRNAs and then chase it into a cross-intron complex. However, *trans*-splicing, where the 5′SS and 3′SS are present on physically distinct molecules, appears to provide an alternative to mimic the conversion from a cross-exon to cross-intron complex. During *trans*-splicing, an A-like complex, in which the U2 snRNP is stably bound to the 3′SS and U1 to a downstream 5′SS, is

assembled on single exon constructs (Grabowski et al., 1991; Robberson et al., 1990). Upon addition of a short 5′SS-containing RNA oligonucleotide (oligo) to these cross-exon complexes, a B-like complex that can be resolved on native gels is formed, and splicing in *trans* to the 5′SS RNA is observed (Ast et al., 2001; Konforti and Konarska, 1995). In this system, the U1 snRNP/ 5′SS interaction is bypassed, with the 5′SS RNA oligo binding directly to the ACAGAG box of the U6 snRNA (Konforti and Konarska, 1994). In current *trans*-splicing models, the tri-snRNP first interacts with the 5′SS RNA oligo and subsequently with complexes formed on the 3′ exon to form splicing-competent spliceosomes. A detailed dissection of the conversion of splicing complexes formed on a single exon construct to a B-like complex might provide insights into the switch from an exon-defined complex to one in which the splice sites are paired across the intron, as occurs during *cis*-splicing.

To learn more about exon definition and the conversion of a cross-exon to a cross-intron splicing complex, we purified cross-exon complexes formed in vitro. Our studies unexpectedly reveal that U4/U6.U5 tri-snRNPs associate already during exon definition. Tri-snRNPs, as part of purified cross-exon complexes, can bind a 5′SS-containing RNA oligo, which in turn stabilizes tri-snRNP association and converts the exon complex into a B-like spliceosomal complex. Our results indicate that cross-exon complexes containing the tri-snRNP can directly engage an upstream 5′SS and thereby lead to the pairing of splice sites across an intron.

RESULTS

Cross-Exon A-like Complexes Form on a MINX Single Exon Construct

To investigate exon definition in more detail, we constructed a derivative of the MINX pre-mRNA substrate, which contains exon 2 flanked upstream by an anchoring site (AS)—a stretch of ~25 nucleotides upstream of the BPS that are recognized in a sequence-unspecific manner by U2-SF3a/b subunits—the BPS, polypyrimidine tract, and 3′SS (Figure 1A; see also Figure S1 available online). Downstream of the exon, a 5′SS and three MS2 loops for affinity purification are present. Upon incubation of this MINX exon RNA with HeLa nuclear extract under splicing conditions for 3 min, a complex comigrating with the cross-intron A complex (formed on MINX pre-mRNA) was detected in native gels after heparin treatment (Figure 1B). No significant increase in these cross-exon complexes was observed after 5 min, indicating that they form efficiently within 3 to 5 min. Similar results were also obtained with a MINX exon RNA lacking MS2 aptamers (Figure S1). Consistent with previous results, formation of cross-exon A-like complexes (hereafter termed exon complexes) was ATP dependent and was greatly reduced upon depletion of U1 or U2 snRNPs from nuclear extract (Figure S1) (Ast et al., 2001; House and Lynch, 2006; Konforti and Konarska, 1995; Robberson et al., 1990).

The U4/U6.U5 Tri-snRNP Is Present in Affinity-Purified Exon Complexes

We next affinity purified complexes formed on the MINX exon RNA. After allowing exon complexes to form, splicing reactions were subjected to glycerol gradient centrifugation. Two peaks containing the ^{32}P-labeled exon RNA were observed, a minor one peaking in fraction 11 and a major one in fraction 15, with S values of 23 and 37, respectively (Figure 1C; see also Figure S1 for MINX exon RNA lacking MS2 sites). In the absence of ATP, solely 23S complexes were observed (Figure 1C). Complexes were then purified by MS2 affinity selection. 23S complexes contained similar amounts of the exon and U1 and U2 RNA—even in the absence of ATP (Figure 1D and data not shown). Thus, this peak likely contains A-like complexes plus some E-like and H complexes. Surprisingly, 37S complexes contained nearly equimolar amounts of not only exon RNA, U1, and U2, but also U4, U5, and U6 snRNA (Figure 1D), indicating that the tri-snRNP also associates with cross-exon complexes. Tri-snRNPs were also found in exon complexes formed on a β-globin exon construct (see below) and on the BSEx4 RNA (Figure S1)—containing exon 4 from the c-src pre-mRNA (Sharma et al., 2008)—indicating that their presence in exon-defined complexes is not limited to the MINX construct. Similar results (i.e., the presence of the tri-snRNP in the exon complex) were also observed via immunoprecipitation of 37S complexes formed on MINX exon RNA lacking MS2 aptamers (Figure S1). Thus, as solely an A-like complex was detected in native gels, tri-snRNP association with the exon complex does not appear to withstand the stringent conditions (e.g., presence of heparin) of native gel electrophoresis.

To test whether tri-snRNP association is influenced by ESEs, we analyzed complex formation on an exon RNA containing a portion of exon 2 of the human β-globin gene and three well-defined ESEs designed by ESEfinder to bind the SRp55 protein (Figure 2A). This β-globin exon is alternatively spliced in vitro and in vivo in an ESE-dependent manner (Labourier et al., 1999). An A-like exon complex also formed on the β-globin construct (Figure 2B), although less efficiently than the MINX A-like complex. Upon mutation of the 5′SS, complex formation was reduced, and after mutation of all ESEs (Figure 2B), it was nearly abolished. Thus, the ESEs play a more prominent role than the downstream 5′SS in stabilizing U2 binding during exon definition. Mainly 23S complexes formed on the β-globin exon RNA, with a small shoulder of complexes at 37S (data not shown). β-globin cross-exon complexes affinity purified from the 37S shoulder contained equimolar amounts of U1, U2, U4, U5, and U6 and exon RNA (Figure 2C). Upon mutation of the ESEs, the 37S shoulder was reduced (data not shown), and the remaining 37S complexes contained only residual amounts of U2, U4, U5, and U6 but an unchanged level of U1 relative to the "wild-type" construct (Figure 2C). The presence of equal amounts of U1 in both complexes, but at the same time reduced levels of U4, U5, and U6 snRNAs in the ΔESE complex, suggests that U1 bound to the downstream 5′SS is not sufficient for tri-snRNP recruitment.

A 5′SS Is Neither Necessary Nor Sufficient for Tri-snRNP Association

To investigate the requirements for tri-snRNP recruitment, we mutated the 5′SS of the MINX exon construct to inhibit U1 snRNP binding. With this mutant, 23S complexes increased, whereas the 37S peak was now visible only as a shoulder

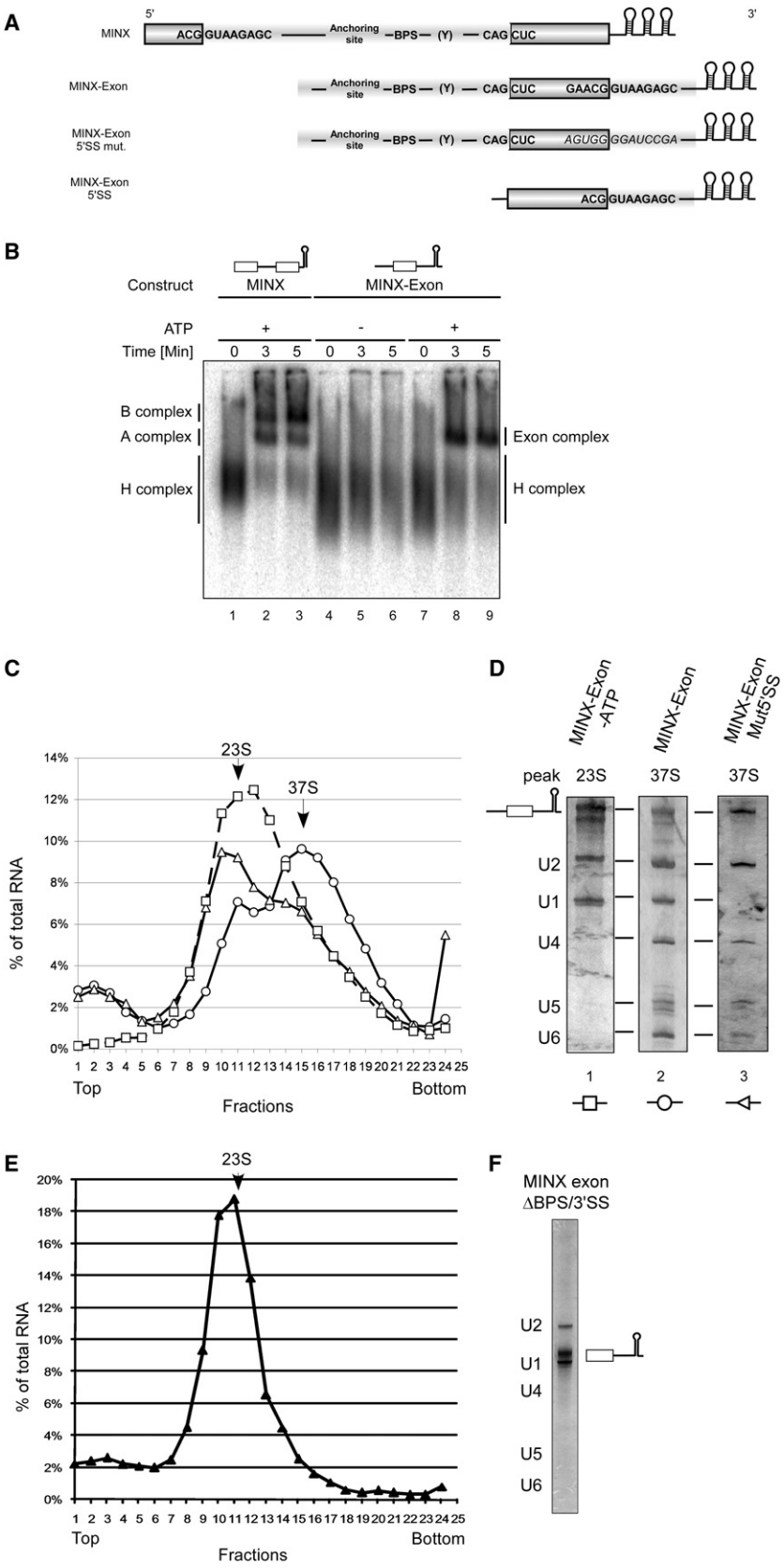

Figure 1. Cross-Exon Complexes Contain U4/U6.U5 Tri-snRNPs in Addition to U1 and U2

(A) Schematic representation of MINX constructs. Exons are depicted as a box, and MS2 RNA aptamers are indicated by stem loops. BPS, branchpoint sequence; (Y), polypyrimidine tract.

(B) Native gel analysis of splicing complexes formed at the indicated time points on ^{32}P-labeled MINX pre-mRNA (lanes 1–3) or MINX exon RNA (lanes 4–9). ATP and creatine phosphate were omitted in lanes 4–6. After addition of heparin, complexes were analyzed on native agarose gels. The identity of the spliceosomal complexes is indicated.

(C) Splicing reactions plus (open circles) or minus (open squares) ATP performed with MINX exon RNA, or MINX exon RNA with a mutated 5′SS (MINX Exon Mut5′SS) (open triangles), were subjected to glycerol gradient centrifugation. The radioactivity in each fraction was determined by Cherenkov counting and expressed as the percent of the total ^{32}P-RNA loaded onto the gradient. S values were determined by comparison with a reference gradient containing prokaryotic ribosomal subunits.

(D) 23S complexes (fractions 10–12) formed in the absence of ATP (lane 1) or 37S complexes (fractions 14–16) formed in the presence of ATP (lane 2) or after mutation of the 5′SS (lane 3) were pooled and subjected to MS2 affinity selection, and their RNA was separated by denaturing PAGE and visualized by silver staining. The positions of the snRNAs and MINX exon RNA are indicated on the left.

(E) Splicing was performed with MINX exon RNA lacking a branch site and polypyrimidine tract (MINX exon ΔBPS/3′SS), and the reaction was subjected to glycerol gradient centrifugation. The radioactivity contained in each gradient fraction was determined as in (C).

(F) Complexes in the 20S peak (fractions 9–11) of (E) were purified and their RNA composition analyzed as in (D).

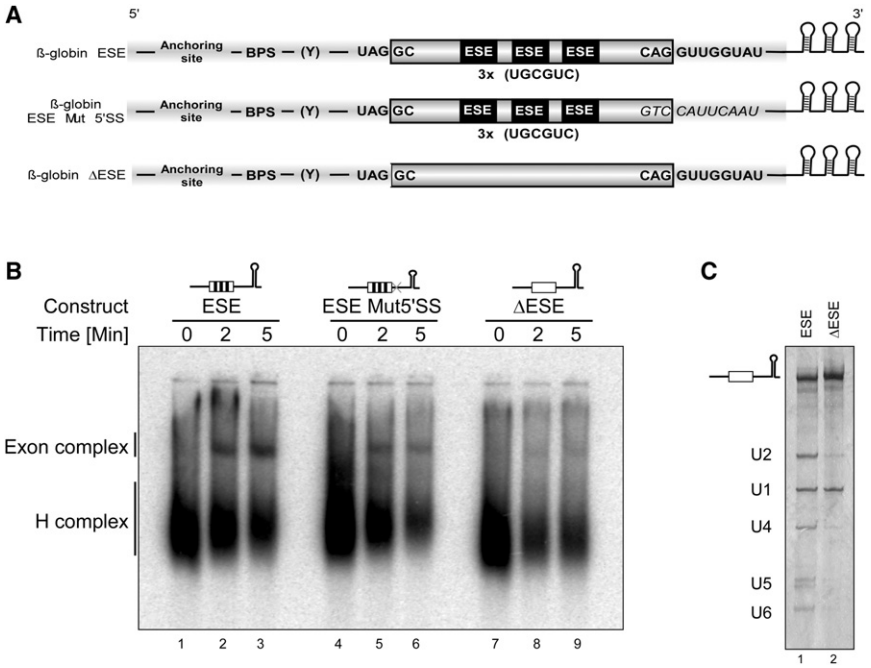

Figure 2. Effect of ESEs on Exon Complex Formation on a β-Globin Exon Construct

(A) Schematic representation of β-globin exon constructs.

(B) ESEs are required for stable exon complex formation on the β-globin exon RNA. Splicing complexes formed at the indicated time points on ^{32}P-labeled β-globin ESE (lanes 1–3), β-globin ESE Mut 5′SS (lanes 4–6) or β-globin ΔESE RNA (lanes 7–9) were analyzed on native gels as described in Figure 1B.

(C) Cross exon complexes formed on β-globin ESE RNA also contain tri-snRNPs. Splicing reactions performed with β-globin ESE exon RNA were subjected to glycerol gradient centrifugation. Complexes sedimenting in the 37S region of the gradient were pooled and affinity selected, and their RNA was analyzed as in Figure 1D.

(Figure 1C). Analysis of purified 37S complexes revealed that although the level of U1 was substantially reduced, nearly equimolar amounts of exon RNA, U2, U4, U5, and U6 were still present (Figure 1D). Similar results were obtained with a 2′OMe oligo against the 5′ end of U1 (Figure S1). Thus, tri-snRNP recruitment does not strictly require a U1/5′SS interaction. Although the amount of 37S complex was reduced when U1 binding was blocked, this is likely an indirect effect due to reduced U2 snRNP binding—i.e., U1 enhances U2 association with single exon RNAs (Figure S1; Robberson et al., 1990).

When all intron nucleotides upstream of the exon were deleted (designated MINX ΔBPS exon RNA), solely 23S complexes (Figure 1E) that contain equimolar amounts of ΔBPS exon RNA and U1, plus lower amounts of U2, but no U4, U5 or U6, were observed (Figure 1F). Thus, a 5′SS plus adjacent exon is not sufficient for tri-snRNP association. The presence of U2 indicates it can still loosely associate presumably via weak interactions with the U1 snRNP. Taken together, these results indicate that tri-snRNP association is not mediated by the 5′SS or U1 snRNP.

U6 Base Pairs with U2 and U4 in the Exon Complex

Association of tri-snRNP with cross-intron B complexes involves a base-pairing interaction between the 5′ end of U2 and the 3′ end of U6 (i.e., U2/U6 helix II) (Nilsen, 1998). To test whether this interaction is also formed in exon complexes, we performed psoralen crosslinking. MS2 affinity-purified MINX exon complexes were irradiated at 365 nm in the presence of psoralen, and crosslinks were analyzed by northern blotting (Figure 3A). A signal from the ^{32}P-labeled exon RNA could be detected without the addition of any probe. In the absence of psoralen, essentially no additional bands were detected. Upon hybridization of probes against the exon RNA or U1, U2, U4, or U6 snRNA, several slower-migrating bands representing RNA-RNA crosslinks were observed in the presence of psoralen. For example, a very slowly migrating band was observed with the U1 probe (lane 7). The same band was also observed with the exon RNA probe but no other snRNA probe (lane 4). This demonstrates crosslink formation between the exon RNA and U1 snRNA, consistent with a U1/5′SS interaction (Figure 3B). Similarly, a crosslink between U2 and exon RNA was also observed, confirming a U2/BPS base-pairing interaction. Significantly, we could not detect any direct interaction of U6 with the MINX exon RNA, which would be expected if U6 base paired with the 5′SS. In contrast, a crosslink between U6 and U2 was clearly visible (lanes 11 and 13); note that the signal from the exon RNA in this region is likely due to internal crosslinks, as crosslinked, naked exon RNA migrates in this region (Figure 3, lane 5). Based on its migration behavior (Hausner et al., 1990) and also its presence in HeLa nuclear extract (lane 14), this U2/U6 crosslink indicates formation of U2/U6 helix II. A U4/U6 crosslink was also observed, suggesting that the U2/U6 interaction can occur without disrupting U4/U6 base pairing. Indeed, triple crosslinks were detected between the exon RNA, U2, and U6, and between U2, U6, and U4, indicating that these interactions take place simultaneously within the same complex. Thus, within the exon complex, U6 is base paired to U4 but also establishes initial contacts with U2, which is stably bound to the BPS of the exon RNA (Figure 3B). These data demonstrate that the tri-snRNP docks to the exon complex, at least in part by interacting with U2 when it is stably bound to the BPS.

Addition of a 5′SS RNA Oligo Generates a Heparin-Resistant B-like Complex

To test whether addition of a 5′SS-containing RNA oligo can convert the MINX cross-exon complex into a stable B-like complex, we performed splicing in the presence of a 16 nt RNA oligo that contains an optimized 5′SS (Figure 4A). On native gels, a complex migrating similarly to the cross-intron B complex (Figure 4B) was observed after 5 min upon addition of a 100-fold

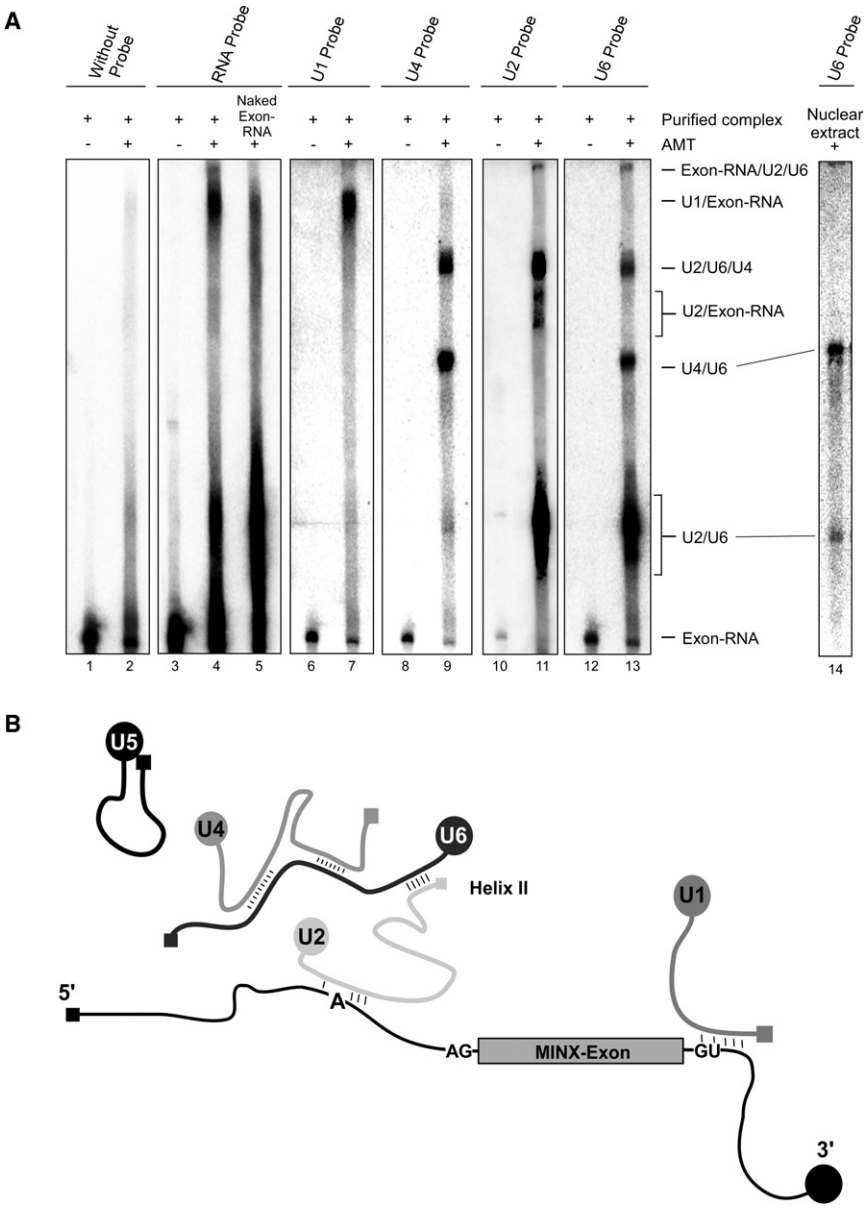

Figure 3. U2 and U6 snRNA Are Base Paired in Exon Complexes

(A) MS2-purified exon complexes (lanes 1–4 and 6–13), naked exon RNA (lane 5) or nuclear extract (lane 14) were irradiated at 365 nm with (+) or without (−) psoralen (AMT). RNA was recovered and transferred to a nylon membrane and sequentially hybridized with ^{32}P-labeled probes against exon RNA (lanes 3–5), U1 (lanes 6 and 7), U2 (lanes 10 and 11), U6 (lanes 12–14), or U4 snRNA (lanes 8 and 9). The identity of the crosslinked species is indicated on the right.

(B) Schematic representation of the RNA-RNA interactions detected in cross-exon complexes. The 5′ end of each U snRNA is represented by a square and the 3′ end by a circle.

molar excess of the 5′SS RNA oligo, but not with a 5′SS RNA oligo containing a mutated 5′SS (Figure 4B). Similar results were obtained with the β-globin exon and BSEx4 RNAs (Figure S1 and data not shown). Consistent with previous data (Konforti and Konarska, 1994), affinity-purified B-like complexes contained the 5′SS RNA oligo (Figure S2). Structure probing studies and the addition of a 2′OMe oligo against the U6 ACAGAG box indicated that the 5′SS RNA oligo interacts with the U6 ACAGAG box in our B-like complexes and that this interaction is required for stable tri-snRNP association (Figure S2). Thus, heparin-resistant tri-snRNP binding occurs after initial association with the exon complex if the tri-snRNP subsequently interacts with a 5′SS in *trans*. The exon complex was still converted with similar efficiency into a B-like complex even when an excess of unlabeled competitor exon RNA was added together with the 5′SS oligo (Figure 4C). This demonstrates that the exon complex can be chased into the B-like complex (i.e., it is a precursor of the B-like complex).

Sedimentation analysis of complexes formed in the presence of the 5′SS RNA revealed an additional peak at ~50S (Figure 4D). Affinity-purified 50S complexes contained similar molar amounts of exon RNA and all five U snRNAs (except for U1, which appeared to be slightly less abundant) (Figure 4E), confirming that they represent the B-like complexes observed on native gels. The reduced levels of U1 likely are due to the fact that excess 5′SS RNA competes with the exon RNA for U1 binding.

Complexes Formed on the MINX Exon RNA Support the Catalytic Steps of Splicing

The conversion of MINX cross-exon complexes to a B-like complex indicates that our cross-exon complexes are functionally competent for subsequent steps of spliceosome assembly. To test whether they also support the catalytic steps of splicing, we assayed for *trans*-splicing after incubating for 60 min under splicing conditions with unlabeled MINX exon RNA (lacking MS2 aptamers) and a ^{32}P-3′-end-labeled 5′SS RNA oligo as previously described by Konforti and Konarska (1995). Significantly, three major radiolabeled bands were observed solely when both the ^{32}P-labeled 5′SS RNA oligo and exon RNA were present (Figure 4F, lanes 1 and 2). Based on their migration behavior, the upper two bands appear to represent *trans*-splicing intermediates (5′SS RNA oligo intron sequences covalently linked to the branch adenosine of the MINX exon RNA), where the faster-migrating band most likely lacks the downstream intron part due to usage of the 5′ss of the MINX Exon RNA in an independent splicing reaction. In contrast, the lower band likely represents one of the

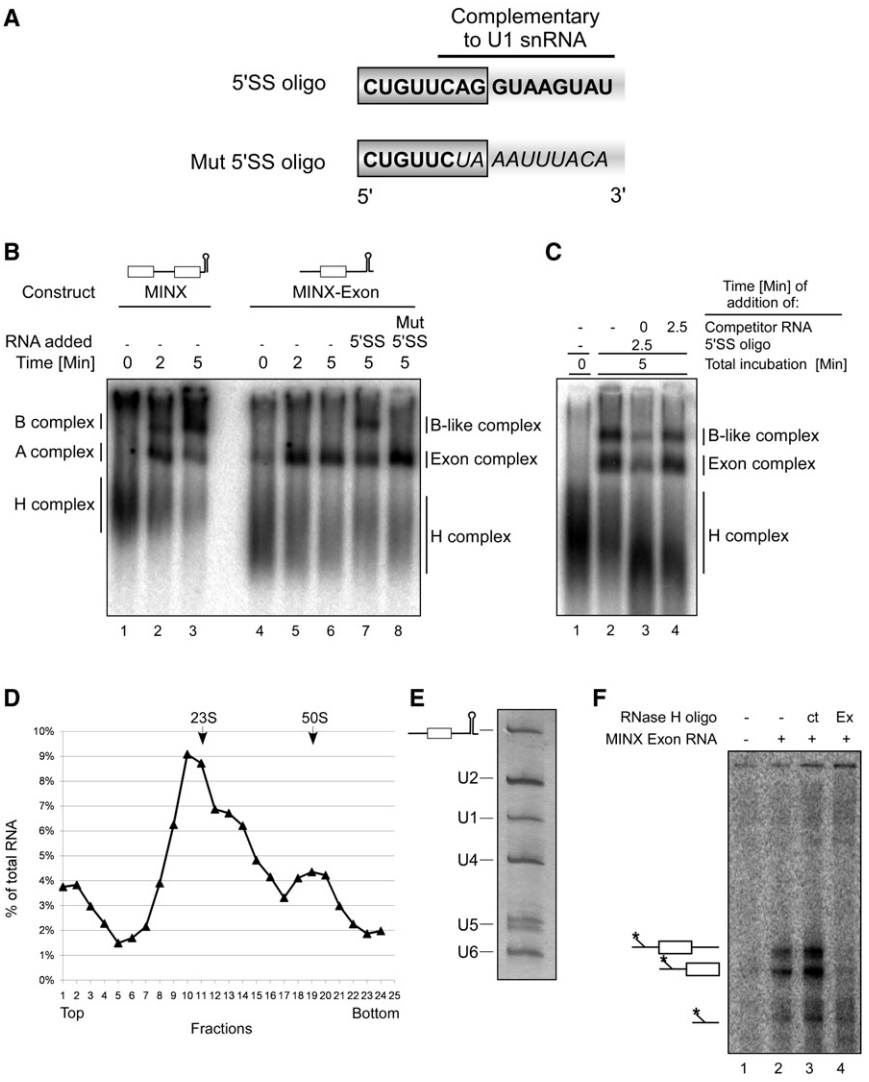

Figure 4. Addition of a 5′SS RNA Oligo Converts the Exon Complex into a B-like Complex

(A) Sequence of the 5′SS RNA oligos. The exon is represented by a black framed box. The black bar indicates the region complementary to the 5′ end of U1 (nucleotides 1–11).

(B) Native gel analysis (in the presence of heparin) of splicing complexes formed at the indicated time points on ^{32}P-labeled MINX pre-mRNA (lanes 1–3), MINX exon RNA (lanes 4–8), or MINX exon RNA plus a 100-fold excess of a 5′SS oligonucleotide (lane 7) or an oligonucleotide with a mutated 5′SS (lane 8).

(C) Exon complexes can be chased into B-like complexes. Splicing was performed with radiolabeled MINX exon RNA, and a 100-fold excess of cold MINX exon RNA was added as competitor either after 0 min (lane 3) or after 2.5 min (lane 4). The 5′SS RNA oligo was added after 2.5 min (lanes 2–4). Complex formation was analyzed on a native gel.

(D and E) Splicing reactions containing MINX exon RNA plus 5′SS oligo were subjected to glycerol gradient centrifugation (D), and complexes sedimenting in the 50S region were pooled, subjected to MS2 affinity selection, and their RNA composition analyzed (E) as in Figure 1D.

(F) B-like complexes can catalyze the first and second step of splicing. Splicing was performed for 60 min in the presence of a ^{32}P-pCp-3′-end-labeled 5′SS RNA oligo alone (lane 1) or together with unlabelled MINX exon RNA (lacking MS2 sites) (lanes 2–4). RNase H digestions were performed with a control DNA oligo (lane 3) or with an oligo complementary to the MINX exon (lane 4). RNA was separated by denaturing PAGE and visualized by autoradiography. The position of the intermediate(s) and product is indicated on the left. The faster-migrating species of the two upper bands most likely lacks the downstream intron due to usage of the 5′ss of the MINX Exon RNA in an independent splicing reaction.

trans-splicing products (i.e., spliced out branched intron). The identity of these bands was confirmed by performing RNase H digestions. Consistent with them containing exon RNA, both of the upper bands, but not the lower one, were hydrolysed in the presence of a DNA oligo complementary to nucleotides of the MINX exon, but not when a control oligo was added (Figure 4F, lanes 3 and 4). Due to their low abundance, reliable sequence information after PCR amplification could not be obtained for the *trans*-spliced RNAs, nor could the branch site be mapped by primer extension. Nonetheless, these data indicate that at least a small subset of our B-like complexes are catalytically active.

Addition of the 5′SS Oligo to Purified Exon Complexes Stabilizes Tri-snRNP Association

The studies described above were performed in nuclear extract, and thus it is possible that during B-like formation the tri-snRNP first interacts with the 5′SS RNA oligo prior to its stable association with the exon complex (Figure 5A). To test whether the tri-snRNP can bind the 5′SS RNA after it associates with the exon complex, we first affinity selected MINX cross-exon complexes. After washing complexes bound to amylose beads with low-salt (65 mM NaCl) buffer, we added the 5′SS RNA. We then assayed for stable tri-snRNP association by washing the matrix-bound complexes with buffer containing 300 mM NaCl. Complexes were then eluted and their RNA composition analyzed. As expected, in the absence of the 5′SS RNA oligo, the eluted exon complexes contained nearly equimolar amounts of all five U snRNAs and the exon RNA when the second wash buffer contained 65 mM NaCl (Figure 5B, lane 1). At 300 mM NaCl, most of U4, U5, and U6 were lost (Figure 5B, lane 2). Upon addition of the 5′SS RNA, but not the mutated 5′SS oligo, complexes washed with 300 mM NaCl still contained similar molar amounts of U1, U2, U4, U5, U6, and exon RNA (Figure 5B, lanes 3 and 4), indicating that tri-snRNP association was stabilized after binding the 5′SS RNA. This indicates that the 5′SS RNA interacts directly with tri-snRNPs already bound to the exon complex and that all factors required for tri-snRNP stabilization are associated with purified exon complexes. Our data, in turn, suggest that the conversion of a cross-exon to cross-intron

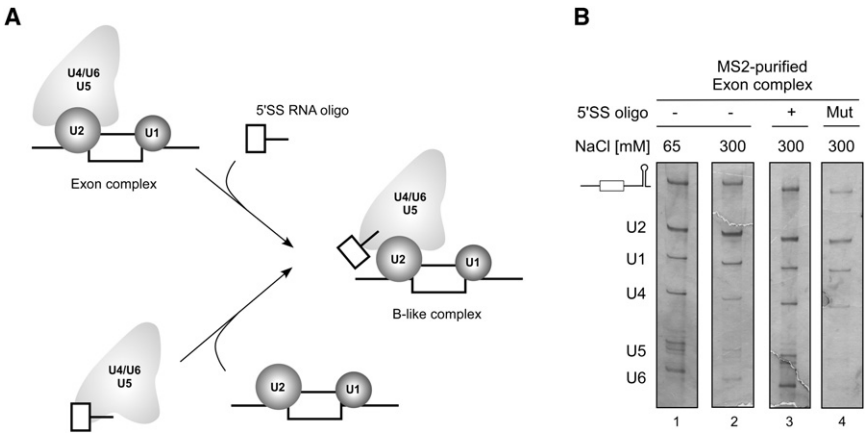

Figure 5. The 5′SS RNA Can Bind to U4/U6.U5 Tri-snRNPs Already Associated with the Exon Complex

(A) Schematic of alternative pathways for B-like complex assembly.

(B) MINX exon complexes containing tri-snRNPs were affinity selcted and washed while bound to the amylose column with buffer containing 65 mM salt. Bound complexes were then incubated in the absence or presence of the 5′SS RNA oligo or an oligo with a mutated 5′SS (Mut) (as indicated) and then washed with buffer containing 65 mM (lane 1) or 300 mM NaCl (lanes 2–4). Complexes were eluted and their RNA composition analyzed as in Figure 1D.

complex (as mimicked here by formation of the B-like complex) does not require initial association of the tri-snRNP with an upstream 5′SS prior to its association with U2 bound to a downstream BPS.

Protein Composition of the Exon and B-like Complexes

We next determined the protein composition of the affinity-purified, 37S MINX exon complexes and 50S B-like complexes by performing liquid chromatography-tandem mass spectrometry (LC-MS/MS). Consistent with its snRNA composition, nearly all proteins of the U1, U2, and U4/U6.U5 snRNPs were found in exon complexes (Table 1). In addition, multiple non-snRNP proteins were detected (Table 1 and Table S1). The vast majority of proteins previously detected in MS2 affinity-purified, human cross-intron A complexes were also found in our cross-exon complexes (Table 1 and Table S1). In contrast, many non-snRNP proteins previously found in affinity-purified B complexes were completely absent or present in lower amounts in the exon complex. For example, components of the hPrp19/CDC5 complex, which plays a crucial role during spliceosome activation (Chan et al., 2003), and hPrp19-related proteins were either underrepresented or absent in cross-exon complexes, based on sequenced peptide numbers (Table 1) or additionally on the results of western blot analyses with antibodies against Prp19, Prl1, AD002, hRBM22, hSyf1, and hSyf3 (Figure 6A).

The vast majority of proteins present in the affinity-purified exon complexes were also found in the B-like complex (Table 1 and Table S1). In contrast, several proteins appeared to be more abundant in the B-like complex or first recruited during B-like complex formation. These included (1) hSnu23 and hPrp38, which are are also found in human cross-intron B complexes (Table 1 and Figure 5A); (2) Npw38 and Npw38BP, which copurify with the human Prp19/CDC5 complex; and (3) the "B complex proteins" UBL5, MFAP1, RED, and hSmu-1 (Table 1). Proteins of the hPrp19/CDC5 complex were also more abundant in the B-like complex as compared to exon complex (Table 1 and Figure 6A), but their levels, as well as those of Prp19-related proteins, were still lower than those observed in cross-intron B complexes (Table 1 and Figure 6A). In summary, exon and B-like complexes have distinct proteomes, with the B-like complex compositionally more similar to the cross-intron B complex.

Addition of the 5′SS RNA Oligo Leads to Phosphorylation of hPrp31 and hPrp6

Phosphorylation of hPrp6 and hPrp31, both tri-snRNP components, occurs concomitant with cross-intron B complex formation and is mediated by hPrp4 kinase (Schneider et al., 2010). As this kinase is present in both exon and B-like complexes, we assayed the phosphorylation status of hPrp6 and hPrp31. Western blotting with antibodies specific for phosphorylated hPrp6 or hPrp31 revealed that these proteins are phosphorylated upon addition of the 5′SS RNA oligo—but not the mutated 5′SS oligo or a 2′Ome RNA oligo against the U6 ACAGAG box—to affinity-purified exon complexes (Figure 6B). Similar results were observed when the B-like complex was generated in the presence of nuclear extract (data not shown). In contrast, in the absence of exon RNA, addition of the 5′SS RNA oligo to nuclear extract, and subsequent incubation under splicing conditions, did not lead to any detectable phosphorylation of hPrp6 or hPrp31 (data not shown). Thus, hPrp6 and hPrp31 are also modified upon stable integration of the tri-snRNP after its association with the exon complex.

DISCUSSION

Here we have affinity purified cross-exon complexes formed during exon definition and demonstrate that they contain the U4/U6.U5 tri-snRNP. The requirements for tri-snRNP recruitment to the exon complex were further dissected, revealing a role for U2, which involved the formation of U2/U6 helix II. Tri-snRNP association with purified exon complexes was stabilized upon addition of a 5′SS-containing RNA in *trans*, suggesting that also during *cis*-splicing the tri-snRNP can interact with an upstream 5′SS after its association with an exon-defined complex. Our data thus provide insights into a potential assembly pathway of spliceosomes generated initially via exon definition.

The U4/U6.U5 Tri-snRNP Interacts with Exon Complexes during Exon Definition

Tri-snRNP association during exon definition was an unexpected finding, as previous MS2 affinity-selected cross-exon complexes formed on exon 4 of the *c-src* pre-mRNA (BSEx4)

Table 1. Protein Composition of Affinity-Purified Exon and B-like Complexes

			Exon Complex					B-like Complex			
Protein	Mol Mass (kDA)	A Comp	#1	#2	#3	#4	B Comp	#1	#2	#3	GenBank Accession Number
Sm Proteins											
B	24.6	+	17	9	4	8	+	7	5	11	gi|4507135
D1	13.3	+	16	6	9	6	+	2	7	8	gi|5902102
D2	13.5	+	17	9	22	11	+	7	9	19	gi|29294624
D3	13.9	+	11	7	9	6	+	7	7	12	gi|4759160
E	10.8	+	8	6	6	3	+	4	6	7	gi|4507129
F	9.7	+	4	7	6	6	+	4	4	9	gi|4507131
G	8.5	+	1	6	2	3	+	3	2	6	gi|4507133
U1 snRNP											
U1-70K	51.6	+	15	9	19	10	+	1	4	6	gi|29568103
U1-A	31.3	+	16	4	13	8	+	–	–	3	gi|4759156
U1-C	17.4	+	1	2	1	–	+	–	–	–	gi|4507127
U1 snRNP Associated											
FBP11	48.5	+	13	7	10	5		1	–	4	gi|113413296
S164	100.1	+	24	3	10	6		2	–	9	gi|55741709
p68	69.2	+	8	11	16	20	+	24	10	33	gi|4758138
CA150 (TCERG1)	124.0	+	–	–	–	–	+	4	–	3	gi|21327715
17S U2 snRNP											
U2A′	28.4	+	33	14	22	25	+	30	8	31	gi|50593002
U2B″	25.4	+	3	5	5	7	+	5	5	15	gi|4507123
SF3a120	88.9	+	37	18	49	54	+	32	25	66	gi|5032087
SF3a66	49.3	+	4	4	5	6	+	3	2	11	gi|21361376
SF3a60	58.5	+	8	9	23	27	+	11	13	25	gi|5803167
SF3b155	145.8	+	61	43	45	64	+	36	36	86	gi|54112117
SF3b145	100.2	+	26	14	32	43	+	24	26	52	gi|55749531
SF3b130	135.5	+	54	37	94	73	+	49	53	83	gi|54112121
SF3b49	44.4	+	2	2	1	1	+	2	1	1	gi|5032069
SF3b14a/p14	14.6	+	13	6	9	10	+	9	4	9	gi|7706326
SF3b14b	12.4	+	5	1	9	7	+	6	7	9	gi|14249398
SF3b10	10.1	+	6	4	1	1	+	1	1	2	gi|13775200
17S U2 Related											
hPRP43	90.0	+	14	8	24	22	+	14	15	36	gi|68509926
SPF45	45.0	+	2	4	5	5	+	6	1	8	gi|14249678
SPF30	26.7	+	3	4	8	6	+	5	3	8	gi|5032113
U2AF65	53.5	+	9	3	9	6	+	4	3	6	gi|6005926
U2AF35	27.9	+	7	4	6	7	+	2	–	7	gi|5803207
hPRP5	117.4	+	26	5	15	2		3	–	2	gi|41327773
SR140	118.2	+	4	4	5	7		4	7	25	gi|51492636
PUF60	59.9	+	31	8	17	4		1	–	6	gi|17298690
U5 snRNP											
hPrp8	273.7		65	68	104	131	+	106	123	204	gi|91208426
hBrr2	244.5		89	57	112	121	+	143	104	155	gi|40217847
hSnu114	109.4		19	32	46	67	+	40	44	96	gi|41152056
40K	39.3		11	9	16	13	+	11	2	21	gi|4758560
hPpr6	106.9		14	24	59	76	+	80	63	93	gi|40807485
hDib1	16.8		–	6	8	7	+	6	7	9	gi|5729802
hPrp28	95.6		22	29	59	66	+	35	26	62	gi|41327771
52K	37.6		2	11	10	9	+	5	5	10	gi|5174409

Table 1. *Continued*

			Exon Complex					B-like Complex			
Protein	Mol Mass (kDA)	A Comp	#1	#2	#3	#4	B Comp	#1	#2	#3	GenBank Accession Number
LSm Proteins											
LSm2	10.8		2	4	3	4	+	–	3	5	gi|10863977
LSm3	11.8		1	2	4	2	+	3	2	4	gi|7657315
LSm4	15.4		2	5	6	6	+	6	5	15	gi|6912486
LSm6	9.1		2	5	8	6	+	7	5	9	gi|5901998
LSm7	11.6		2	1	4	3	+	3	4	4	gi|7706423
LSm8	10.4		2	–	2	2	+	3	2	3	gi|7706425
U4/U6 snRNP											
hPrp3	77.6		12	21	42	48	+	24	19	57	gi|4758556
hPrp4	58.4		8	16	26	40	+	34	16	46	gi|24431950
CypH	20.0		10	10	14	10	+	15	5	18	gi|5454154
hPrp31	55.4		3	9	18	18	+	26	11	35	gi|40254869
15.5K	14.2		3	2	5	4	+	4	3	10	gi|4826860
U4/U6.U5 Tri-snRNP											
hSnu66	90.2		6	29	47	49	+	35	38	56	gi|10863889
hSad1	65.4		31	17	41	30	+	23	17	33	gi|56550051
27K	18.9		2	3	6	6		–	–	2	gi|24307919
hSnu23	23.6		–	–	–	–	+	–	1	8	gi|21389511
hPRP38	37.5		–	3	3	2	+	8	4	21	gi|24762236
B Complex Proteins											
MFAP1	51.9		1	–	4	2	+	11	9	24	gi|50726968
RED	65.6		1	4	11	10	+	18	12	32	gi|10835234
hSmu-1	57.5		2	4	17	15	+	29	22	51	gi|8922679
UBL5	8.5		–	–	–	–	+	3	2	5	gi|13236510
THRAP3	108.8		1	–	2	2		2	–	7	gi|55665950
hPRP19/CDC5L											
hPRP19	55.2	+	5	4	13	12	+	19	19	30	gi|7657381
CDC5L	92.2	+	4	4	6	3	+	10	14	33	gi|11067747
SPF27	21.5	+	1	5	2	–	+	4	3	12	gi|5031653
PRL1	57.2		1	1	3	–	+	6	6	20	gi|4505895
Hsp73	70.4	+	1	–	3	1		2	3	10	gi|5729877
AD-002	26.6	+	–	2	–	3	+	4	–	2	gi|7705475
CTNNBL1	65.1	+	–	–	2	–	+	1	–	5	gi|18644734
Npw38BP	70.0	+	–	–	–	–	+	14	11	24	gi|7706501
Npw38	30.5	+	–	–	–	–	+	3	2	3	gi|5031957
hPRP19/CDC5L Related											
hSyf1	100.0		5	1	1	–	+	–	–	2	gi|55770906
hSyf3	100.6		2	1	4	3	+	1	–	5	gi|30795220
hIsy1	33.0		2	–	1	1	+	1	–	2	gi|20149304
SKIP	51.1		–	–	3	3	+	7	3	14	gi|6912676
KIAA0560	171.3		7	1	2	2		1	–	5	gi|38788372
PPIase-like 1	18.2		1	–	–	–	+	2	–	1	gi|7706339
G10	17.0		–	–	–	–	+	1	–	3	gi|32171175

Proteins identified by LC-MS/MS after separation by PAGE in at least two of three preparations are shown. Note that only a subset of proteins identified are shown here, with the remainder shown in Table S1. Proteins not reproducibly detected are summarized in Table S2. Proteins generally accepted to be common contaminants, such as ribosomal proteins, are not shown. The presence of a protein is indicated by a number which represents the absolute number of peptides sequenced for that protein in a particular preparation (i.e., #1, #2, #3, or #4). A "+" represents the presence of a protein in purified human A (Behzadnia et al., 2007) or B (Deckert et al., 2006) complexes. Proteins are grouped in organizational and/or functional subgroups.

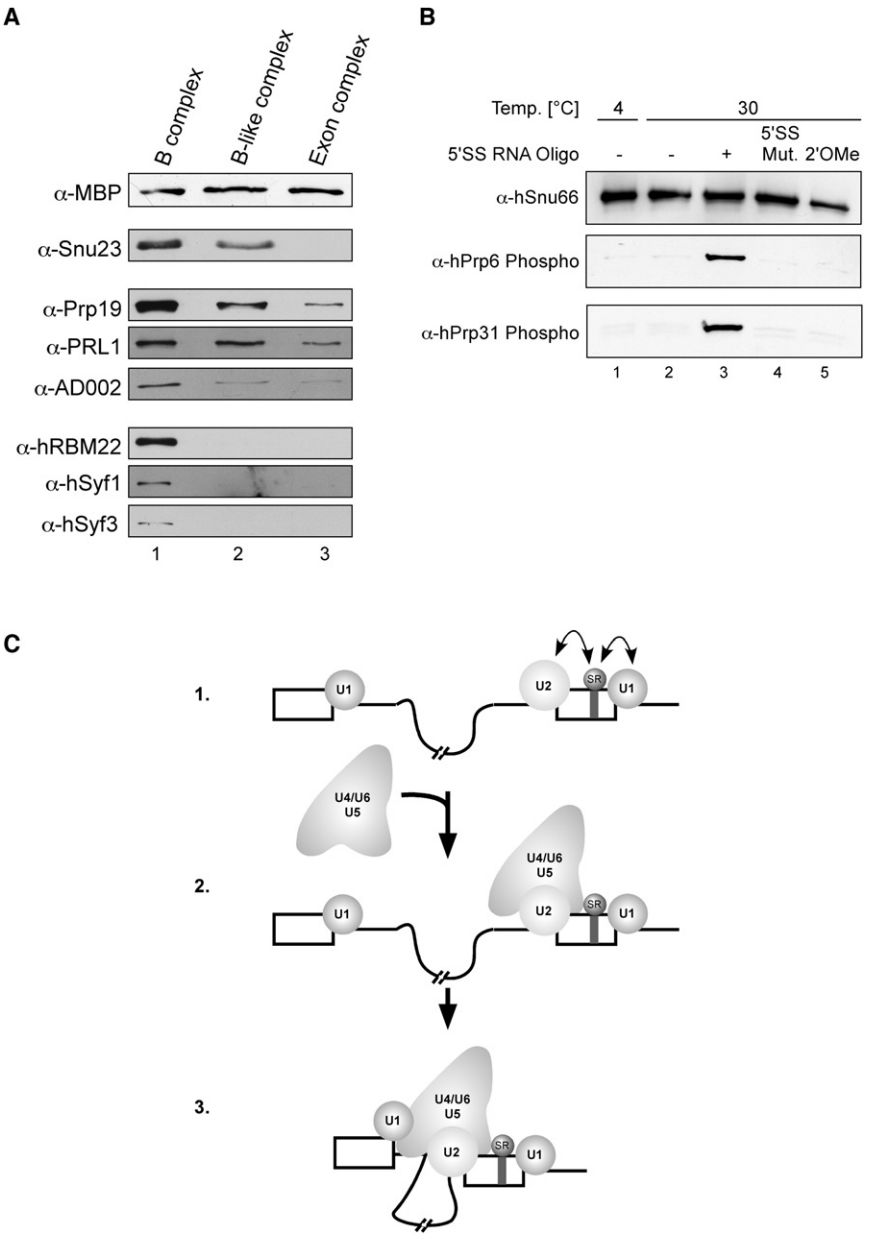

Figure 6. Composition of Exon and B-like Complexes and Phosphorylation Status of hPrp31 and hPrp6

(A) B, B-like, or exon complexes were purified and analyzed by western blotting with the indicated antibodies.

(B) Purified exon complexes containing tri-snRNP were incubated with ATP minus oligo (lanes 1 and 2), or plus 5′SS RNA oligo (lane 3), mutated 5′SS RNA oligo (lane 4), or 2′OMe oligo against the U6 ACAGAG box (lane 5). Western blotting was performed with the indicated antibodies.

(C) Model of exon complex formation and conversion of the exon-defined complex into a cross-intron complex, where the 5′SS and 3′SS are paired across the upstream intron.

contained solely U1 and U2 snRNPs (Sharma et al., 2008). However, in our hands, tri-snRNPs also interacted with BSEx4, as well as a β-globin exon RNA, indicating that their presence is a more general phenomenon. Addition of the 5′SS oligo to purified exon complexes stabilized tri-snRNP association and also triggered phosphorylation of the tri-snRNP proteins Prp6 and Prp31 (Figure 6), modification events that also accompany stable association of the tri-snRNP during cross-intron B complex formation (Schneider et al., 2010). Stabilization of tri-snRNP association also led to the recruitment of a subset of proteins specifically or predominantly associating first during the conversion of a cross-intron A complex to B complex. Finally, complexes formed on the MINX exon RNA catalyzed *trans*-splicing after addition of the 5′SS oligo. However, as the efficiency of *trans*-splicing was quite low, we cannot rule out that a small subpopulation of complexes, not related to the B-like complex, are responsible for this catalytic activity. Taken together, these results suggest that the tri-snRNP is associated in a functional manner with the exon complex. Whether the tri-snRNP associates concomittantly with the U1 and U2 snRNPs during exon complex formation is not clear. A 23S exon complex containing solely U1 and U2 is detected in glycerol gradients, consistent with the idea that it may be a precursor of the exon complex that additionally contains the tri-snRNP. However, additional experiments are needed to clarify this point.

Recruitment of the Tri-snRNP to the Exon Complex Occurs via the U2 snRNP

U2, as opposed to U1, plays a key role in recruiting the tri-snRNP to the exon complex. In complexes where U1 binding is unaltered but U2 no longer stably binds (i.e., MINX ΔBPS exon RNA or β-globin ΔESE RNA), tri-snRNP association is completely abolished (Figures 1 and 2). Psoralen crosslinking demonstrated that the U6 snRNA base pairs with U2 (forming U2/U6 helix II) within the cross-exon complex (Figure 3), confirming a direct interaction between U2 and a tri-snRNP subunit. Additional contacts involved in tri-snRNP docking are presently not known but likely include protein-protein interactions. Indeed, recruitment of the tri-snRNP to the cross-intron A complex is mediated by several proteins that are also found in purified cross-exon complexes, including SR proteins (Roscigno and Garcia-Blanco, 1995) and the U2-associated SPF30 protein (Meister et al., 2001).

The association of the tri-snRNP with purified exon complexes was stabilized upon addition of a 5′SS RNA that binds the ACAGAG box of the U6 snRNA. 5′SS binding thus appears

to induce a conformational/compositional change that leads to/enables stable tri-snRNP association with the U2 snRNP and possibly other components of the exon complex. Binding of the 5′SS RNA oligo induces phosphorylation of hPrp6 and hPrp31, and it is likely that this phosphorylation event stabilizes contacts between these tri-snRNP proteins and other components of A or exon complexes.

The Proteome of Exon and B-like Complexes

Exon complexes contained essentially all snRNP proteins, plus numerous non-snRNP spliceosomal proteins (Table 1 and Table S1). The protein composition of complexes formed on exon 4 of the *c-src* pre-mRNA, which contained solely the U1 and U2 snRNPs, was recently reported (Sharma et al., 2008). Nearly all of the proteins detected by MS in this cross-exon complex were also found in our complexes; thus these shared proteins appear to be "core" components of exon complexes. The vast majority of proteins detected in affinity-purified, cross-intron A complexes (Behzadnia et al., 2007) were also found in our cross-exon complexes. Unexpectedly, there did not appear to be any obvious candidates for proteins that associate solely with exon complexes.

A relatively small number of proteins are recruited to the exon complex upon its conversion to a B-like complex. These include MFAP1, RED, hSmu-1, and UBL5, which are also recruited during formation of the human cross-intron B complex. Homologs of these proteins are not found in the budding yeast *S. cerevisiae*, where alternative splicing events are essentially nonexistent. Thus, they may be involved in regulated splicing events in higher eukaryotes. Interestingly, UBL5 in *S. pombe* is required for splicing and interacts with Snu66, a component of the tri-snRNP (Wilkinson et al., 2004). Thus, this protein could potentially help recruit the tri-snRNP to the exon complex. hSnu23 was detected in the stabilized B-like complex, but not in the exon complex, and hPrp38 appeared to be more abundant in the former. hSnu23 and hPrp38 are present in the yeast but not human tri-snRNP (Gottschalk et al., 1999) but are also found in human cross-intron B complexes. Thus, they may also be involved in stabilizing the tri-snRNP interaction with the exon complex.

The protein compositions of the B-like and cross-intron B complex were very similar, but notable differences exist such as the absence or underrepresentation of components of the hPrp19 complex and Prp19-related proteins in the B-like complex (Table 1 and Figure 6). The less stable association of these proteins suggests that the B-like complex represents an earlier stage of spliceosome assembly compared to the B complex. Alternatively, the molecular organization of the B-like complex might generally differ somewhat from the B complex, as it was directly generated from a cross-exon complex, whereas the cross-intron B complex is generated from a cross-intron A complex. Although it is the product of a splicing reaction in which the 5′SS is added in *trans*, the B-like complex may be more representative of the majority of spliceosomes whose assembly is initiated by the formation of a cross-exon complex during exon definition.

Recent data from our lab demonstrate a crucial role for human Prp4 kinase during tri-snRNP integration into cross-intron B complexes (Schneider et al., 2010). The fact that hPrp6 and hPrp31 are phosphorylated when affinity-purified cross-exon complexes are incubated with the 5′SS RNA oligo further supports the idea that they are phosphorylated directly by hPrp4 kinase in B and B-like complexes, as this kinase is detected in both complexes (Table 1). This further suggests that base pairing of the U6 ACAGAG box with the 5′SS in some unknown way triggers this phosphorylation event.

Insights into the Conversion from Cross-Exon to Cross-Intron Complexes

Although pre-mRNA splicing normally involves splice sites present in *cis*, the *trans*-splicing system used here to generate B-like complexes nonetheless provides insights into a possible pathway for the conversion of cross-exon to cross-intron splicing complexes. Indeed, it is difficult to study this transition using pre-mRNA substrates in which exons are physically linked, as splicing snRNPs/factors will assemble simultaneously on different regions of the pre-mRNA. During exon definition, both ends of the exon are recognized in a coordinated manner, with factors binding the 3′SS (i.e., U2, U2AF) communicating (in many cases via SR protein-bound ESEs) with factors bound to the downstream 5′SS (i.e., U1) (Berget, 1995; Robberson et al., 1990). In current models, the next assembly step entails that contacts across an exon be broken to allow new contacts across the intron between the 3′SS (bound by U2) and upstream 5′SS (bound initially by U1) (Robberson et al., 1990). Afterwards, the tri-snRNP is proposed to bind the cross-intron A complex, forming the B complex. Subsequently, U1 is displaced from the 5′SS by the mutually exclusive base-pairing interaction of U6 with the 5′SS. In the *trans*-splicing system used here, the 5′SS is directly bound by U6, as excess 5′SS oligo sequesters the U1 snRNP in the extract. Thus, in our *trans*-splicing system, U6 can apparently substitute for U1 in establishing cross-intron interactions and also in pairing the 5′SS and 3′SS, indicating that splice site pairing can potentially occur downstream of cross-intron A complex formation.

Our data demonstrate that the tri-snRNP associates already at the time of exon definition. Thus, the establishment of interactions between U2 and U1 bound to an upstream 5′SS (i.e., across the intron) are not a prerequisite for or must temporally precede tri-snRNP binding. Our data also indicate that the tri-snRNP can engage the 5′SS as part of the exon complex and that this in turn leads to the stable, apparently productive integration of the tri-snRNP (i.e., B complex formation) (Figure 6C). They further suggest that a cross-exon complex can be directly converted to a cross-intron B complex in which the splice sites are paired, and that the switch from a cross-exon complex to a cross-intron complex in which an upstream 5′SS is paired with a downstream 3′SS can occur without major rearrangement of the "early" tri-snRNP interactions that are formed in the exon-defined complex. Indeed, initial structure probing studies comparing cross-exon complexes with the 5′SS oligo-induced B-like complexes suggest that this transition does not involve large changes in RNP conformation (Figure S2 and data not shown). In contrast to previous models, the cross-exon to cross-intron transition may not necessarily require the disruption of cross-exon interactions between U2 and U1 at the downstream 5′SS.

Rather, both cross-intron and cross-exon bridging interactions could theoretically be maintained.

Although the data provided here support the assembly pathway depicted in Figure 6C, they do not exclude alternative pathways for the steps occurring subsequent to exon definition. Indeed, in light of the lower cellular levels of U4, U5, and U6 relative to U1 and U2, functionally active exon complexes containing U1 and U2, but lacking tri-snRNPs, may also potentially form. Indeed, tri-snRNPs can also associate already with cross-intron A complexes formed on single intron pre-mRNAs (Maroney et al., 2000), but functional cross-intron A complexes can nonetheless form in the absence of tri-snRNPs, indicating the existence of alternative assembly pathways for the cross-intron A complex (Behzadnia et al., 2006).

Implications for Regulated Splicing Events

The proposed direct conversion of an exon complex to a cross-intron B complex in which the splice sites are paired has important implications for regulated splicing events. Recently, it was shown that regulation can occur at the time of stable tri-snRNP addition after exon definition. A block in the transition from cross-exon to cross-intron B complexes appears to be mediated by the RBM5 protein, which represses the inclusion of exon 6 of the apoptotic factor FAS pre-mRNA (Bonnal et al., 2008), by PTB, which represses the inclusion of the neuron-specific N1 exon of the c-src pre-mRNA (Sharma et al., 2008), and by hnRNP L, which prevents inclusion of exon 4 of the CD45 gene (House and Lynch, 2006). In light of our results showing that tri-snRNPs associate with exon complexes, it is possible that in some of these cases, the specific regulatory protein involved blocks the association of the tri-snRNP with the exon complex and that this leads to a block in subsequent assembly steps. Taken together, our data suggest an alternative pathway for spliceosome assembly after initial exon definition.

EXPERIMENTAL PROCEDURES

In Vitro Transcription

Templates for the MINX and β-globin exon RNAs were generated by PCR from pMINX (Zillmann et al., 1988) and the pspDupS4 series (Labourier et al., 1999), respectively. Mutations were introduced via PCR and confirmed by sequencing. For detailed sequence information, see Figure S1. Transcription of uniformly ^{32}P-labeled RNA was performed in vitro (Deckert et al., 2006), except that m^{7}G (5′) ppp (5′) G cap was omitted.

In Vitro Splicing Assays

HeLa nuclear extract was prepared according to Dignam et al. (1983). In vitro splicing reactions contained 40% nuclear extract, 8 mM HEPES-KOH (pH 7.9), 8% (v/v) glycerol, 0.08 mM EDTA, 0.4 mM DTE, 0.2 mM PMSF, 3 mM $MgCl_2$, 65mM KCl, 2 mM ATP, 20 mM creatine phosphate, and 10 nM of ^{32}P-labeled pre-mRNA or exon RNA. After 2.5 min, formation of B-like complexes was induced by addition of 1 μM (final concentration) RNA oligo containing a 5′SS (CUGUUCAGGUAAGUAU, exonic sequence underlined), and incubation was continued for 2.5 min at 30°C (Ast et al., 2001). Spliceosomal complexes were analyzed on agarose gels (Behzadnia et al., 2006) after adding heparin (final concentration of 0.1 μg/μl). For *trans*-splicing assays, 100 nM of exon RNA were incubated for 2.5 min at 30°C before the addition of ^{32}P-pCp-3′-end-labeled 5′SS RNA oligo. Reactions were further incubated for 60 min at 30°C. RNase H digestion was performed for 10 min at 30°C with an oligo against the 3′ end of the exon (TCGAAAGACCGC) or a control oligo (GTCTTGTAAGCT).

MS2 Affinity Purification and Psoralen Crosslinking

Exon and B-like complexes were purified via MS2 affinity selection as described previously (Deckert et al., 2006), with the following modifications: the final RNA concentration was 10 nM; splicing reactions were incubated for 2.5 min at 30°C; and in the case of the B-like complex, a 5′SS-containing RNA oligo was added and splicing was continued for an additional 2.5 min. In vitro splicing reactions were loaded onto linear 10%–30% (v/v) glycerol gradients containing G65 buffer (20 mM HEPES-KOH [pH 7.9], 65 mM NaCl, 1.5 mM $MgCl_2$), and ultracentrifugation was performed for 130 min at 374,000 × g in a Sorvall TH660 rotor at 4°C. The distribution of ^{32}P-labeled RNA was determined by Cherenkov counting, and complexes were further purified by affinity selection on an amylose Sepharose column. Unless noted otherwise, all purification steps were performed at 65 mM NaCl. RNA was recovered from the purified complexes, analyzed by denaturing PAGE, and visualized by silver staining. The stoichiometry of the snRNAs and exon RNA was determined by visual inspection of the silver-stained bands. Psoralen crosslinking of purified exon complexes, northern blotting, and generation of ^{32}P-labeled DNA probes were as described (Behzadnia et al., 2007).

5′SS RNA Oligo-Induced Stabilization of Purified Exon Complexes

Exon complexes containing the tri-snRNP were purified via MS2 affinity selection as described above. Complexes bound to amylose beads were washed with G65 buffer. Subsequently, 2 column volumes of G65 buffer containing the 5′SS RNA oligo or a mutant version thereof (end concentration 10 μM) were added, followed by incubation for 5 min at 4°C. Samples were then washed with G300 (G65 buffer with 300 mM NaCl) buffer, as indicated. Complexes were eluted and their RNA analyzed as described above.

Mass Spectrometry

Proteins were recovered from purified exon or B-like complexes, separated by PAGE, and entire lanes of the Coomassie-stained gels cut into 25 slices of equal dimensions, irrespective of the visual presence or absence of a protein band. MS was performed after digestion of proteins in-gel, and peptides were analyzed on an LC-coupled ESI Q-ToF (Waters) mass spectrometer and proteins identified as described (Deckert et al., 2006).

Immunoblots

Exon and B-like complexes were purified as described above. Protein (1 pmol) was recovered and separated on a 10%/13% SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed with the indicated antibodies using an ECL detection kit (Pierce).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, two tables, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2010.02.027.

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