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The 5′ End of U2 snRNA Is in Close Proximity to U1 and Functional Sites of the Pre-mRNA in Early Spliceosomal Complexes

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SUMMARY

Recognition and pairing of the correct 5′ and 3′ splice sites (ss) of a pre-mRNA are critical events that occur early during spliceosome assembly. Little is known about the spatial organization in early spliceosomal complexes of the U1 and U2 snRNPs, which together with several non-snRNP proteins, are involved in juxtapositioning the functional sites of the pre-mRNA. To better understand the molecular mechanisms of splice-site recognition/pairing, we have examined the organization of U2 relative to U1 and pre-mRNA in spliceosomal complexes via hydroxyl-radical probing with Fe-BABE-tethered U2 snRNA. These studies reveal that functional sites of the pre-mRNA are located close to the 5′ end of U2 both in E and A complexes. U2 is also positioned close to U1 in a defined orientation already in the E complex, and their relative spatial organization remains largely unchanged during the E to A transition.

INTRODUCTION

Most introns are removed from nuclear pre-mRNAs by the U2-type spliceosome, which consists of the U1, U2, U4/U6, U5 snRNPs and a large number of non-snRNP proteins (Will and Lührmann, 2006). The two-step splicing reaction begins with a nucleophilic attack by the pre-mRNA's branchpoint (BP) adenosine at the 5′ splice site (ss), generating free 5′ exon and intron-3′ exon in the form of a lariat. In the second step, the 3′ OH of the 5′ exon carries out a nucleophilic attack at the 3′ss, and concomitantly, the intron is released and the 5′ and 3′ exons are ligated together. Splice-site recognition and pairing, as well as the juxtaposing of the reactive sites of the pre-mRNA, are mediated by multiple interactions between short conserved sequences of the pre-mRNA—the 5′ and 3′ss, BP sequence (BPS), and polypyrimidine tract—and components of the spliceosome during its stepwise assembly. The decision process for juxtaposing the correct 5′ and 3′ss, i.e., commitment to splicing, has been shown to occur already in the E complex (Das et al., 2000; Michaud and Reed, 1993), the earliest detectable spliceosomal complex. The E complex forms by the ATP-independent association of the U1 snRNP with the 5′ss. It also contains the U2 snRNP and members of the SR protein family, as well as additional non-snRNP factors such as SF1/mBBP, U2AF65, and U2AF35, which bind the BPS, downstream polypyrimidine tract, and 3′ss AG dinucleotide, respectively (Abovich and Rosbash, 1997; Reed, 2000; Staknis and Reed, 1994). The latter, together with SR and U1 snRNP proteins and possibly FBP11 (the metazoan counterpart of Prp40p), are involved in crossintron protein-protein interactions that bridge the 5′ and 3′ ends of the intron (Abovich and Rosbash, 1997; Kao and Siliciano, 1996). Although the U2 snRNP is loosely associated at this stage, E complex formation is dependent on its presence (Das et al., 2000; Dönmez et al., 2004).

In the subsequently formed A complex, the U2 snRNP stably interacts with the BPS in an ATP-dependent manner. This interaction involves base pairing of the U2 snRNA with the BPS and multiple contacts between components of the U2-associated SF3a/b heteromeric protein complexes, and both the BPS and so-called anchoring site (AS, located 6–25 nucleotides (nt) upstream of the BP; [Gozani et al., 1996]). The U2/BPS interaction results in the bulging out of the BP adenosine, thereby specifying it as the nucleophile for the first catalytic step of splicing (Query et al., 1996). After these early events, the U4/U6.U5 tri-snRNP is recruited together with a host of additional factors, resulting in the B complex (Deckert et al., 2006; Will and Lührmann, 2006). An intricate series of RNA-RNA rearrangements and remodeling events lead to catalytic activation of the spliceosome, and the catalytic steps of splicing ensue. During activation, the U6 snRNA engages in base pairing interactions with the 5′ end of the U2 snRNA, and the resulting U2/U6 snRNA structure is thought to form the catalytic center of the spliceosome and thus be directly involved in catalysis (Nilsen, 2002; Staley and Guthrie, 1998).

The U2 snRNP appears to play a central role in splice-site recognition/pairing during the early stages of the splicing process. However, relatively little is currently known about the 3D structural organization of early spliceosomal complexes, in particular the organization of U2 relative to the functional sites of the pre-mRNA and to the U1 snRNP.

Hydroxyl-radical probing with Fe-BABE-tethered pre-mRNA recently revealed that the BP and the 3′ ss are located within ~20 Å of the 5′ss in the E complex (Kent and MacMillan, 2002). However, the structural organization of the main subunits of the E and A complexes (i.e., U1 and U2) remains unknown.

To learn more about the 3D architecture of early spliceosomal complexes and thereby enhance our limited understanding of the molecular mechanisms of splice-site pairing, we have attached the hydroxyl-radical probe Fe-BABE to the 5′ end of the U2 snRNA and mapped regions of the pre-mRNA and U1 snRNA that are in close proximity to U2 both in E and A spliceosomal complexes. In E complexes, hydroxyl-radical-cleaved nucleotides were detected in the polypyrimidine tract/3′ss, AS, 5′ss, and 5′ exon of the pre-mRNA, demonstrating that these regions are located near the functionally important 5′ end of U2 and also to one another. The U1 snRNA was also cleaved, and mapping of these cleavages onto the U1 snRNA and U1 snRNP 3D structures revealed a distinct orientation of U2 relative to U1. Thus, already in the E complex, the U2 and U1 snRNPs are in close proximity and "bridge" the 5′ and 3′ end of the intron. Similar cleavage patterns were observed in A complexes, indicating that the spatial organization of U1 relative to U2 remains largely unchanged during A complex formation.

RESULTS

Generation and Characterization of BABE-Modified U2 snRNPs

Hydroxyl-radical probing has been successfully used to investigate RNA neighborhoods in ribosomes, by tethering Fe-BABE to RNA or protein (Culver and Noller, 2000; Joseph and Noller, 2000), or more recently, in spliceosomes, by attaching Fe-BABE to the pre-mRNA (Kent and MacMillan, 2002; Rhode et al., 2006). Hydroxyl radicals will cleave the phoshodiester backbone of nucleic acids within ~20 Å of their site of generation (Han and Dervan, 1994; Wang and Cech, 1992) and, thus, are useful for studying local RNA structure. To investigate the 3D architecture and dynamic organization of early spliceosomal complexes, we probed RNA proximities by site-directed generation of hydroxyl radicals from Fe-BABE covalently attached to the U2 snRNA. As the m_3G cap of U2 snRNA was found to be dispensable for splicing (Dönmez et al., 2004), we targeted Fe-BABE to the 5′ terminal nucleotide. To this end, a six-carbon spacer containing a sulfhydryl group was inserted during the chemical synthesis of an oligomer that comprises the 5′ end of U2 snRNA (i.e., nt 1–24) and contains all natural modifications required for U2 function (Figure 1A, see also Dönmez et al. [2004]). The oligomer was first derivatized with Fe-BABE (~40% yield, Figure 1B). The reaction product was then purified and ligated to in vitro-transcribed RNA comprising the remaining portion of U2 (Figure 1A). U2 snRNPs were then reconstituted in vitro first by incubating with purified snRNP proteins (to form 12S particles) and then in nuclear extract specifically depleted of 12S U2 snRNPs to form the functional 17S form (Dönmez et al., 2004). In vitro reconstitution of 17S U2 snRNPs with 5′ Fe-BABE U2 snRNA was as efficient as with nonderivatized U2 snRNA; in both cases, ~80% of the U2 snRNA was converted into a 17S particle.

We first investigated whether in vitro-reconstituted 5′ Fe-BABE U2 snRNPs supported spliceosomal complex formation and splicing. E complexes did not form on MINX pre-mRNA in U2-depleted (ΔU2) extract (Figure 1C, lane 1). However, when 12S U2 snRNPs containing Fe-BABE were added to the ΔU2 extract (and 17S U2 snRNPs were allowed to form), E complex formation was observed with an efficiency comparable to that of native nuclear extract (Figure 1C, lanes 3 and 4) after incubating for 10 min under splicing conditions (but in the absence of ATP). Fe-BABE U2 snRNPs also supported A and B complex formation in the presence of ATP as efficiently as in vitro-reconstituted U2 snRNPs lacking BABE (Figure 1D, cf. lanes 6–10 and 11–15); the efficiency and kinetics of complex formation in both cases were comparable to that observed with native nuclear extract (Figure 1D, lanes 1–5). Finally, whereas no splicing was observed with ΔU2 extract alone or when complemented with U2 snRNPs reconstituted from U2 snRNA lacking natural modifications (Figure 1E, lanes 2 and 4), MINX pre-mRNA was spliced at wild-type levels (lane 1) upon addition of U2 snRNPs reconstituted from either purified HeLa U2 snRNA (containing all modifications) or chimeric U2 snRNA with natural modifications within nt 1–24 (lanes 3 and 5). 5′ Fe-BABE U2 snRNPs also complemented splicing (lane 7), albeit at a reduced efficiency (~45% when compared to reconstituted U2 snRNPs lacking BABE, lane 5). This reduction is probably due to the bulky Fe-BABE group, as the six-carbon spacer with the sulfhydryl group alone did not inhibit splicing (cf. lanes 5 and 6). In subsequent experiments, the latter RNA was used as a "minus BABE" control.

Site-Directed Hydroxyl-Radical Probing of Pre-mRNA in E Complexes

We next investigated proximities of the 5′ end of U2 snRNA relative to the pre-mRNA in the E complex. E complex assembly was performed with U2 snRNA lacking or bearing Fe-BABE at its 5′ end, and hydroxyl-radical production was subsequently initiated by addition of H_2O_2. Primer extension was then carried out with primers complementary to the intron (nt 83–100, Figure 2B) or exon 2 (nt 199–215, Figure 2A) of the pre-mRNA (see also Figure 2D for summary of cleavages). Bands observed in lane 4 ("+BABE+H_2O_2") that are missing or less intense in the control lanes 1–3 arise from cleavage of the pre-mRNA by hydroxyl radicals generated by Fe-BABE and indicate that the cleaved nucleotide is in proximity to the 5′ end of U2.

Cleavage intensities were quantified in this and all subsequent experiments, as explained here for a portion of the gel shown in Figure 2B (i.e., nt 40–61). The intensity

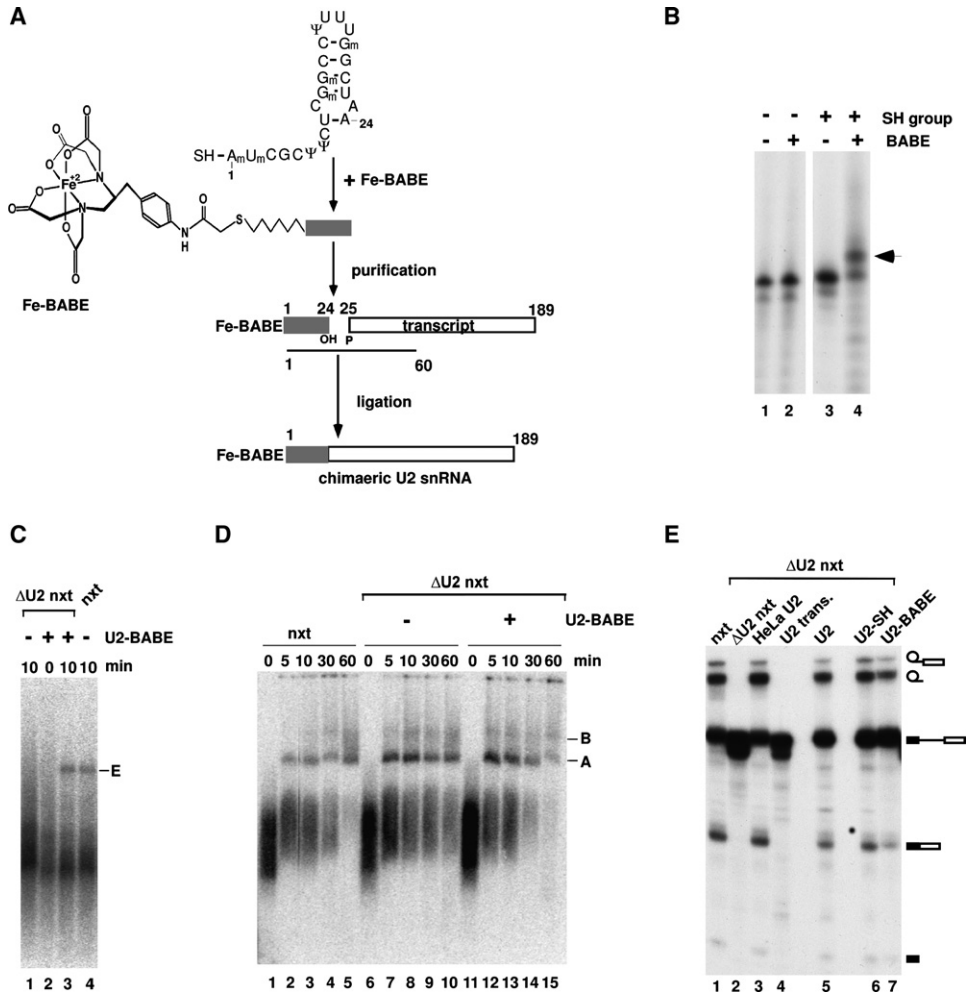

Figure 1. Synthesis and Function of Fe-BABE-Modified Human U2 snRNA

(A) An oligoribonucleotide corresponding to the first 24 nt of U2 snRNA with an SH group at its 5′ end was chemically synthesized and derivatized with Fe-BABE. After purification, the modified oligonucleotide was ligated to in vitro-transcribed U2 snRNA comprising the remaining nt 25–189.

(B) ^{32}P-labeled synthetic oligoribonucleotides with (lanes 3 and 4) or without (lanes 1 and 2) the 5′ SH group were derivatized with Fe-BABE (lanes 2 and 4) and analyzed by denaturing PAGE. The arrow (right) shows the Fe-BABE-modified RNA product.

(C–E) Fe-BABE-modified, chimeric U2 snRNA supports the formation of spliceosomal E, A, and B complexes and pre-mRNA splicing. (C) E complex formation in untreated HeLa nuclear extract (lane 4) and U2-depleted extract (ΔU2) either alone (lane 1) or complemented with in vitro-reconstituted U2 snRNPs containing Fe-BABE-modified U2 snRNA (lanes 2 and 3) after incubating in the absence of ATP for the indicated times. Complexes were analyzed on a 1.5% low-melting agarose gel. (D) Time course of splicing complex formation in untreated extract (lanes 1–5) and ΔU2 extract complemented with U2 snRNPs containing either non-BABE-modified (lanes 6–10) or BABE-modified, chimeric U2 snRNA (lanes 11–15). Complex formation was analyzed as above, and the positions of the A and B complexes are indicated on the right. (E) In vitro splicing with untreated extract (lane 1) and ΔU2 extract alone (lane 2) or complemented with U2 snRNPs containing endogenous HeLa or in vitro-transcribed U2 snRNA (lanes 3 and 4), or either unmodified (lanes 5 and 6) or Fe-BABE-modified (lane 7) chimeric U2 snRNA. Splicing intermediates/products (shown schematically on the right) were analyzed by denaturing PAGE.

of the bands in the +BABE+H_2O_2 and −BABE−H_2O_2 lanes was determined by densitometric scanning (Figure 2C, blue and green lines), and the difference in intensity of each band was subsequently calculated (Figure 2C, red line). Nucleotides where this difference amounted to 50 or more arbitrary intensity units were considered to be cleaved and further classified, essentially according to Joseph et al. (2000), as weak (50–99 units; e.g., C42 and C43 in Figure 2B), medium (100–149 units; e.g., C57), or strong (150 units and up; e.g., C50, C52, and C53) cleavages. Experiments were repeated three to four times, and the error of the mean cleavage intensity values was generally 5%–10%.

As a control, we also performed hydroxyl-radical probing with 5′ Fe-BABE U2 snRNA lacking internal modifications, which therefore cannot be incorporated into the E complex (Dönmez et al., 2004). Only three H_2O_2-induced cleavages above background (as determined by

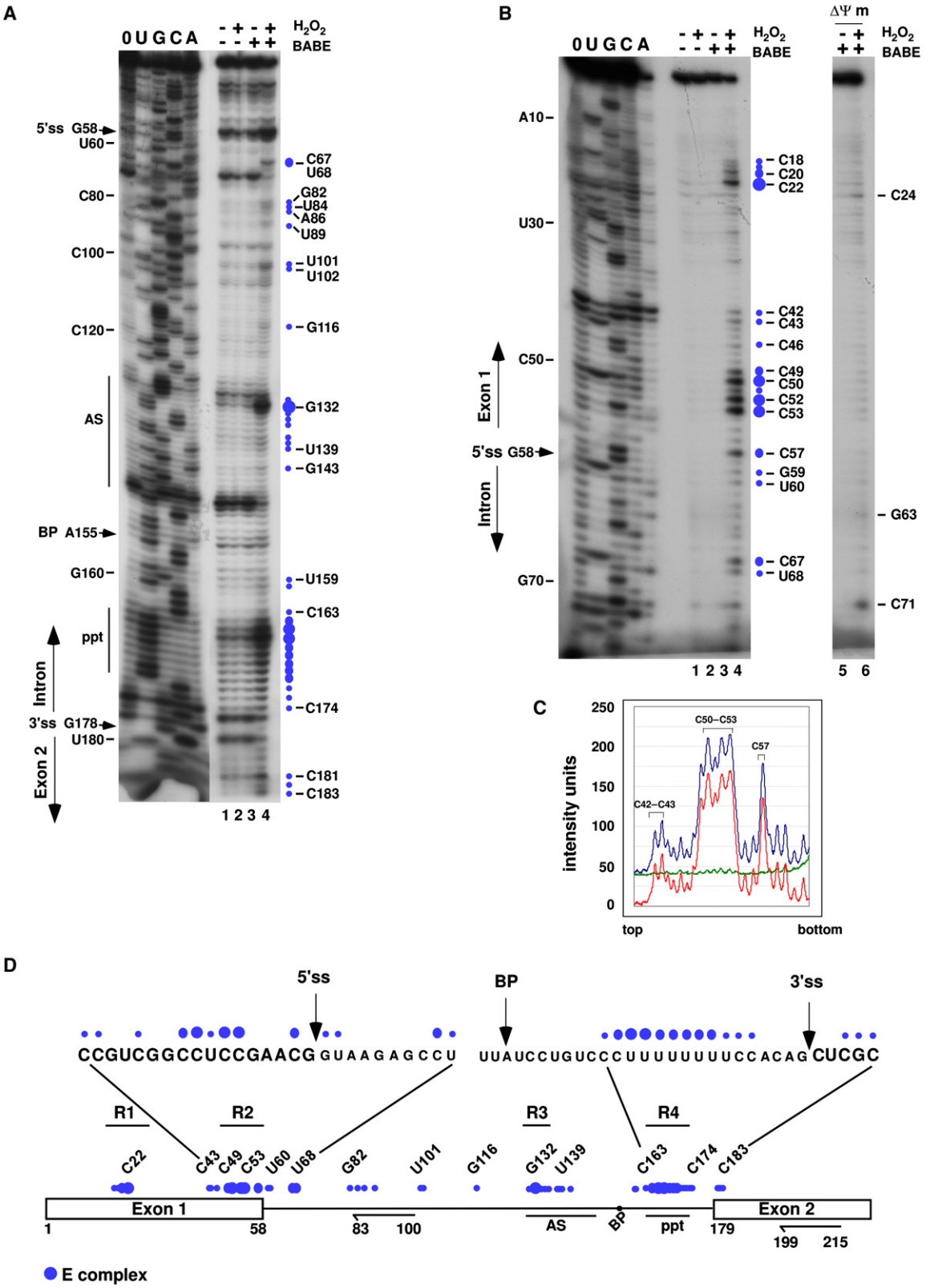

Figure 2. Site-Directed Hydroxyl-Radical Probing of Pre-mRNA in E Complexes

(A) Cleavage sites on the 3′ half of the pre-mRNA were determined by primer extension with a primer complementary to nt 199–215 of exon 2. Hydroxyl-radical-cleaved nucleotides are indicated by blue dots on the right. The size of the dots reflects the intensity of the cleavages, which is

densitometric scanning) were observed (Figure 2B, cf. lanes 5 and 6; data not shown), demonstrating that nearly all of the cleavages observed in lane 4 are not due to "free" Fe-BABE U2 snRNPs in the reaction mixture. In addition, we performed hydroxyl-radical probing with MINX pre-mRNA lacking the BPS (ΔU150-C157), which can undergo E, but not A, complex formation (Das et al., 2000). Significantly, a nearly identical cleavage pattern was observed with this mutant (Figure S1 in the Supplemental Data available with this article online), confirming that the cleavages detected in lane 4 solely arise from pre-mRNA present in E complexes.

Strong cleavages were observed in four main regions of the pre-mRNA (Figure 2D, R1–R4), including a portion of the polypyrimidine tract (R4) and the so-called AS located upstream of the BPS (R3). In addition, weak cleavages were detected at the 3′ end of the polypyrimidine tract and in exon 2 (Figure 2A). Strong cleavages were also found close to the 5′ss (C50, C52, and C53) near the 3′ end of exon 1 (R2) and less intense cleavages both 5′ and 3′ of R2 (Figure 2B). An additional strong cleavage (R1) was observed in exon 1 (C22) (Figure 2B). Scattered weak cleavages were also detected in the central part of the intron (Figure 2D). Taken together, our data demonstrate that regions bordering both the 5′ and 3′ss, as well as the polypyrimidine tract and AS of the pre-mRNA, are near the 5′ end of U2 snRNA already in the E complex.

To determine whether the observed proximities are dependent on the U1 snRNP, we prevented U1 binding by deleting the 5′ss (i.e., ΔA56-G65) of the pre-mRNA. This Δ5′ss pre-mRNA was completely inactive in E complex formation, and no pre-mRNA cleavages were detected when site-directed hydroxyl-radical probing was performed (data not shown). Thus, U1 snRNP bound to the 5′ss is essential for the functional interaction of U2 snRNP with the pre-mRNA during E complex formation.

Site-Directed Hydroxyl-Radical Probing of Pre-mRNA in the A Complex

To determine whether the spatial organization of U2 relative to the pre-mRNA changes during the E to A transition, we performed hydroxyl-radical probing with A complexes containing 5′ Fe-BABE U2 snRNA. Splicing was performed in the presence of ATP and a 2′-O-Me oligonucleotide complementary to the ACAGAG box of U6 snRNA (i.e., nt 27–46). The latter blocks tri-snRNP integration, thereby inhibiting B complex formation and enhancing the accumulation of A complexes (Frilander and Steitz, 1999). Primer-extension analyses (Figure 3) revealed cleavages in the pre-mRNA's polypyrimidine tract, regions surrounding the 5′ and 3′ss, and in the AS. Thus, the global distribution of cleavages was similar to that observed in the E complex (see Figure 3C for comparison). However, several notable differences can be discerned. For example, cleavages in the polypyrimidine tract were shifted upstream, and novel cleavages were detected in the BPS, including the BP adenosine at position 155. Furthermore, cleavages around the 3′ss (A177 to C183) were enhanced. Most strikingly, cleavages near the middle of exon 1 (region R1 and C18–C22, Figure 2D) were completely lost, whereas those at the 3′ end of exon 1 were significantly reduced. Conversely, cleavages at the 5′ end of the intron were enhanced in the A complex (e.g., A64 and C67). The loss of cleavages in the R1 region indicates that they are E complex specific and further demonstrates that the splicing conditions used here have led to the formation of A complexes that are devoid of E complexes. In contrast to the aforementioned differences, cleavages in the anchoring sequence did not change significantly.

Taken together, these data reveal that the global organization of functionally important RNA elements is similar in the E and A complex: regions bordering both splice sites, the polypyrimidine tract, and the AS are close to the 5′ end of the U2 snRNA. On the other hand, they suggest that during A complex formation either (1) the 5′ end of U2 has moved relative to the pre-mRNA's BPS and 5′ exon or that (2) a rearrangement and/or the addition of other spliceosomal factors has led to a differential accessibility of these regions to hydroxyl radicals.

Proximity of the 5′ End of U2 snRNA to U1 snRNA in Early Spliceosomal Complexes

The observed cleavages in the vicinity of the 5′ss prompted us to examine whether the 5′ end of the U2 snRNA is also in close proximity to the U1 snRNP. We thus performed hydroxyl-radical footprinting with E and A complexes containing 5′ Fe-BABE U2 snRNA, as described above (see Figures 2 and 3), and analyzed the U1 snRNA for hydroxyl-radical-induced cleavages via primer extension (Figures 4A–4D; see also Figure 5A for summary). Strong cleavages were observed in stem I (nt 39, 41,

classified as strong, medium, or weak. 0, input RNA; U, G, C, A, dideoxy sequencing reactions of input RNA. Spontaneous reverse transcriptase stops or cleavages due to nuclease activity other than Fe-BABE in the absence (lane 1) or presence of Fe-BABE (lane 3). Lane 2, background cleavage of pre-mRNA in the absence of Fe-BABE U2 after addition of H_2O_2. Lane 4, hydroxyl-radical cleavages in the presence of Fe-BABE U2 after addition of H_2O_2. Abbreviations: ss, splice site; BP, branchpoint; AS, anchoring site; and ppt, polypyrimidine tract.

(B) Analysis of hydroxyl-radical cleavages in the 5′ half of the pre-mRNA with a primer complementary to nt 83–100 of the intron. Lane designations are as in (A). Lane 6, hydroxyl-radical cleavage of pre-mRNA in the presence of "free" Fe-BABE U2 snRNPs not incorporated into the E complex due to the lack of natural modifications in U2 nt 1–24 (ΔΨm) and corresponding background cleavage control (lane 5).

(C) Quantification of cleavage intensities in a portion of the gel from (B) by densitometric scanning (see text for detailed description). The band intensities of the +BABE+H_2O_2 lane (blue line), −BABE−H_2O_2 lane (green line), and the difference in their intensities (red line) are shown.

(D) Summary of the cleaved nucleotides on the pre-mRNA in the E complex. R1, R2, R3, and R4 indicate regions where strong cleavages are observed. Note that due to the major cleavage at position 132 (which leads to a lower amount of cDNA that encompasses the 5′ end of the pre-mRNA), cleavages at nt 60–67 are much less prominent in (A) than in (B).

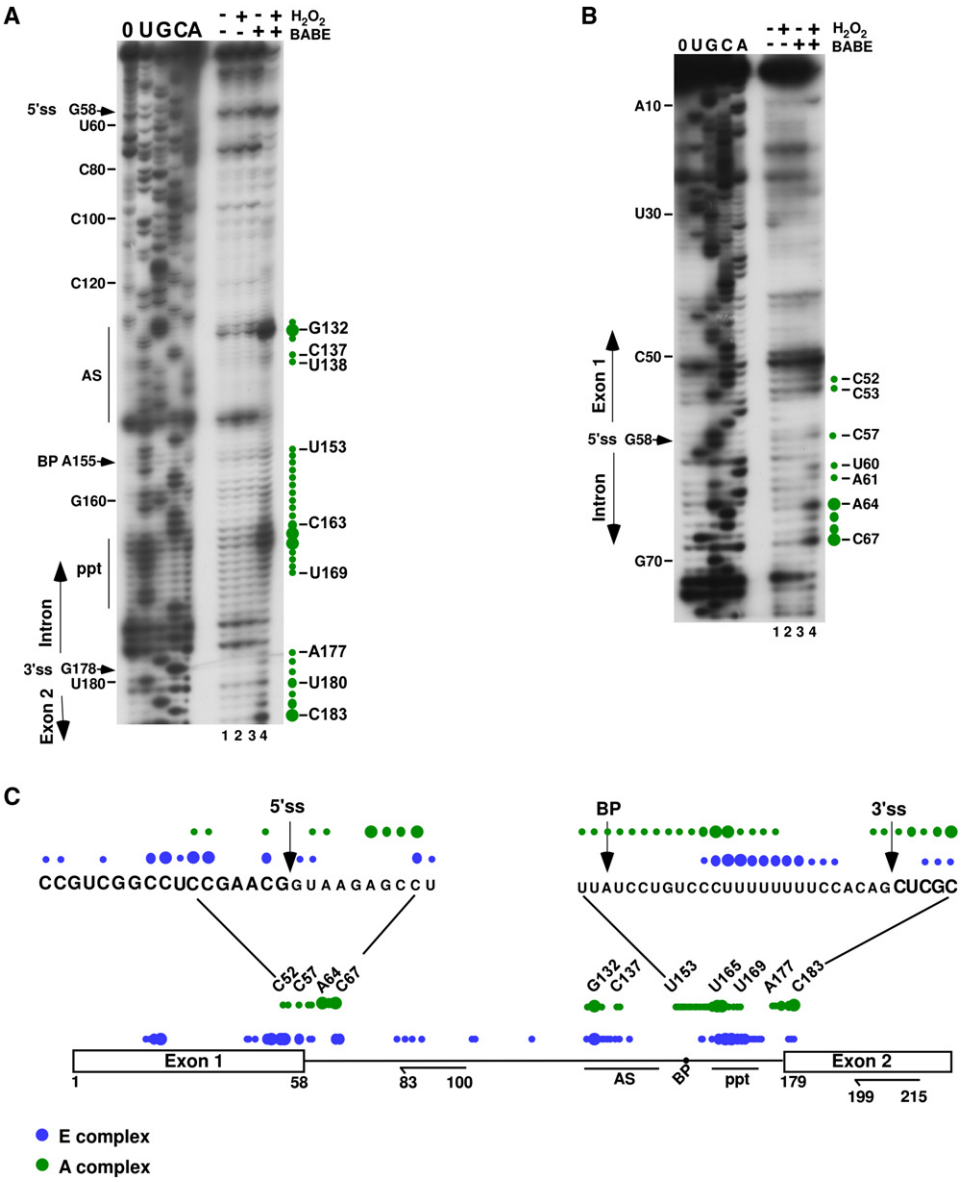

Figure 3. Site-Directed Hydroxyl-Radical Probing of Pre-mRNA in A Complexes

(A and B) Hydroxyl-radical cleavages on the 3′ half (A) (primer complementary to nt 199–215) or 5′ half (B) (primer complementary to nt 83–100) of the pre-mRNA. Lane designations are as in Figure 2. Cleaved nucleotides are indicated by green dots.

(C) Summary of cleaved nucleotides on the pre-mRNA in the E and A complex.

and 42) and stem-loop III (nt 100, 101, and 110). Moderate cleavages were found in the upper part of stem II (nt 73–77) and the internal loop of stem II (nt 52–56), whereas weak cleavages were detected at the 5′ end of U1, which base pairs with the 5′ss, and also at a limited number of nucleotides in stem I, stem-loop II, and stem-loop III. Significantly, no U1 cleavages above background were detected when the pre-mRNA was omitted (Figure 4E) or if MINX pre-mRNA lacking the 5′ss was used (data not shown), confirming that the observed cleavages are dependent on splicing complex formation. These data indicate that the 5′ end of the U2 snRNA is indeed in close proximity to the U1 snRNP, already at the time of E complex formation. Interestingly, the cleavage pattern did not differ dramatically between the E and A complex (Figures 4A–4D and 5A), indicating that the close spatial relationship between the U1 snRNP and the 5′ end of the U2 snRNA does not change significantly during the E to A complex transition. Most of the limited number of changes in the U1 cleavage pattern were concentrated in the four-way junction and stem-loop III, as well as helix H, to which the 5′ss interacting sequence is directly connected.

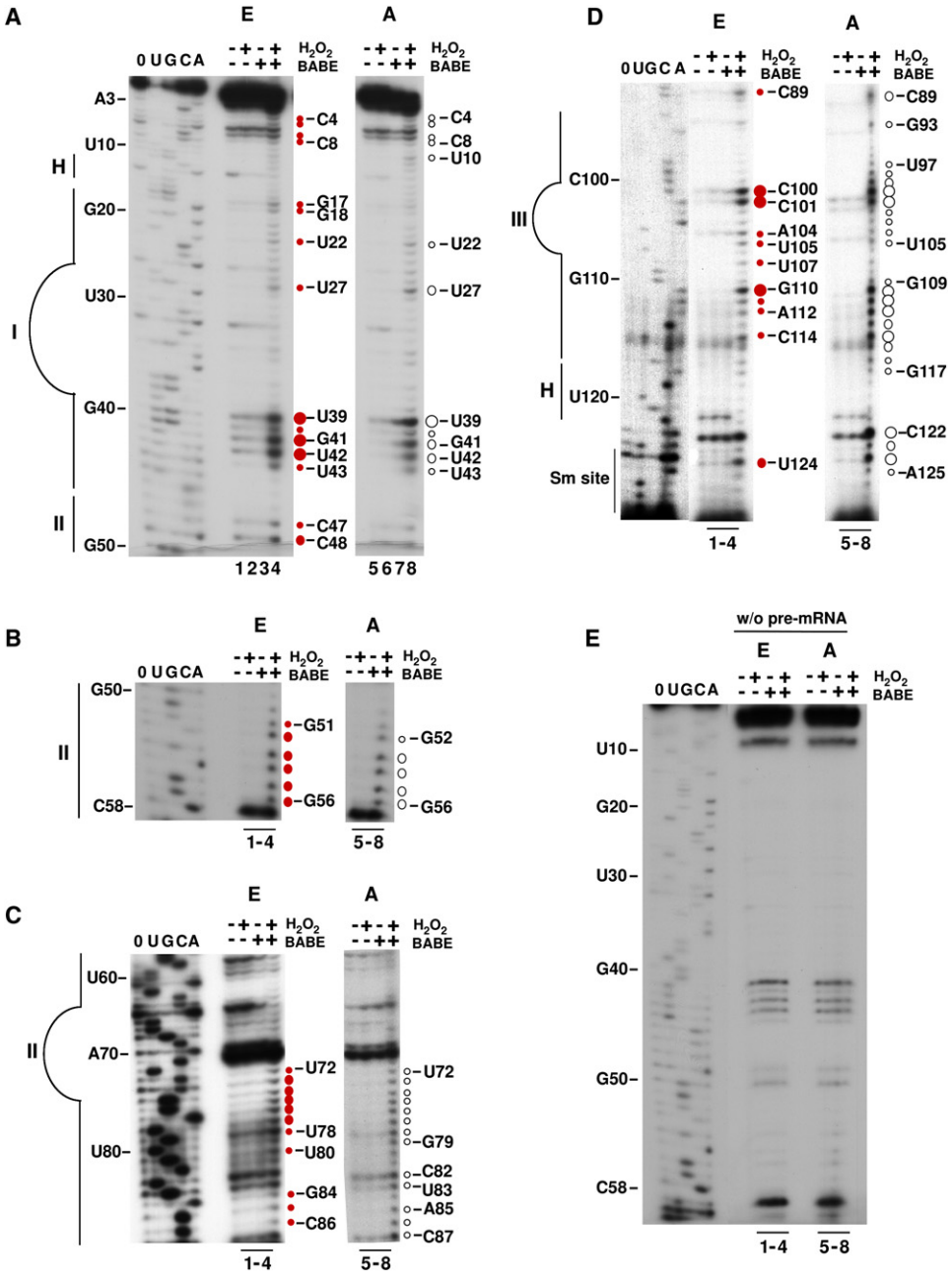

Figure 4. Site-Directed Hydroxyl-Radical Probing of U1 snRNA in E and A Complexes

(A) Fe-BABE U2-induced cleavages within the first 50 nt of U1, including stem H, I, and the lower part of stem II, were analyzed by primer extension (primer complementary to nt 63–76) in E (lanes 1–4) and A (lanes 5–8) complexes. U1 cleavages above background in E and A complexes are indicated by red and white dots, respectively. Lane designations are as in Figure 2A. Analysis of cleavages in nt 50–58 (stem II) of U1 RNA with the primer used in (A) is shown in (B), in nt 58–87 (stem II) with a primer complementary to nt 101–119 in (C), and in nt 88–126 (stem III and H) with a primer complementary to nt 134–152 in (D).

(E) Splicing was performed under conditions normally generating E (lanes 1–4) or A complexes (lanes 5–8), but in the absence of pre-mRNA. Cleavages within the first 58 nt of U1 were analyzed with the primer used in (A). No cleavages above background were detected.

The U1 cleavage pattern is not random, but rather cleavages are limited to selected regions of the molecule, suggesting that U1 and U2 are oriented in a specific manner. Significantly, when we subjected purified U1 snRNPs (either in nuclear extract or in buffer) to hydroxyl radicals produced from Fe-EDTA in solution and analyzed the cleavage pattern as described above, many additional U1 nucleotides were cleaved and the relative cleavage

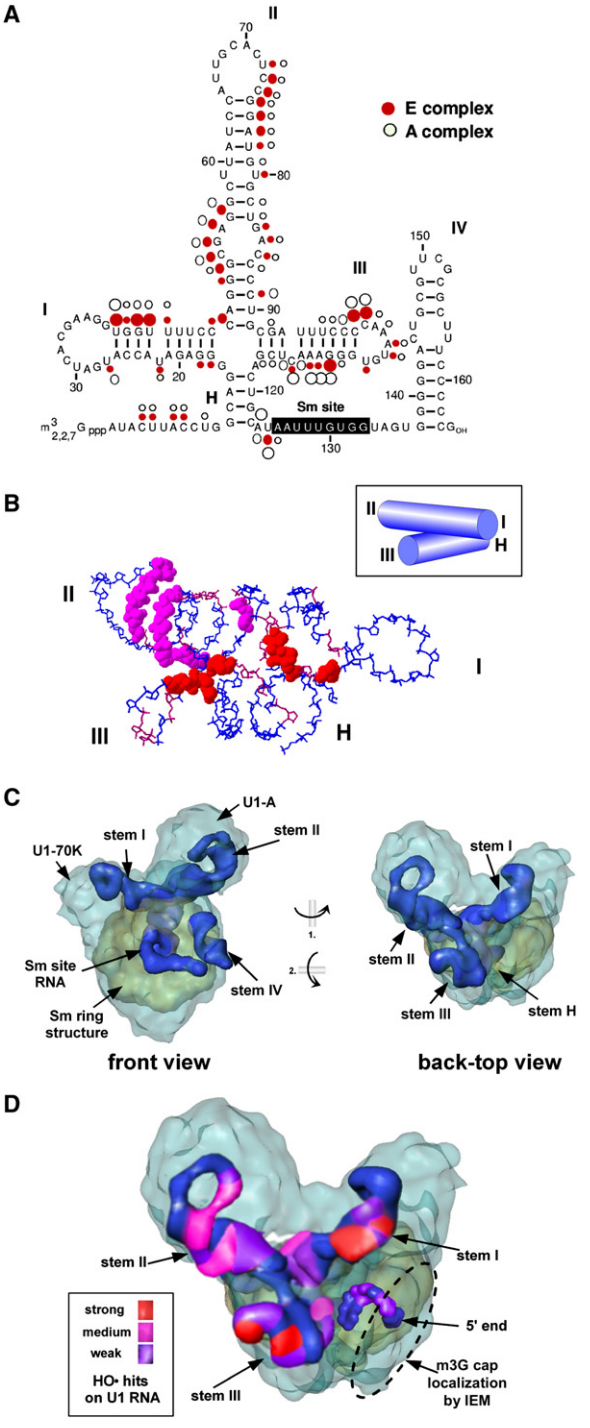

Figure 5. U1 and U2 snRNPs Are Positioned in a Defined Orientation Relative to Each Other in Early Spliceosomal Complexes

(A) Secondary structure of the human U1 snRNA with summary of Fe-BABE-U2-induced cleavages in the E and A complex (red and white dots, respectively). Stem-loops I–IV and stem H are indicated.

(B) 3D structure model of the human U1 snRNA (Krol et al., 1990), where uncleaved (blue) and weakly cleaved (red) nucleotides are modeled as sticks, and strongly and moderately cleaved nucleotides detected in the E complex as space filling entities. Strongly and moderately cleaved nucleotides are highlighted red and pink, respectively. Inset, the stacking scheme of RNA double helices in the four-way junction, with stems I, II, III, and the 5′-3′ connecting helix H.

(C) 3D model of the U1 snRNP (Stark et al., 2001). The classical "frontal" view is shown on the left and a "back-top" view is shown on the right.

(D) Mapping of the U1 hydroxyl-radical cleavages observed in the E complex, onto the U1 snRNA in the U1 snRNP 3D model. The dashed line indicates the region where the 5′ end of U1 should be located based on immuno-EM studies with anti-m_3G antibodies. Note that the position of the 5′ end of U1 in this region is depicted arbitrarily.

intensities in several regions differed from those observed in the E and A complex (data not shown). These data are consistent with the idea that not only those U1 regions that are accessible to hydroxyl radicals were cleaved in E and A complexes.

To learn more about the spatial relationship of the U1 and U2 snRNAs, we first mapped those cleavages observed in the E complex onto the protein-free, 3D model proposed for the human U1 snRNA (Krol et al., 1990) that was later supported experimentally (Duckett et al., 1995) (Figure 5B). This model posits a four-way junction with coaxial stacking of helices I and II and helices H and III. Significantly, nearly all cleavages are located on one side of each of the coaxial helices, and these sides face in the same direction (Figure 5B and Figure S2). Thus, the cleaved nucleotides are oriented such that they could face a single point source of hydroxyl radicals, consistent with the idea that the 5′ end of U2 is located on one side of the U1 snRNA.

We next extended our analysis to the proposed 3D structure of the human U1 snRNP (Stark et al., 2001) (Figure 5C), which not only is based on the single-particle cryo-electron microscopy (cryo-EM) 3D reconstruction of the native U1 snRNP but also incorporates all biochemical and additional structural information that was available at that time, including the U1 snRNA helical stacking model. The classical "frontal" view of the U1 model is shown at the left of Figure 5C, where the position of the Sm protein ring (highlighted green) and other U1 snRNP proteins (e.g., U1-70K and U1-A) are indicated, together with the U1 snRNA (dark blue). This model posits that the Sm site of the U1 snRNP winds through the hole of the Sm protein ring and stem-loop IV is positioned on top of the ring. To accommodate the U1 snRNA model into the 3D density map of the U1 snRNP obtained by cryo-EM, the stem I/II coaxial stacking helix was bent upward into a crescent and stem III was tilted toward stem II (Stark et al., 2001). A "back-top" view of the U1 snRNP, looking at stem-loops I, II, and III, is shown on the right; due to the inability to precisely locate the 5′ end of U1, note that this part of the snRNA was not included in the model.

When we map our cleavage data onto the slightly modified RNA structure in the U1 snRNP model, cleaved nucleotides are still found on one side of the RNA at the "back" of the U1 snRNP, and moreover, the cleaved regions are brought even closer together (Figure 5D, red

and pink patches). Uncleaved nucleotides are mostly on the opposite side of the U1 snRNP. These data indicate that the U2 snRNP interaction domain is located predominantly at the "back" of the U1 snRNP and, furthermore, support the apparent structure/position of the U1 snRNA in the U1 snRNP 3D model. The largest distance between strong and medium cleavages, those on the coaxial stems I/II, still spans ~50 Å. Thus, the 5′ end of the U2 snRNA must indeed be very close to the U1 snRNP (presumably at an approximately equal distance from the most strongly cleaved nucleotides), considering the migration distance of hydroxyl radicals (~20 Å) and the length of the spacer arm (~10 Å).

EM immunolocalization studies previously indicated that the m_3G cap of U1 (and thus its 5′ end) is positioned somewhere in the lower right-hand region of the particle as depicted in Figure 5D (Kastner et al., 1992). However, as there was not enough biochemical and structural information to place it on a particular "side" of the U1 particle, this portion of the U1 snRNA was not included in the original U1 snRNP 3D model (Stark et al., 2001). The detection of hydroxyl-radical cleavages at the 5′ end of the U1 snRNA suggests that this functionally important region is also positioned on the same side of the U1 snRNP as the other cleaved nucleotides (Figure 5D). Furthermore, our data also are consistent with the formation of a base-paired U1/5′ss helix. That is, when cleaved nucleotides at the pre-mRNA's 5′ss and those in the 5′ss interacting region of U1 are mapped onto a double-stranded RNA helix, cleaved nucleotides of both RNA species could potentially be hit by a single point source of hydroxyl radicals. In summary, our data show that, as early as in the E complex, the U2 and U1 snRNPs are in close proximity and in a defined orientation relative to one another.

DISCUSSION

We have examined U1 and pre-mRNA nucleotides in the neighborhood of the functionally important 5′ end of the U2 snRNA in early spliceosomal complexes by site-directed hydroxyl-radical footprinting. Our data provide important insights into the structural organization of U2 relative to U1 and the pre-mRNA in the E and A complex and suggest that U2 is a central component of the molecular bridge connecting the 5′ and 3′ ends of the intron in the E complex. They further indicate that nucleotides near the 5′ end of U2, which later form part of the catalytic center of the spliceosome, are already positioned near the functional sites of the pre-mRNA at the earliest stage of spliceosome assembly. This suggests that major rearrangements in their organization relative to the pre-mRNA are not required for subsequent catalytic activation and catalysis.

The 5′ End of U2 Is Near the Pre-mRNA's Functional Sites

Of the regions of the pre-mRNA hit by hydroxyl radicals, the polypyrimidine tract and the 3′ end of exon 1 are among those showing the strongest cleavages (Figure 2D), indicating proximity of these two elements in the E complex. Previous RNA proximity data obtained by attaching Fe-BABE to the pre-mRNA (Kent and MacMillan, 2002) showed that the nucleotide at position −6 relative to the BP and also the 3′ss are near the 5′ end of the intron (including the 5′ss), already in the E complex. These data thus indicated that the pre-mRNA's functional sites (i.e., the 5′ss, BPS, and 3′ss) are spatially preorganized in the E complex. Consistent with these results, our data demonstrate that nucleotides immediately bordering these functional sites are in close proximity. Thus, they further delineate additional constraints on the relative orientation of these RNA elements in the E complex. Data presented here firmly place the 5′ end of the U2 snRNA within the 3D space where functional sites of the pre-mRNA are concentrated. Both the 5′ and 3′ss are close to the 5′ end of U2 snRNA, as nucleotides neighboring them are hit by hydroxyl radicals. The same holds for the polypyrimidine tract, which connects the 3′ss and the BPS and is known to be bound by U2AF65 (Banerjee et al., 2003; Singh et al., 2000; Zamore et al., 1992). Recent data indicate that the U2AF65 interaction leads to bending of the polypyrimidine tract such that the 3′ss becomes spatially juxtaposed to the BP (Kent et al., 2003).

The AS, which is an important spliceosome assembly element, is also positioned close to the 5′ end of U2 snRNA already at the stage of E complex formation. It is contacted in the A complex by several U2-associated proteins, including subunits of SF3a and SF3b, which thereby help to tether the U2 snRNP to the BPS in the A complex (Gozani et al., 1996). Whether these proteins contact the AS in E complexes isolated under mild conditions (i.e., at low salt) has not been investigated; indeed, there is generally a paucity of crosslinking data regarding pre-mRNA contacts in E complexes containing the U2 snRNP. Thus, it is not clear whether components of the U2 snRNP directly contact the pre-mRNA at this stage. The 5′ end of U2 is positioned very near to a portion of the AS, and this spatial organization appears to be relatively fixed, with no major change detected upon formation of the A complex. Interestingly, the very 5′ end of the U2 snRNA (i.e., positions 1, 2, and 6) is contacted by SF3b49, both in isolated 17S U2 snRNPs and after U2 incorporation into the A complex (Dybkov et al., 2006). In the A complex, SF3b49 has also been shown to contact the AS at a position 1 nt downstream of G132, the most strongly cleaved nucleotide in this region. Thus, it is conceivable that it also plays an important role in juxtapositioning the 5′ end of U2 near the AS already in the E complex. Alternatively or additionally, U2 may be held in a position near the AS via interaction with U2AF65 bound to the nearby polypyrimidine tract (Gozani et al., 1998). At any rate, our data suggest that the U2 snRNA is poised for its subsequent base pairing interaction with the BPS during A complex formation, already in the E complex.

Curiously, nucleotides in exon 1 centered around C22 and located some 36 nt from the 5′ss are also positioned

close to the 5′ end of U2 snRNA. As the U1 snRNP is known to interact with the cap binding proteins bound to the m^7G cap at the 5′ end of the pre-mRNA (Lewis et al., 1996), it is conceivable that this interaction "bends" exon 1 such that its 5′ end is located closer to the 5′ss. This would in turn place nucleotides in exon 1 closer to the BPS and 3′ss and also the 5′ end of U2. Indeed, our data are consistent with previous crosslinking studies that demonstrated that U2AF65 contacts an equivalent region in exon 1 (Chiara et al., 1996).

Close Spatial Relationship of the U1 and U2 snRNPs

Multiple cleavages were found in distinct regions of the U1 snRNA in both E and A complexes (Figures 4 and 5), confirming that U1 and U2 are in close proximity at the earliest stage of spliceosome assembly and may even contact one another. Indeed, the relative spatial organization between U1 and the 5′ end of U2 does not change significantly during A complex formation, further suggesting a direct, spatially fixed interaction. Communication between U1 and U2 within spliceosomal complexes is likely mediated directly by the U2-associated, DEAD-box protein hPrp5 (Xu et al., 2004) and/or indirectly by members of the SR protein family (Boukis et al., 2004; Fu and Maniatis, 1992). The fact that a functional 5′ss is required for E complex formation, but not the BPS (data not shown; Das et al., 2000), suggests that U2 snRNP assists in joining/pairing the ends of the intron by interacting on one side with the U1 snRNP at the 5′ss and on the other side with U2AF65 at the polypyrimidine tract and/or directly through SF3a/b protein interactions with the AS (see above).

Recent studies have provided evidence that U1 and U2 snRNPs can form a di-snRNP complex in the absence of pre-mRNA at least in yeast (Xu et al., 2004). However, when we performed hydroxyl-radical probing under conditions excluding the incorporation of Fe-BABE U2 snRNPs into spliceosomal complexes (e.g., when pre-mRNA was omitted), no U1 cleavages above background were detected. This suggests that in HeLa nuclear extract the vast majority of U2 snRNPs do not form a complex with U1 snRNPs (at least under the conditions employed in these studies). Alternatively, U1 and U2 may interact in the absence of pre-mRNA, but their spatial orientation differs from that in the E and A complex, such that the 5′ end of U2 is not located near the U1 snRNP.

Mapping of the U1 snRNA cleavages onto the U1 snRNA and U1 snRNP 3D structural models provided valuable information as to the orientation of U1 relative to the 5′ end of U2. The distribution of cleavages on the U1 snRNA in these 3D models confirmed that there is a defined orientation of U1 and U2: that is, the 5′ end of U2 appears to be located on a specific "side" of the U1 snRNP, which for historical reasons we have designated as the "back" of the particle (Figure 5). Our cleavage data also help to more precisely localize the position of the 5′ end of U1 within the U1 snRNP. As only limited experimental data were available, including immunolabeling with anti-m_3G antibodies followed by EM, previously it had proven difficult to precisely localize the 5′ end of the U1 snRNA within the U1 snRNP. Data presented here clearly indicate that the 5′ end of U1 is located on the same "side" of the particle as other regions of U1 hit by hydroxyl radicals (i.e., the "back" side).

Potential Structural Changes Accompanying the E to A Complex Transition

During the E to A transition, the U2 snRNP becomes stably associated with the pre-mRNA, engaging in a base pairing interaction with the BPS. Aside from the establishment of additional U2 contacts with the pre-mRNA, it is likely that this step is also accompanied by 3D spatial rearrangements involving U2. We did not, however, detect much change in the spatial relationship between U1 and U2 in the A versus E complex, indicating that the apparent interaction/positioning of these particles is relatively "fixed." Likewise, global rearrangements in U2 relative to the pre-mRNA were not observed, with the notable exception of the central region of exon 1. Nonetheless, we did observe some clear differences in the pre-mRNA cleavage pattern between these two spliceosomal complexes. For example, cleavages in the 5′ exon were completely lost or reduced, whereas moderate increases in cleavages at the 5′ss and BPS/PPT were observed. This suggests that stabilization of the pre-mRNA/U2 interaction during A complex formation is indeed accompanied by small rearrangements in the organization of the 5′ end of U2 relative to the pre-mRNA.

Alternatively, many of the observed changes in the pre-mRNA cleavage pattern could be due to alterations in the accessibility of the pre-mRNA's backbone due to recruitment or loss of spliceosomal proteins (i.e., local RNP remodeling events). For example, concomitant with A complex formation, U2AF65 becomes less tightly bound to the polypyrimidine tract (Bennett et al., 1992; Champion-Arnaud et al., 1995; Chiara et al., 1997; Michaud and Reed, 1993), and SF1/mBBP is released (Chiara et al., 1996; MacMillan et al., 1994). These events could potentially bring about the observed differential accessibilities of the BPS and the polypyrimidine tract to hydroxyl radicals in the E versus A complex.

Conserved Early Spatial Organization of snRNPs in the Major and Minor Spliceosome

The close spatial organization of the 5′ end of U2 both with U1 and functional sites of the pre-mRNA suggests similarities in the general structural organization of U2- and U12-type prespliceosomes. The minor U12-type spliceosome, which splices a rare class of pre-mRNA introns, is comprised of the U11, U12, U5, and U4atac/U6atac snRNPs, where U11 and U12 are the functional analogs of U1 and U2, respectively (Patel and Steitz, 2003). Assembly of the minor spliceosome is largely homologous to the major U2-type spliceosome, with the notable exception that the U11 and U12 snRNPs form a highly stable di-snRNP and thus interact with the pre-mRNA as a preformed complex (Wassarman and Steitz, 1992). U11 and U12 interact with

the 5′ss and BPS, respectively, in a cooperative manner, and their close association ensures that the reactive sites of the pre-mRNA involved in the first step of splicing are "bridged" already at the earliest step of minor spliceosome assembly (Frilander and Steitz, 1999). Our data indicate that the U1 and U2 snRNPs are also in very close proximity and likely contact each other in early spliceosomal complexes. Thus, they may form a molecular bridge between the ends of the intron, much like the U11/U12 di-snRNP. Recent studies indicate that the 5′ end of the U12 snRNA not only base pairs with the BPS but also contacts the penultimate nucleotide of the 5′ exon in early spliceosomal complexes (Frilander and Meng, 2005). Thus, the 5′ and 3′ ends of U12-type introns are in close proximity to the 5′ end of U12 and to each other in the minor prespliceosome, similar to the situation with U2 in the U2-type prespliceosome.

Preorganization of U2 and Its Impact on Subsequent Assembly Steps

The 3D organization of the functional sites of the pre-mRNA and the 5′ end of U2 may remain essentially unchanged upon incorporation of the U4/U6.U5 tri-snRNP during the next step of spliceosome assembly (i.e., B complex formation). Although Fe-BABE was tethered to the 5′ terminal nucleotide of U2, our data suggest that nt 11–20 of human U2 that form a catalytically important U2/U6 helix after incorporation of the U4/U6.U5 tri-snRNP into the spliceosome might also be located not far from the 5′ss and the BP, which react during the first step of splicing. Thus, a major rearrangement in the 5′ end of U2 relative to the reactive functionalities of the pre-mRNA might not be required to generate catalytically active spliceosomes in the subsequent steps leading to the first step of splicing. Furthermore, our data are consistent with the idea that at least part of the 3D interaction interface (i.e., involving U2) that mediates tri-snRNP addition is established already during the early steps of spliceosome assembly.

EXPERIMENTAL PROCEDURES

U2 snRNA Preparation and Fe-BABE Modification

The oligoribonucleotide 5′-SH-AmUmCGCΨΨCUCGmGmCCΨUUUGmGCUAA-3′, (where SH indicates a sulfhydryl group plus linker), corresponding to the first 24 nt of human U2 RNA, was purchased from Dharmacon. 2.5 μM oligo was cleaved with 111 mM TCEP trialkylphosphine tris (2-carboxyethyl) phosphine (TCEP) for 1 hr at 55°C in order to reduce disulfides and then precipitated with EtOH. Fe-BABE was prepared from aminobenzyl-EDTA (Dojindo, Japan) as described (DeRiemer and Meares, 1981) and allowed to react with the SH group of the oligo as described previously (Newcomb and Noller, 1999). The Fe-BABE-modified oligo was gel purified and ligated to in vitro-transcribed RNA comprising the remaining nucleotides of the U2 snRNA as described (Dönmez et al., 2004). The resulting chimeric Fe-BABE-modified U2 snRNA was gel purified prior to use.

Pre-mRNA Splicing and Complex Formation

Plasmids encoding MINX pre-mRNA lacking the BP sequence (ΔBPS) or 5′ss (Δ5′ss) were generated by PCR-based techniques from the pMINX plasmid (Zillmann et al., 1988). HeLa nuclear extract, U2-depleted extract, and in vitro-reconstituted 17S U2 snRNPs were prepared as described (Dönmez et al., 2004). In vitro splicing, as well as E and A complex formation, was performed with in vitro-transcribed, ^{32}P-labeled MINX pre-mRNA as described previously (Dönmez et al., 2004). To enhance A complex formation (and prevent assembly of B complexes), a 2′-O-methylated oligoribonucleotide complementary to nt 27–46 of U6 snRNA (5′-CUCUGUAUCGUUCCAAUUUU-3′) was added to the splicing reaction to a final concentration of 5 μM, and the mixture was incubated for 10 min at 30°C prior to addition of pre-mRNA. Splicing products were analyzed by denaturing PAGE, whereas E and A complexes were analyzed on native agarose gels (Das et al., 1999).

Hydroxyl-Radical Cleavage

Splicing reactions (15 μl) were diluted to 200 μl on ice with buffer containing 50 mM cacodylic acid-KOH (pH 7.0), 1.5 mM $MgCl_2$, and 100 mM KCl. 0.8 μl ascorbic acid (500 mM) was added, and the formation of hydroxyl radicals was initiated with 2 μl H_2O_2 (0.4% v/v). The reaction was allowed to proceed for 10 min on ice and was then stopped by addition of glycerol to a final concentration of 2.5% (v/v). Proteinase K digestion was performed, and the RNA was recovered. Control reactions were treated in an identical manner, except ascorbic acid and H_2O_2 were omitted.

Primer-Extension Analysis

Primer extension was performed as described (Hartmuth et al., 1999) with primers complementary to nt 83–100 (5′-GCTTGGGCGCAGGTAAC-3′) or 199–215 (5′-CTGGAAAGACCGCGAAG-3′) of the MINX pre-mRNA. U1 snRNA was analyzed with primers complementary to nt 63–76 (5′-CCGGAGUGCAAUGG-3′), 101–119 (5′-GTCGAGTTTCCCACATTTG-3′), or 134–152 (5′-CGAACGCAGTCCCCCACTA-3′). Radioactively labeled pre-mRNA was removed by adding NaOH (final concentration 20 mM) and incubating at 60°C for 1 hr. Primer-extension products were analyzed on a 9.6% denaturing polyacrylamide gel. Densitometric analysis was performed with BioRad Quantity 1 software.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.molecule.org/cgi/content/full/25/3/399/DC1/.

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