The Prp19-associated Complex Is Required for Specifying Interactions of U5 and U6 with Pre-mRNA during Spliceosome Activation*

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Activation of the spliceosome involves a major structural change in the spliceosome, including release of U1 and U4 small nuclear ribonucleoprotein particles and the addition of a large protein complex, the Prp19-associated complex. We previously showed that the Prp19-associated complex is required for stable association of U5 and U6 with the spliceosome after U4 is released. Changes within the spliceosome upon binding of the Prp19-associated complex include remodeling of the U6′ splice site interaction and destabilization of Lsm proteins to allow further interaction of U6 with the intron sequence. Here, we further analyzed interactions of U5 and U6 with pre-mRNA at various stages of spliceosome assembly from initial binding of tri-small nuclear ribonucleoprotein complex to the activated spliceosome to reveal stepwise changes of interactions. We demonstrate that both U5 and U6 interacted with pre-mRNA in dynamic manners spanning over a large region of U6 and the 5′ exon sequences prior to the activation of the spliceosome. During spliceosome activation, interactions were locked down to small regions, and the Prp19-associated complex was required for defining the specificity of interaction of U5 and U6 with the 5′ splice site to stabilize their association with the spliceosome after U4 is dissociated.

Splicing of precursor mRNA takes place on a large, dynamic ribonucleoprotein complex, the spliceosome, which is constituted of five small nuclear RNAs, U1, U2, U4, U5, and U6, and numerous protein components (reviewed in Refs. 1–3). These components associate with pre-mRNA in a sequential manner to assemble the spliceosome into a functional complex, which can catalyze the splicing reaction (4). During spliceosome assembly, U1 first interacts with the 5′ splice site, followed by association of U2 with the branch site to form a stable complex called the prespliceosome. The preformed U4/U6/5′ tri-snRNP1 is then recruited to the spliceosome to form a complex containing all five small nuclear RNAs. A subsequent structural rearrangement of the spliceosome releases U1 and U4, leading to the activation of the spliceosome.

Base pair interactions play important roles in mediating splice site recognition and alignment by snRNPs in the spliceosome (reviewed in Refs. 1, 2, and 4). During spliceosome assembly, the 5′ splice site is recognized by U1 in part via base pairing with the 5′-end of U1 RNA (5–7). Binding of U2 to the branch site is mediated by base pairing between U2 RNA and the branch site sequence (8, 9). The association of tri-snRNP with the spliceosome appears to also involve RNA-RNA interactions as demonstrated by isolation of cross-linked products of U4 small nuclear RNA to the second residue of the 5′ splice site using a Saccharomyces cerevisiae in vitro trans-splicing system (10).

Interactions of U5 and U6 with pre-mRNA in the spliceosome have been extensively studied. U5 interacts with exon sequences at the 5′ and 3′ splice sites through the conserved loop 1 sequence (11–16). The contacts between U5 and the 5′ exon are established in pre-mRNA and persist through both catalytic steps (12). After the first catalytic step, U5 further interacts with the 3′ exon near the splice junction to align the 5′ and 3′ exons for ligation (12, 16). U6 has been demonstrated to be a key element in formation of the catalytic core of the spliceosome by simultaneously base pairing with the 5′ splice site and with the U2 RNA (17–20). Two types of U6′ splice site base pairing, differed by a shift of 5 bases, were proposed based on biochemical and genetic analysis, respectively (10, 19, 20). Both types of interaction, as shown in Fig. 1, were later shown to exist but take place at different stages of spliceosome assembly, one after the dissociation of U4 but prior to the binding of the Prp19-associated complex and the other after the spliceosome is activated (21). In addition, a novel interaction between the 3′-terminal U-tract of U6 and the intron sequence was also detected that requires prior destabilization of Sm-like proteins (Lsm) in the active spliceosome (21).

The activation of the spliceosome involves a series of structural rearrangement of snRNPs, including dissociation of U1 and U4 and formation of base pairs between U6 and U2 and between U6 and the 5′ splice site. Factors that mediate such structural rearrangement have not been directly demonstrated, although Brr2 was implicated in the unwinding of U4/U6 and Prp28 in displacing U1 from the 5′ splice site (22, 23). A protein complex consisting of at least eight protein components, identified in the yeast S. cerevisiae as the Prp19-associated complex or NTC (nineteen complex) (24–28), is added to the spliceosome after the dissociation of U1 and U4 during spliceosome activation. NTC is required for stable association of U5 and U6 with the spliceosome after U1 and U4 are released (21).

NTC-dependent stabilization of U6 is mediated through remodeling of U6 snRNP, which includes the switch of U6′ splice site base pairing, and destabilization of Lsm from U6 to allow the interaction of the 3′ end of U6 with the intron sequence (Fig. 1). How stabilization of U5 is mediated remains to be determined.
To gain further insights into the rearrangement of RNA-RNA interactions during the activation of the spliceosome, we examined the interactions of U5 and U6 with pre-mRNA at different stages of spliceosome assembly from their initial binding as tri-snRNP until the spliceosome was activated. We surprisingly found that both types of U6/5’ splice site base pairing previously identified in the spliceosome after U4 dissociation existed in the preactivated spliceosome. Upon U4 dissociation, one of the two forms predominated, depending on whether NTC was present. This suggests that U6 might dynamically interact with the 5’ splice site over a broader region of U6 sequence during the association of tri-snRNP with the spliceosome. Similarly, the interaction between U5 and pre-mRNA also spanned a large region of the 5’ exon upon binding of tri-snRNP and post-U4 dissociation and became better defined only after the binding of NTC. We suggest that the association of tri-snRNP with the spliceosome involves dynamic interactions between U5 and U6 with pre-mRNA, and NTC plays a role in determining a stable form of base pairing between U6 and the 5’ splice site and defining specific interactions between U5 and pre-mRNA to stabilize the association of U5 and U6 with the spliceosome after U4 is released, leading to the activation of the spliceosome.

**EXPERIMENTAL PROCEDURES**

** Yeast Strains—**The following yeast strains were used: BJ2168 (MATa pro1 prb1 pep4 leu2 trp1 ura3), YSCC1 (MATa pro1 prb1 pep4 leu2 trp1 ura3 PRP19-HA), and YSCC43 (MATa pro1 prb1 pep4 leu2 trp1 ura3 PRP4-HA).

*Oligonucleotides—*The following oligonucleotides were used for directed RNA H cleavage and primer extension: U5-A, GTTCTTGATG-TTACCT; U5-B, CCTATCCTTTAGAAGTTCTC; U5-C, ACCGGAGT-GGTTCTG; U5-D, ACAGCTTTACCTGTTTC; U5-E, TGGCAAGCCC-AGA; U6-A, CCTATCTTTTAAGCAGG; Pre-I, CAGAACTCATTGG- TTAAT; Pre-II, TCTTAGCTTAAATGGGAGT; Pre-III, CAATTG- GACCGTGCACCTTCT; Pre-VI, TTTGACTATTGGGAAG; Pre-VII, AAG- CAGTAAGCCTGGA; Pre-VIII, CTAAAAGGGGATCC.

The following 5’-biotinylated oligonucleotides (Qiagen) were used for affinity selection: U5-Cbio, BioTEG-ACCCGGATGTTCTG; U6-Abio, BioTEG-TCTCATTTTGAAACGG; Pre-IVbio, BioTEG-CCAGGTTATG-GAGAGTG.

Antibodies and Reagents—Anti-HA antibody 8G5F is a monoclonal antibody produced by immunizing mice with a keyhole limpet hemocyanin-conjugated peptide of the HA epitope. Anti-Nte85, anti-Ntc20, and anti-Smd1 are polyclonal antibodies produced by immunizing rabbits with the corresponding recombinant proteins expressed in *Escherichia coli*. Streptavidin-Sepharose was purchased from Sigma, protein A-Sepharose from Amersham Biosciences, proteinase K from MD Bio Inc., RNase H from Promega, and SuperScript II from Invitrogen.

Splicing Extracts, Substrates, and Reactions—Splicing extracts were prepared according to Cheng et al. (29) using yeast strain BJ2168, YSCC1, or YSCC43. A truncated form of actin pre-mRNA, Ac/Cla, in which only 5 nucleotides were retained after the branch point was used in this experiment. Splicing substrates were prepared by in vitro transcription with SP6 RNA polymerase of pSiPAC6–88 linearized with ClaI. Splicing reactions were performed as described by Lin et al. (30) on a large scale.

*Depletion of NTC—*PRP19-HA extracts from strain YSCC1 were used for depletion of NTC with the anti-HA antibody 8G5F. Each 1 ml of the splicing extract was incubated with 3 μl of 8G5F conjugated to a 0.5-ml bed volume of protein A-Sepharose at 4 °C for 1 h. After centrifugation to remove the bound material, the supernatant was used as the NTC-depleted extract.

**UV Cross-linking, RNase H Mapping, and Primer Extension—**For UV cross-linking, each 2 ml of the splicing reaction mixture was pretreated with the anti-Snd1 antibody (400 μl of serum coupled to a 500-μl bed volume of protein A-Sepharose). The bound material was resuspended in 4 ml of Buffer E (12 mM Hepes-KOH, pH 7.9, 30 mM KCl, 3 mM MgCl2, 0.12 mM EDTA, and 12% (v/v) glycerol) and distributed into two wells of a 6-well culture plate. The plate was placed on ice 10 cm underneath the UV lamp in a UV Stratalinker (Stratagene Inc.) and irradiated at an energy level of 0.125 J. After deproteinization, RNA was precipitated and resuspended in 79 μl of H2O and then mixed with 10 μl of *E. coli* tRNA (10 mg/ml), 10 μl of Buffer S (0.2 mM Hepes-KOH, pH 7.9, and 0.5 mM KCl) and 1 μl of desired 5’-biotinylated oligonucleotide U5-Cbio, U6-Abio, or Pre-IVbio at 300 μl. The mixture was boiled for 2 min, slow cooled to room temperature, and precipitated with a 50-μl bed volume of streptavidin-Sepharose by incubation at 4 °C for 1 h. The precipitate was heated for 2 min to release the selected RNAs, which were fractionated on a 5% acrylamide, 8% urea gel. Individual cross-linked products were excised from gels and eluted. Following precipitation, the amount of RNA was estimated by counting in a scintillation counter (LS6500; Beckman Coulter). For RNase H digestion, 0.015–0.03 fmol of cross-linked RNA dissolved in 4 μl of H2O was mixed with 1 μl of 30 μl desired oligonucleotide, 1 μl of 10 mg/ml tRNA, 1 μl of Buffer S; heated on a hot plate for 2 min; and chilled on ice. Digestion was carried out at 37 °C for 30 min following the addition of 1 μl of 0.1 mM MgCl2, 1 μl of 10 mM dithiothreitol, and 1 μl of diluted RNase H (0.5 unit/μl). For primer extension, 0.075–0.15 fmol of cross-linked RNA and 106 cpm of 5’-radiolabeled oligonucleotide was mixed in a 5-μl annealing mixture containing 50 mM Tris- HCl, pH 8.0, and 75 mM KCl, heated on a hot plate for 2 min, and immediately transferred to a 45 °C water bath and then mixed with a 5-μl premelled reaction mixture containing 50 mM Tris-HCl, pH 8.0, and 20 mM dithiothreitol, 1 μl each of four dNTPs, 30 units of RNasin, and 50 units of SuperScript II reverse transcriptase and incubated at 45 °C for 1 h.

**RESULTS**

Dynamic Interactions of U6 with the 5’ Splice Site in the Preactivated Spliceosome—We previously demonstrated that two different modes of base pairing between U6 and the 5’ splice site can form in the spliceosome, one being NTC-de-
pended and the other NTC-independent (21). We proposed that these base pairings occur at different stages of the spliceosome assembly process after the dissociation of U4 and that NTC might play a role in mediating the base-pairing switch (Fig. 1). To determine whether NTC-independent pairing occurs even before the dissociation of U4, we analyzed the interaction of U6 and the 5' splice site in the preactivated spliceosome by UV cross-linking. It has been demonstrated that the U4-containing spliceosome can be arrested and accumulated if the splicing reaction is carried out at lower concentrations of ATP (31). Fig. 2A shows that U4 was in the base-paired form with U6, presumably in the di-snRNP or tri-snRNP complex, under such conditions when RNA isolated from the spliceosome was analyzed on nondenaturing gels (lane 3). Therefore, using extracts prepared from a strain in which the U4 snRNP protein Prp4 was tagged with the HA epitope, the U4-containing spliceosome could be isolated by performing the splicing reaction at low ATP concentrations, followed by immunoprecipitation with the anti-HA antibody. To block splicing catalysis after activation of the spliceosome, actin pre-mRNA Ac/Cla, truncated 5 bases after the branch point (32), was used in this experiment as in our previous analysis (21). The isolated spliceosome was irradiated with UV light at 254 nm, and pre-mRNA cross-linked to U6 was selected with a 5'-biotinylated oligonucleotide complementary to U6 RNA and analyzed on denaturing polyacrylamide gels (21). For comparison, splicing reactions were also carried out at high concentrations of ATP in either wild-type or NTC-depleted extracts, and total spliceosome or the active spliceosome was isolated by immunoprecipitation with the anti-Smd1 antibody or with the anti-Ntc85 antibody, respectively.

As shown in Fig. 2B, the U6-cross-linked products X1 and X2 appeared in the spliceosome formed at a high concentration of ATP (lanes 1–3) as reported previously (21). X1, appearing only in the active spliceosome, contains cross-links between the Lsm binding site of U6 and the intron sequence in a region near the conserved ACAGA box (10). X2a and X2b are cross-linked products between the 5' splice site and U6 in a region near the conserved ACAGA box (10). X2a, previously identified as dX2, accumulated in the spliceosome formed in NTC-depleted extracts (lanes 2), which also appears only in the active spliceosome (lanes 1 and 3), was not seen in the spliceosome formed in NTC-depleted extracts (lane 2). These results led us to propose that during spliceosome activation, base pairing represented by X2a forms immediately after dissociation of U4 and switches to the form represented by X2b upon binding of NTC. When splicing was carried out at a low concentration of ATP in Prp4-HA-tagged extracts and the spliceosome was precipitated with U6-Abio, it has been demonstrated that the U4-containing spliceosome can be isolated by immunoprecipitation with the anti-Ntc85 or anti-HA antibody, respectively. Following UV-irradiation and deproteinization, U6-cross-linked products were affinity-selected with U6-Abio.
analysis confirmed that X2a and X2b from the preactivated spliceosome were cross-linking at similar positions as X2a and X2b isolated from the spliceosome formed at a high concentration of ATP. As shown in Fig. 2C, a mixture of similar extension stops of X2a and X2b from the preactivated spliceosome was seen, except that X2b had more heterogeneous stops between C13 and U10 of U6 (lane 10) than those isolated from the active spliceosome, in which a stop at A44 predominated (lane 9). A cross-link to G39 observed in both X1 and X2, speculated to be cross-linking to a certain spliceosomal protein component in an NTC-dependent manner (21), was not detected in the preactivated spliceosome (lane 10), further supporting the requirement of NTC for this cross-linking.

The presence of both X2a and X2b in the preactivated spliceosome was better revealed when comparing spliceosomes at various ATP concentrations, as shown in Fig. 3. Splicing at lower concentrations of ATP accumulates more prespliceosome and preactivated spliceosome, whereas at higher ATP concentrations it forms more activated spliceosome. In wild-type extracts, both X2a and X2b were present at lower concentrations of ATP (lanes 1–5) and 2.0 mM of ATP (lanes 6–10) in either wild-type (WT; lanes 1–5) or NTC-depleted (lanes 6–10) extracts by immunoprecipitation with the anti-Smd1 antibody. U6-cross-linked products were isolated and analyzed as in Fig. 2A.

FIG. 3. U6-cross-linked products identified from splicing reactions carried out at different concentrations of ATP. Total spliceosome was isolated from splicing reactions carried out at 0.1, 0.2, 0.5, 1.0, and 2.0 mM of ATP (lanes 1–5 and lanes 6–10) in either wild-type (WT; lanes 1–5) or NTC-depleted (lanes 6–10) extracts by immunoprecipitation with the anti-Smd1 antibody. U6-cross-linked products were selected with a 5’-biotinylated oligonucleotide complementary to U5 RNA. Fig. 4A shows two major (Y1 and Y3) and two minor (Y2 and Y4) U5-specific cross-linked products identified from total spliceosome formed in wild-type extracts when precipitated with the anti-Smd1 antibody (lane 1). Only Y1 and Y3 were detected in the active spliceosome when precipitated with the anti-Ntc85 antibody (lane 3). In NTC-depleted extracts, Y2 and Y4 became more prominent with a dramatic decrease in the relative amounts of Y1 and Y3. Furthermore, Y1 appeared to be a more heterogeneous mixture of different products (lane 2). Interestingly, all four products were also detected in the preactivated spliceosome (lane 4), indicating that all of these interactions between U5 and the pre-mRNA occurred prior to the release of U4. The generation of the U5-cross-linked products was compared in wild-type and NTC-depleted extracts by performing the splicing reaction at various ATP concentrations (Fig. 4B).

The relative amounts of these products from wild-type and NTC-depleted extracts were similar at lower ATP concentrations, when more preactivated spliceosome accumulated (lanes 1, 2, 6, and 7). At higher ATP concentrations that promoted the dissociation of U4, Y2 and Y4 species accumulated in larger amounts at the expense of Y1 and Y3 in NTC-depleted extracts but not in wild-type extracts. In addition, Y1 became more discrete in wild-type extracts (lanes 3–5) but more heterogeneous in NTC-depleted extracts (lanes 8–10). These results indicate that there are multiple forms of U5 interaction, with pre-mRNA occurring early in spliceosome assembly. Upon dissociation of U4, the interaction becomes more specific in the presence of NTC.

To elucidate the modes of interaction between U5 and pre-mRNA, the cross-linking sites of individual cross-linked products, affinity-selected and gel-purified, were mapped by RNase H digestion with oligonucleotides complementary to different regions of U5 or actin pre-mRNA. Fig. 5A shows the relative position of the oligonucleotides used to the pre-mRNA. Each oligonucleotide directed the cleavage of the 32P-labeled pre-mRNA into two fragments, indicated as R and L, representing fragments on the right side and the left side of the oligonucleotide, respectively. Within each pair of fragments produced from cross-linked products, the one containing the cross-link will have slower mobility with respect to its cognate uncross-linked species. Judging whether the right or left fragment is up-shifted allows one to locate the cross-link between specific oligonucleotides. As shown in Fig. 5B, the cross-linking sites of Y1 and Y3 isolated from the spliceosome formed in wild-type extracts were mapped between oligonucleotides I and II of the pre-mRNA in the 5′ splice site region, since the right-sided fragments from oligonucleotides I, VI, VII, and VIII were up-shifted, whereas the left-sided fragment from oligonucleotide II was up-shifted. Y1 and Y3 showed identical digestion patterns, except that the cross-link-containing fragments from Y1 migrated slower than those from Y3, suggesting that they were cross-linked at the same positions of the pre-mRNA to the long form and short form of U5 (U5L and U5S), respectively (see below). Y1 and Y3 isolated from the spliceosome formed in NTC-depleted extracts had identical mapping pattern to those from wild-type extracts (data not shown). The
Cross-linking sites of Y2 and Y4 isolated from the spliceosome formed in NTC-depleted extracts were mapped to a region upstream of oligonucleotide VII (lanes 21–30), since none of the right-sided fragments were shifted, and Y2 and Y4 were possibly cross-linked pre-mRNA to U5L and U5S at the same positions, respectively. Y2 showed a mixture of digestion pattern of Y2 and Y3 due to close mobility of these two cross-linked products. Y2 and Y4 isolated from the spliceosome formed in wild-type extracts had identical patterns to those from NTC-depleted extracts (data not shown).

Since U5 has not been shown to interact with the pre-mRNA in any region other than the 5' splice site prior to catalytic steps, it was surprising to find cross-linking of Y2 and Y4 near the 5'-end of the pre-mRNA. RNase H mapping with oligonucleotide VIII further upstream revealed that the cross-linking sites were located in the region between oligonucleotides VII and VIII. As also shown in Fig. 5C the large, right-sided fragment VIII_R was up-shifted (lanes 5 and 8), excluding the possibility of cross-linking at the very 5'-end of the pre-mRNA. A change of the sequence in the 5' exon revealed that such interaction occurred in a region 50–60 bases upstream of the 5' splice site with no sequence specificity (data not shown). These results suggest that besides the previously observed interactions between the conserved loop and the 5' splice junction, U5 also interacts with the 5' exon of pre-mRNA in a region far upstream from the 5' splice site upon its binding to the spliceosome and that this interaction persisted after the dissociation of U4 if NTC was absent. In the presence of NTC, this interaction was not accumulated as the spliceosome progressed to the active form.

Precise cross-linking sites on Y1 and Y3 were determined by primer extension analysis using primer Pre-II. Y1 and Y3 were purified from the spliceosome isolated from various sources for primer extension analysis. Total spliceosome was formed in wild-type and NTC-depleted extracts and precipitated with the anti-Smd1 antibody. The active spliceosome was formed in wild-type extracts and precipitated by the anti-Ntc20 antibody.

The preactivated spliceosome was formed at low ATP concentration in Prp4-HA extracts and precipitated with the anti-HA antibody. As shown in Fig. 6, Y1 and Y3 had identical extension patterns in the presence or absence of NTC showed distinct cross-linking patterns. In the presence of NTC, cross-linking occurred predominately at the last and penultimate nucleotide of the 5' exon (lanes 5, 6, 9, and 10). The absence of NTC, cross-linking was more heterogeneous and extended from position 1 to 10 (lanes 7 and 8), indicating that U5 may interact with the 5' exon in multiple forms near the 5' splice junction prior to binding of NTC. Indeed, a similar cross-linking pattern was seen in the preactivated spliceosome coprecipitated with Prp4-HA (lanes 11 and 12) that showed multiple cross-linking sites extended only up to A^-6 but contained an extra cross-linking at the first nucleotide of the intron G^1. Cross-linked products in the active spliceosome as precipitated by the anti-Ntc20 antibody showed a similar pattern to the total spliceosome (lanes 5, 6, 9, and 10), since the majority of the spliceosome isolated is the activated form under normal splicing conditions. These results suggest that during spliceosome assembly, U5, in association with U4/U6, can interact with the pre-mRNA in a broad region spanning from the first nucleotide of the intron to position –6 in the 5' exon, possibly in a dynamic fashion. Upon dissociation of U4 but prior to the binding of NTC, the interaction was shifted slightly upstream, excluding G^1 but extending to A^-10. The interaction is confined to the last 2 nucleotides of the 5' exon upon binding of NTC, which might play a role in mediating specific interaction between U5 and the 5' splice site.

Cross-linking sites on U5 were mapped with five oligonucleotides, whose positions to U5 are shown in Fig. 7A, by RNase H digestion. The lengths of U5 on each side of individual oligonucleotides are shown in the inserted table for estimating the size of produced fragments. Since U5L and U5S differ in their

**Fig. 4.** U5-cross-linked products identified in the active spliceosome and the preactivated spliceosome. A, spliceosome was isolated and irradiated with UV light as described in Fig. 2A, and U5-cross-linked products were selected using U5-C_H11002. B, splicing reactions were carried out at different concentrations of ATP (0.1, 0.2, 0.5, 1.0, and 2.0 in lanes 1–5 and lanes 6–10) in either wild-type (WT, lanes 1–5) or NTC-depleted (lanes 6–10) extracts, and U5-cross-linked products were isolated. Δ, depletion; H, high concentration; L, low concentration; S, α-Smd1; N, α-Ntc85; A, α-HA.
3’-end by 35 bases, digestion with each oligonucleotide will produce the same 5’ fragments but different 3’ fragments between U5L and U5S (see the table in Fig. 7A). Digestion of cross-linked products with U5 oligonucleotides will affect the relative mobility of the 32P-labeled pre-mRNA due to the removal of either the 5’ or 3’ fragment of cross-linked U5, and the same cross-link to U5L and U5S will give the same digested product if the cross-link is located in the 5’ fragment. As shown in Fig. 7B, the cross-link-containing fragments produced from Y1 with oligonucleotides C, D, and E were of the same size as those produced from Y3, suggesting cross-linking in the 5’ fragments, and those from oligonucleotides A and B were different in size, suggesting cross-linking in the 3’ fragments. We concluded from these results that the cross-linking sites were located between B and C, where the conserved loop 1 is. The digestion patterns of Y1 and Y3 from the spliceosome formed in wild-type extracts and NTC-depleted extracts were similar except that those from NTC-depleted extracts were more heterogeneous, possibly due to multiple cross-linking sites on the pre-mRNA. Attempts to determine precise cross-linking sites on U5 by primer extension analysis did not yield results due to a high background in extension stops in the loop 1 region of U5 (data not shown).

When Y2 and Y4 from the spliceosome formed in NTC-depleted extracts were digested with RNase H, each cross-link-containing fragment appeared as a smeared band, indicating that there might be multiple cross-linking sites on pre-mRNA and/or U5. Since cross-linking on the pre-mRNA was mapped near the 5’-end of the pre-mRNA, it is possible that the removal of half of U5 after RNase H digestion resulted

![FIG. 5. RNase H mapping of U5 cross-linking sites on pre-mRNA. A, a diagram depicting relative positions on the pre-mRNA of oligonucleotides used to direct RNase H digestion. Open box, the 5’ exon; solid line, the intron; A, the branch point nucleotide. B, mapping with oligonucleotides Pre-I, -II, -VI, and -VII. Pairs of the fragments of pre-mRNA generated after RNase H digestion are indicated on the side as L and R for left- and right-sided fragments. Y1 and Y3 were isolated from the spliceosome formed in wild-type extracts (WT), and Y2 and Y4 were isolated from NTC-depleted extracts (ΔNTC), as described in Fig. 4A. Each cross-linked product was purified from gels and subjected to RNase H digestion. The circles and arrows on Y1 and Y3 mapping indicate fragments up-shifted (from circle to arrow) due to the presence of cross-links, but only digestion with Pre-I and Pre-II that contained cross-links to opposite sides of the oligonucleotides is indicated (lanes 9 and 10 and lanes 14 and 15). The boxed regions represent the unshifted right-sided fragments from oligonucleotides I, VI, and VII (lanes 22–24 and 27–29) as an indication of cross-links on the left-sided fragment. C, mapping of Y2 and Y4 with oligonucleotides Pre-VII and -VIII as in B. The left-sided fragments of the digested product are not seen due to their low content of radiolabel. The circles and arrows indicate fragments up-shifted (from circle to arrow in lanes 5 and 8).}
in a less branched structure, which migrated faster on polyacrylamide gels. Judging from the relative mobility of fragments from Y2 and Y4, cross-linking sites were also mapped between oligonucleotides B and C in the region of the conserved loop. Despite the difficulty in determining the precise cross-linking sites, these results indicate that in addition to the 5' splice site, the U5 conserved loop can also interact with the 5' exon in a region 50–60 bases upstream of the 5' splice site upon binding of tri-snRNP. After U4 is dissociated from the spliceosome, the interaction with the 5' splice site was not greatly perturbed if NTC was not present. In the presence of NTC, the interaction with the 5' splice site becomes dominant with concomitant loss of upstream interactions.

**Fig. 6.** Primer extension analysis to determine precise U5 cross-linking sites on pre-mRNA. Y1 and Y3 were purified as in Fig. 5B and subjected to primer extension analysis using oligonucleotide Pre-II. A pre-mRNA dideoxynucleotide-sequencing ladder is shown (lanes 1–4) for comparison, and the sequence is marked to the left. Negative and positive numbers represent positions of the nucleotide upstream and downstream of the 5' splice site (5' SS), respectively. Mapped cross-linking sites are marked by asterisks beside the sequence. Non-cross-linked RNA without (RNA) or with (RNA + UV) exposure to UV was subjected to the same analysis for comparison (lanes 13 and 14). Δ, depletion; H, high concentration; L, low concentration; S, α-Smd1; N, α-Ntc20; A, α-HA.

**DISCUSSION**

Although interactions between small nuclear RNAs and pre-mRNA during spliceosome assembly have been extensively studied, the precise stage of the assembly at which each interaction occurs has never been clearly defined. Based on the results of biochemical and genetic studies, two different modes of base pairing between U6 and the 5' splice site were proposed (19, 20). By UV-cross-linking analysis using NTC-depleted extracts, we subsequently found that these proposed interactions might represent interactions between U6 and the 5' splice site at different stages of spliceosome assembly, one before and the other after the binding of NTC post-U4 dissociation (21).

In the report presented here, we have identified several UV-cross-linked products, which might represent dynamic RNA-RNA interactions between U6 and the intron and between U5 and pre-mRNA in the U4-containing preactivated spliceosome. The X2a and X2b types of interaction were previously characterized to both involve base pairing of the 5' splice site with the U6 sequence near the conserved ACAGA box with a shift of 5 bases between them. Since the X2b type of interaction was found in the active spliceosome when NTC was present, whereas the X2a type was found in the spliceosome after U4 dissociation but in the absence of NTC, we previously suggested that NTC might play a role in mediating a switch of base pairing from the X2a to the X2b type of interaction (21). In this study, we interestingly found that both X2a and X2b cross-linked products could be isolated from the preactivated spliceosome prior to the dissociation of U4, suggesting that both types of interaction occurred during the association of tri-snRNP with the spliceosome. Thus, the X2b type of interaction, as well as the X2a type, occurs early in spliceosome assembly independent of NTC function. The fact that cross-linking sites were more heterogeneous in both X2a and X2b in the preactivated spliceosome, as revealed by primer extension analysis, further suggests that U6 might interact with the 5' splice site in a dynamic manner upon association with the spliceosome in the form of tri-snRNP. We propose that whereas U6 interacts dynamically with the 5' splice site over a broader region near the ACAGA box, the dissociation of U4 results in a structural change to U6 and confines its interaction with the 5' splice site to a specific region. Base-paired interactions upstream of the ACAGA box predominate in the absence of NTC, whereas downstream interactions are stabilized by NTC, leading to the activation of the spliceosome. Nevertheless, the possibility remains that dynamic interactions in the preactivated spliceosome are confined to the X2a type of interaction immediately following the dissociation of U4 and then switch to the X2b type upon binding of NTC to the spliceosome.

Another set of interactions between U6 and the intron, as represented by the cross-linked product X1, also requires the function of NTC, since X1 was neither seen in the splicing reaction carried out in NTC-depleted extracts nor in reactions performed at lower concentrations of ATP (Figs. 2A and 3). Destabilization of Lsm complex from U6 is required for such interactions, which involve the Lsm binding site of the U6 3' terminal U-tract with the intron in a region ~30 bases downstream of the 5' splice site. It is not clear whether the selection of a proper base-pairing mode at the 5' splice site is a consequence or prerequisite for this interaction. It is also possible that after the dissociation of U4, U6 anRNP is remodeled in such a way as to coordinate interactions in both regions so as to stabilize the association of U6 with the spliceosome. Since NTC is constituted of at least eight protein components, it remains to be seen whether NTC components mediate these events in a cooperative manner or individual components contribute differentially in modulating specific interactions.
The interactions of U5 with pre-mRNA have been studied by both biochemical and genetic methods and demonstrated that the U5 conserved loop 1 can interact with the 5' and 3' exons of pre-mRNA near the splice junctions (11–14, 16, 33). By site-specific cross-linking, it was shown that U5 loop 1 contacts the last residue of the 5' exon before and after the first catalytic reaction and also contacts the first two residues of the 3' exon in the second catalytic reaction, presumably serving to align the two exons for ligation (12). Although it was later demonstrated that the loop 1/5' exon interactions are not necessary for the first catalytic step of splicing but are critical for the second step in the yeast spliceosome (16), it is critical for U5 to hold the 5' exon following cleavage from the pre-mRNA prior to the step 2 splicing reaction. By UV-cross-linking analysis using Ac/Cla pre-mRNA, we were able to analyze the interaction between U5 and the pre-mRNA in broader regions prior to catalytic steps. Consistent with previous results, we detected strong cross-links at the last and penultimate residues of the 5' exon with U5 loop 1, represented by cross-linked products Y1 and Y3, in the active spliceosome. In extracts depleted of NTC, multiple cross-links in the 5' exon extending from −1 to −10 were detected, suggesting that NTC was required for targeting specific interactions of U5 with the 5' exon sequence of pre-mRNA in the 5' splice site. These results are not only consistent with a previous report that U5[96–99] on U5 loop 1 cross-linked to the 5' splice site from −6 to +2 when replaced with photoactivatable 4-thiouridine (33) but gives further insights into dynamic change in U5/5' splice site interaction at different stages of spliceosome assembly.

In addition to this conventional 5' splice site/U5 loop 1 interaction, novel interactions between loop 1 and the 5' exon in a region 50–60 bases upstream of the 5' splice site, represented by cross-linked products Y2 and Y4, were detected. Cross-linking identified in this region appeared to spread over a broader region, in which a high photochemical background was observed, thus making it difficult to determine precise cross-linking sites by primer extension analysis. Experiments with pre-mRNA containing different sequence in this region suggested that distance dependence from the 5' splice site rather than sequence specificity was important for this set of cross-linking (data not shown). Interestingly, cross-linking sites on U5 were also mapped to the loop 1 region. Thus, the U5 loop 1 not only appears to interact with the 5' splice site in a dynamic manner but may also interact with the 5' exon of pre-mRNA further upstream.

Dynamic interactions were also seen in the U4-containing prereactivated spliceosome, from which a similar pattern of U5 cross-linking to the pre-mRNA was observed. Both sets of cross-link, Y1/Y3 and Y2/Y4, were detected, although the cross-linking site of Y1 and Y3 were slightly downstream, extending from +1 to −6. Thus, dissociation of U4 did not cause a dramatic change in the interaction of U5 with the pre-mRNA, but binding of NTC eliminated the upstream interaction and promoted specific interaction at the 5' splice site. How U5 loop 1 can interact with two regions of the pre-mRNA 50–60 bases apart in the absence of NTC function is not clear. It is possible that during the association of tri-snRNP with the spliceosome, the U5 loop 1 might interact dynamically with the 5' exon over a broad region down to the 5' splice site but accessible to cross-linking only at both ends of the region upon UV-irradiation. Alternatively, this upstream region could be brought close to the 5' splice site due to pre-mRNA secondary structure or through interaction with protein components. Nevertheless, this dynamic interaction might serve to hold U5 in a position awaiting proper configuration of the pre-mRNA to target the site for specific interaction. We previously demonstrated that NTC is required for stabilization of U5 and U6 on the spliceosome after U4 dissociation (21). However, the stability of U5 is affected to a lesser extent in a stability assay in which, when the majority of U6 is dissociated, 50% of U5 remains associated in the absence of NTC (21). This suggests that the dynamic interactions of U5 and the 5' exon might contribute in part or act in concert with protein components of U5 snRNP in stabilizing the association of U5 with the spliceosome.

![Diagram of the yeast U5 RNA and cross-linking sites](image-url)
U4/5’ splice site interaction has been demonstrated in the yeast spliceosome using an in vitro trans-splicing system. The +2 residue of the intron can cross-link to residues 75, 78, and 82 of U4 downstream of the U4/U6 helix I independent of the U2 association with the branch point, indicating that U4 RNA also contacts the pre-mRNA during its association with the spliceosome (10). By carrying out splicing at lower concentrations of ATP in a conventional cis-splicing reaction, we have been able to isolate the preactivated spliceosome and detected multiple cross-linked products of U4 to the pre-mRNA (data not shown). The fact that U4 dissociation resulted in changes in U6/5’ splice site interaction to be confined to a smaller region suggests cooperative interactions of U4 and U6 with the 5’ splice site in the preactivated spliceosome. It is likely that both U4 and U6 are dynamically in close contact with the 5’ splice site during the establishment of the tri-snRNP/pre-mRNA interaction, and U4 may play an important role in aligning U6 to the 5’ splice site as previously suggested (10). The dissociation of U4 may position U6 to specific sites in the presence of NTC function. In the absence of NTC, the U6/5’ splice site interaction, although also more confined, is not correctly positioned to allow the activation of the spliceosome.

Besides U4 and U6, the conserved splicing factor Prp8 was also shown to cross-link to the 5’ splice site at the +2-position independent of prior binding of U2 snRNP to the branch point (34). Given that Prp8 is a component of U5 snRNP, it was proposed that U1 and U5 snRNP functionally collaborate to recognize and define the 5’ splice site at early steps of spliceosome assembly. Coupling of U1 snRNP and tri-snRNP recognition of the 5’ splice site has also been suggested by study of the U4 cold-sensitive mutant, U4-cs1, which contains triple nucleotide substitution immediately downstream of the U4/U6 stem 1, resulting in extended pairing of stem 1, thus masking the region in U6 that ultimately pairs with the 5’ splice site (35). Spliceosomes formed in the U4-cs1 mutant at 16 °C fail in unwinding of U4/U6 and accumulate higher levels of U1, suggesting simultaneous association of U1 snRNP and tri-snRNP with the 5’ splice site and coupling between displacement of U1 from and pairing of U6 with the 5’ splice site (36). Supporting this notion, interactions between protein components of U1

**Fig. 8.** A summary of dynamic interactions of U5 and U6 with pre-mRNA and the role of NTC in the process of spliceosome activation. Ac/Cla pre-mRNA is indicated by a black filled box for the 5’ exon and a black line for the intron with sequences near the 5’ splice site and the branch point (bp) shown. X, nonconserved exon nucleotide. Multiple drawings of U5 and U6 indicate dynamic interactions with pre-mRNA. Orange open box: the conserved ACAGA of U6; red block, documented base pairings between U6 and the 5’ splice site; red bar, cross-linked residues identified by primer extension analysis; zigzags, cross-linking by UV; red dashed line, cross-linking mapped by RNase H digestion. The question mark indicates that it is not known whether base pairings between U2 and U6 form in the absence of NTC.
snRNP and tri-snRNP that may contribute to stabilization of tri-snRNP binding to the spliceosome have also been documented (37–39). Our finding of dynamic interactions between U5 RNA and the 5' exon suggests a role of U5 snRNP in the association of tri-snRNP with the spliceosome through RNA-RNA interactions. Since the dissociation of U4 did not perturb dynamic interactions of U5 with the pre-mRNA to any great extent, the U5/pre-mRNA interaction is largely independent of the interaction of U4, and perhaps also U6, with the pre-mRNA. It was shown that inactivation of either U6 or U4 snRNP prevents the association of Prp8 with the 5' splice site (34). This may suggest that the integrity of U4/U6 might be required for the initial interaction of tri-snRNP to target U5 snRNP to the spliceosome but is no longer important once the preliminary dynamic interactions between U5 and the pre-mRNA are established.

Taken together, we demonstrate dynamic interactions between U5 and the 5' exon, and between U6 and the 5' splice site during the association of tri-snRNP with the spliceosome. A proposed scheme for such interactions is shown in Fig. 8. The initial U6/5' splice site interaction involves dynamic interactions of two sets of ACA trinucleotides, 42–44 and 47–49, on U6 with the UGU at positions +4 to +6 of the intron. Upon dissociation of U4, the interaction between U6 and the 5' splice site becomes more confined to one of the ACAs, depending on whether NTC is present. In the absence of NTC, the upstream 42ACA44 is used. In the presence of NTC, the 47ACA49 within the conserved ACAGA box base pairs with the UGU to form the active spliceosome. This proposed equilibrium between the two states of ACA/UGU pairing agrees with the previous result of U4-cs1 trans-acting suppressors in that mutations within 42ACA44 of U6 small nuclear RNA were identified (35). These suppressors were interpreted to act by preventing inappropriate pairing of 42ACA44 with the UGU sequence of the 5' splice site when 47ACA49 is masked by extended pairing in U4-cs1. An alternative explanation for the action of these suppressors in the view of equilibrium between the two pairing states is that mutations within 42ACA44 would result in shift of the equilibrium toward pairing with 47ACA49, thus facilitating formation of the active spliceosome. In addition to a shift between the two ACA/UGU pairing states after U4 is dissociated, the U6 snRNP undergoes further structural rearrangement to destabilize Lsm to bring about a novel interaction between the 3' end of the Lsm binding site and the intron sequence ~50 bases down-stream of the 5' splice site. U5 also interacts with the 5' exon dynamically over a broad region at its initial binding to the pre-mRNA. Such interactions were not perturbed to a great extent after U4 dissociation until the binding of NTC, which plays an exclusive role in determining specific interactions of U5 with the 5' splice site. All together, NTC may function as specificity factor in defining base-pairing interactions of U5 and U6 with pre-mRNA in the active spliceosome. Base-pairing interaction between U2 and U6 constitutes an important part of the catalytic core of the spliceosome. Such an interaction arises only after U4 is dissociated, since the same region of U6 that base-pairs with U2 is used to base-pair with U4 in U4/U6 di-snRNP. Although it seems certain that U2/U6 base pairing is in the active spliceosome, whether formation of such base pairing requires the function of NTC remains to be elucidated.

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