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Proximity of the U12 snRNA with both the 5′ Splice Site and the Branch Point during Early Stages of Spliceosome Assembly†

Mikko J. Frilander* and Xiaojuan Meng

Institute of Biotechnology, Program on Developmental Biology, PL56 (Viikinkaari 9), 00014 University of Helsinki, Finland

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U12 snRNA is required for branch point recognition in the U12-dependent spliceosome. Using site-specific cross-linking, we have captured an unexpected interaction between the 5′ end of the U12 snRNA and the −2 position upstream of the 5′ splice site of P120 and SCN4a splicing substrates. The U12 snRNA nucleotides that contact the 5′ exon are the same ones that form the catalytically important helix Ib with U6atac snRNA in the spliceosome catalytic core. However, the U12/5′ exon interaction is transient, occurring prior to the entry of the U4atac/U6atac.U5 tri-snRNP to the spliceosome. This suggests that the helix Ib region of U12 snRNA is positioned near the 5′ splice site early during spliceosome assembly and only later interacts with U6atac to form helix Ib. We also provide evidence that U12 snRNA can simultaneously interact with 5′ exon sequences near 5′ splice site and the branch point sequence, suggesting that the 5′ splice site and branch point sequences are separated by <40 to 50 Å in the complex A of the U12-dependent spliceosome. Thus, no major rearrangements are subsequently needed to position these sites for the first step of catalysis.

During pre-mRNA splicing, the 5′ splice site (5′ss) and the branch point sequence (BPS), located at the opposite ends of each intron, must come into close proximity to facilitate the chemistry of the first catalytic step (4). In metazoan cells, two different types of introns, namely, the U2 type and U12 type, are recognized by their different 5′ss and BPS sequences. The U2-type introns comprise >99.5% of all spliceosomal introns and contain relatively poorly conserved 5′ss and BPS regions. In contrast, these sequences are highly conserved in the low-abundance U12-type introns (13, 47).

Distinct U2- and U12-dependent spliceosomes facilitate the removal of the two different intron types. These two spliceosomes differ in their composition of small nuclear ribonucleoproteins (snRNPs), but their overall assembly pathways and catalytic mechanisms are similar. The U1, U2, U4, and U6 small nuclear RNAs (snRNAs) are specific to the U2-dependent spliceosome, whereas U11, U12, U4atac, and U6atac snRNAs are functionally analogous components in the U12-dependent spliceosome. Both spliceosomes utilize the U5 snRNA (42, 56).

A widely accepted paradigm of spliceosome assembly states that snRNPs and protein components associate with the pre-mRNA in a stepwise manner (3, 4, 16). In the U2-dependent spliceosome, this process starts with intron recognition in complex E (the commitment complex in yeast), in which the 5′ss is recognized by the U1 snRNP, whereas the BPS, polypyrimidine tract (PPT), and 3′ splice site (3′ss) are recognized by the protein factors SF1/BBP, U2AF65, and U2AF35, respectively (1, 36, 37, 44, 68, 75). In the first ATP-dependent stage (complex A, or prespliceosome), SF1/BBP at the BPS is replaced by the U2 snRNP (43, 74). A spliceosome (complex B) is formed upon entry of the U4/U6.U5 tri-snRNP (20, 24). Additional rearrangements of RNA-RNA interactions (39, 50), and changes in protein composition (33) result in the final catalytically active spliceosome (complex C) in which the U2 and U6 snRNAs together with the pre-mRNA form the catalytic core (6, 17, 32). In the catalytic core, U5 snRNA and Prp8/220-kDa protein interact with exon sequences near the 5′ss (38, 49, 57, 69), while U6 and U2 snRNAs base pair with the 5′ss and BPS, respectively, and with each other to achieve a structure that can carry out likely RNA-based catalysis (21, 25, 59, 60, 74). During the assembly, U1 and U4 snRNPs are released from the final spliceosome prior to catalysis. However, this traditional view of spliceosome assembly has been challenged recently by the identification of a penta-snRNP structure in yeast cell extracts that exhibits splicing activity (52). These data suggest that individual spliceosomal complexes that have been most commonly analyzed by native gels may represent dissociation products of the dynamic preassembled penta-snRNP complex in different conformational stages (3, 40). Although this question is not yet fully resolved, the identification of the U5/5′ exon interactions in early spliceosomal complexes (35, 49) and the detected interaction between U1 and U5 snRNPs (1, 2) suggest that larger spliceosomal complexes may indeed be involved already in intron recognition, an idea that is consistent with the penta-snRNP model of assembly.

The assembly of the U12-dependent spliceosome follows the sequential assembly model of the U2-dependent spliceosome (41), although the data are also compatible with the dynamic penta-snRNP model. As in the assembly process described above, the 5′ss and BPS of a U12-type intron initially base pair with U11 and U12 snRNAs, respectively, to form a prespliceosome complex A (14, 23, 55, 72). In contrast to the U2-dependent system, a complex E has not been described, but rather at the earliest stage a preformed U11/U12 di-snRNP (61, 63) cooperatively recognizes U12-type introns. In particular, the

* Corresponding author. Mailing address: Institute of Biotechnology, Program on Developmental Biology, PL56 (Viikinkaari 9), 00014 University of Helsinki, Finland. Phone: 358-9-191-59509. Fax: 358-9-191-59366. E-mail: Mikko.Frilander@Helsinki.Fi.

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U12-BPS interaction requires a prior U11-5′ss interaction (10). Following initial intron recognition, the entry of the U4atac/U6atac.U5 tri-snRNP to the nascent spliceosome promotes global structural rearrangements that lead to catalytic core formation. As in the U2-dependent spliceosome, the U6atac and U12 snRNAs are base paired both together and with the 5′ss and BPS in the catalytic core, respectively, thus holding the critical 5′ss and BPS regions in proximity for the first step of catalysis (9, 19, 48, 54).

In this study, we investigated in vitro recognition of the 5′ss using site-specific cross-linking methods. Unexpectedly, we captured an interaction between the 5′ end–most region of the U12 snRNA and the −2 position of the P120 splicing substrate. This transient interaction takes place early during spliceosome assembly. The region of the U12 interacting with the −2 position also forms the catalytically important U12/U6atac helix Ib in the catalytic core. We also captured a similar interaction using a cross-linking substrate derived from an unrelated SCN4a splicing substrate. Our data suggest that this region of U12 snRNA is positioned near the 5′ss before the tri-snRNP-specific interactions occur, further suggesting that catalytically important structures may start forming at the earliest stages of spliceosome assembly.

MATERIALS AND METHODS

2′-*O*-Methyl RNA oligonucleotides. The 2′-*O*-methyl RNA (2ome) oligonucleotides were purchased from the Keck Oligonucleotide Synthesis Facility, Yale University. The 2ome oligonucleotides used to pretreat the nuclear extracts were U2b and $U1_{1\text{-}14}$, both used to block U2-dependent splicing (27, 55); $U12_{1\text{-}16}$, complementary to helix I forming region of U12 snRNA (54); U12-BPS, complementary to nucleotides 11 to 28 of U12 snRNA (55); $U6atac_{1\text{-}20}$, complementary to the 5′ss binding region of U6atac snRNA (9, 54); and the 5′ exon oligonucleotides complementary to nucleotides 6 to 22 ("-6 oligo") (9) or 8 to 24 ("-8 oligo") upstream of the 5′ss in the P120 splicing substrate.

Splicing substrates. Capped, full-length P120 splicing substrates were produced by in vitro transcription as described before (9). The chimeric P120-SCN4a substrate contains a P120 5′ exon and intron plus 3′ exon sequences from the SCN4a substrate (15). The construction of P120 substrates with two ^{4S}U residues (P120[−2/+2]) has been described previously (9, 11). P120 substrates containing a single ^{4S}U substitution (P120[−2] or P120[+2]) were constructed essentially as was P120[−2/+2]. The ^{4S}U-containing SCN4a cross-linking substrates were constructed essentially as the P120 cross-linking substrate described above. The SCN4a cross-linking substrates contain 5′ exon, intron, and 3′ exon sequences derived from SCN4a-ENH1 splicing substrate (15), but the 5′ exon has been truncated and is similar in size as in P120 splicing substrate (41 versus 46 nucleotides) and modified to contain a single ^{4S}U at the −2 position and a short sequence complementary to 5′ end of U12 snRNA. The P120 splicing substrates containing mutations in the site interacting with U12 snRNA and the substrates containing duplicated 5′ splice sites were constructed by PCR and verified by sequencing.

Splicing reactions. In vitro splicing reactions were performed as described before (10) using ^{32}P-labeled P120 substrate. Splicing of the chimeric P120-SCN4a substrate was accomplished as described before (67), except that 500 nM $U1_{1\text{-}14}$ 2ome oligonucleotide was used to inhibit the U2-dependent spliceosome. Cross-linking reactions included 10 nM trace-labeled ^{4S}U-containing P120 substrates or 20 nM SCN4a substrates; for the P120 substrates the cross-linking reaction conditions were as described previously (9), while with the SCN4a substrates the cross-linking reactions are carried out in splicing conditions described by Wu and Krainer (67), except that the substrate concentration was 20 nM. Native gel analysis was performed as described before (10) using 25 μg/ml heparin in the loading buffer, unless otherwise indicated.

Cross-linking and identification of cross-linked RNA species. The conditions for performing and processing psoralen and ^{4S}U cross-linking reactions were described previously (9, 10). In time course experiments and in mapping (see Fig. 2B and C and 4A), a saturating concentration (20 nM) of splicing substrate was used to maximize amount of the cross-linked product. In other reactions the substrate concentration was 5 to 10 nM. A 365-nm UV lamp (UVL-225D; UVP, Cambridge, United Kingdom) or a 337-nm N_2 excimer laser (PSX-100; Neweks Ltd., Tallinn, Estonia) was used to excite the ^{4S}U residues during cross-linking reactions. Cross-linked RNAs were separated in denaturing 5 to 8% polyacrylamide gels and detected by Northern blotting (9, 54).

The location of the U12/5′ exon cross-link on U12 snRNA was determined using P120[−2] substrate. A large-scale cross-linking reaction (250 μl) was performed in the presence of 5′ exon oligonucleotide. The sample was irradiated for 3 min on ice with the 337-nm UV laser (100 Hz pulse rate, 0.3 mJ/pulse) to produce sufficient amounts of the U12-specific cross-link for mapping purposes. Following irradiation, the sample was treated with proteinase K, extracted with phenol, and precipitated with ethanol. The U12 snRNA and cross-linked products thereof were specifically 3′ end labeled with ^{32}P-dCTP using the "splint-labeling" method (17) and were subsequently gel purified. End-labeled RNAs were partially hydrolyzed with RNase T_1 or PhyM (0.5 U of either RNase, incubated for 20 min at 50°C in a buffer containing 6.72 M urea, 2 mM EDTA, 0.24 mg/ml yeast tRNA, 14.4 mM morpholineethanesulfonic acid, pH 6.0), and analyzed directly on a 5% denaturing polyacrylamide gel followed by autoradiography.

RESULTS

U12 snRNA interacts with 5′ exon sequences. We previously used site-specific UV cross-linking with a modified P120 splicing substrate (P120[−2/+2]) containing two 4-thio-uridine (^{4S}U) residues at the −2 and +2 positions relative to the 5′ss to investigate the in vitro recognition of the 5′ss by the components of the U12-dependent spliceosome in HeLa nuclear extract (9). Figure 1A illustrates the snRNA/pre-mRNA interactions near the 5′ss that were previously captured using the P120[−2/+2] substrate. However, our present work yielded unexpected results when the P120[−2/+2] substrate was used in psoralen cross-linking experiments followed by northern hybridization with a U12 snRNA–specific probe. In addition to the expected psoralen-induced U12-BPS and U12-U6atac helix I cross-links (9), we detected an additional cross-linked band (Fig. 1B, lane 4). This cross-link was dependent on ^{4S}U residues in the substrate (compare lanes 3 and 4) and was inhibited by 2′-*O*-methyl RNA (2ome) oligonucleotides complementary to the U12 snRNA (lanes 5 and 6), suggesting that the novel cross-link is formed between U12 snRNA and either one of the −2 or +2 ^{4S}U residues in the splicing substrate. This was confirmed by an RNase H targeting experiment in which UV cross-linking was performed in the absence of psoralen, followed by DNA oligonucleotide–directed RNase H cleavage. RNase H cleavage of the cross-link-containing band was observed only with DNA oligonucleotides complementary to the U12 snRNA or the P120 splicing substrate (Fig. 1C, lanes 2, 6, and 7).

To delineate which of the two ^{4S}U residues was cross-linked to U12 snRNA, we constructed two additional splicing substrates, each containing a single ^{4S}U residue either at the −2 position (P120[−2]) or the +2 position (P120[+2]). These substrates were subsequently used in cross-linking experiments in parallel with P120[−2/+2]. Northern analysis revealed that a single ^{4S}U residue at the −2 position was sufficient for the U12-specific cross-link formation (Fig. 2A, compare lanes 4 and 10), whereas no U12-specific cross-link was detected using P120[+2] (data not shown).

To confirm the identity of the ^{4S}U-containing substrates, blots from the above experiments were stripped and reprobed for U5 snRNA (U5 interacts with the −2 site) and U11 snRNA (U11 interacts with the +2 site). As expected, P120[−2/+2] produced cross-links that were detected with both the U5- and

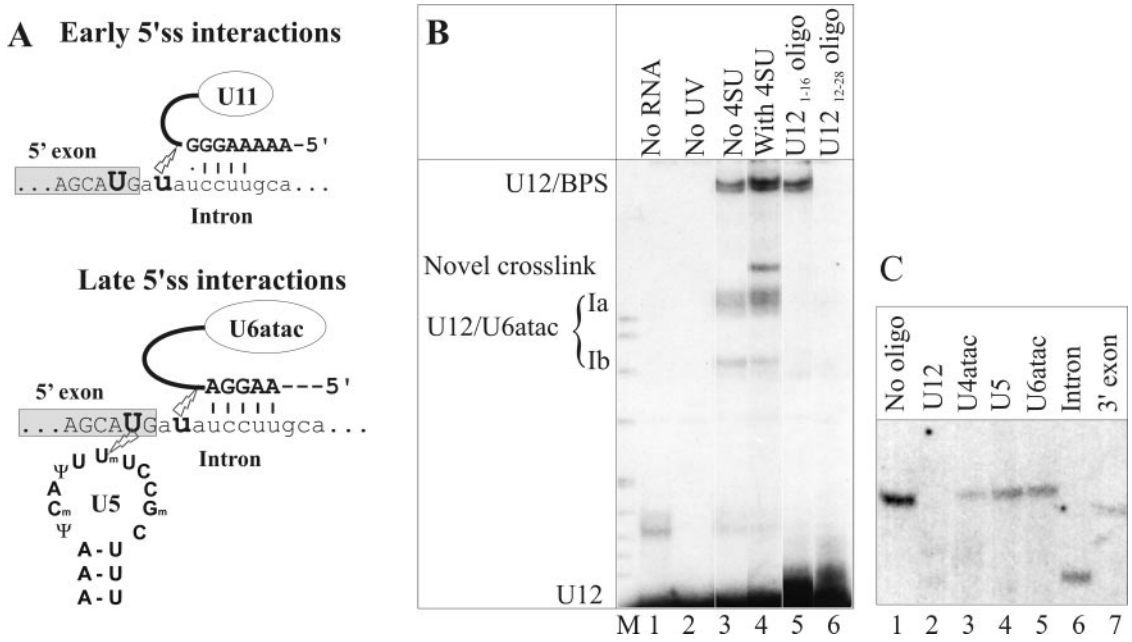

FIG. 1. (A) Reported snRNA/pre-mRNA interactions near the 5′ss. At early stages of spliceosome assembly, U11 can be cross-linked to the +2 position of the 5′ss consensus sequence in the intron. At late stages of assembly, the +2 position interacts with U6atac whereas the −2 position (in the exon) interacts with U5. Predicted cross-links are shown as lightning bolts, but the exact nucleotides within each snRNA shown have not been identified. (B) Combined psoralen and ^{4S}U cross-linking analysis. The ^{4S}U-containing P120[−2/+2] splicing substrate was incubated in HeLa nuclear extract under splicing conditions for 90 min. 4′-aminomethyl-4,5′,8-trimethyl-psoralen was then added to each reaction, followed by irradiation at 365 nm and Northern analysis using a U12-specific probe as described in Materials and Methods. The conditions for individual reactions are indicated above each panel, and the positions of the cross-linked RNA species are indicated on the left. The control substrate in lane 3 did not contain ^{4S}U. (C) Identification of U12-specific cross-links by RNase H cleavage. The reactions were performed as for panel B except that psoralen was omitted and the incubation time was 60 min. The cross-linked RNA samples were incubated with RNase H plus 100 pmol of DNA oligonucleotides complementary to snRNAs or P120 splicing substrate as indicated above each lane. Subsequently, cross-links were visualized by Northern blotting with a U12-specific probe as described for panel B.

U11-specific probes (Fig. 2A, lane 4, middle and upper panel), whereas with P120[−2] only the U5-specific cross-links were observed (lane 10, upper panel). Furthermore, both substrates produced U12-specific cross-links that had virtually identical intensities and responded similarly to various 2ome oligonucleotides used to block spliceosome assembly (see below). Therefore, based on RNase H targeting and the use of splicing substrates containing a single ^{4S}U residue, we conclude that U12 snRNA is in close proximity with the −2 position of the P120 splicing substrate during spliceosome assembly in nuclear extracts.

To further investigate the requirements for the U12/5′ exon interaction, we arrested spliceosome assembly at various stages using 2ome oligonucleotides complementary to either the P120 splicing substrate or the snRNA components of the U12-dependent spliceosome (see Materials and Methods). Both P120[−2/+2] and P120[−2] were used to test the effect of the 2ome oligonucleotides, and both substrates displayed identical U12 snRNA-specific cross-linking patterns (Fig. 2A). The most intriguing results were obtained by including a P120 5′ exon oligonucleotide, which stimulated U12/5′ exon cross-link formation by approximately three- to fivefold compared with the control (Fig. 2A, compare lanes 4 and 5 and 10 and 11). Furthermore, the U12/5′ exon cross-link was insensitive to the U6atac$_{1\text{-}20}$ oligonucleotide (compare lanes 4 and 6 and 10 and 12). Together the data above suggest that the U12/5′ exon interaction could take place early during spliceosome formation, since both the U6atac$_{1\text{-}20}$ and the 5′ exon 2ome oligonucleotides also prevent the U6atac/5′ss interaction (9 and data not shown) and either abolish or reduce the extent of the U5/5′ exon interaction (Fig. 2A, U5 panel, lanes 4 and 5 and 10 and 11).

In contrast, each of the two U12-specific oligonucleotides used in this experiment prevented the formation of the U12/5′ exon cross-link. The lack of cross-link with the U12-BPS oligonucleotide (Fig. 2A, lanes 8 and 14) was most likely due to inhibition of prespliceosome formation, suggesting that the U12/5′ exon interaction requires prior U12/BPS interaction. This is supported by similar negative cross-linking results from experiments with a 3′-truncated P120[−2] substrate lacking the BPS, 3′ss, and the 3′ exon (data not shown). The above results suggest, together with ATP requirement for cross-link formation (Fig. 2B), that the U12-5′ exon interaction takes place after the intron recognition but prior to tri-snRNP entry to the spliceosome. The observed inhibition of the cross-link formation with the U12$_{1\text{-}16}$ oligonucleotide (Fig. 2A, lanes 7 and 13), which prevents the formation of the U12/U6atac helix I but not the U12/BPS interaction (Fig. 1B, lane 5), is in contrast most likely due to a direct or indirect blockage of the U12 region that interacts with the 5′ exon (see below).

An important question is whether the U12/5′exon interaction takes place in functional spliceosomal complexes, in which

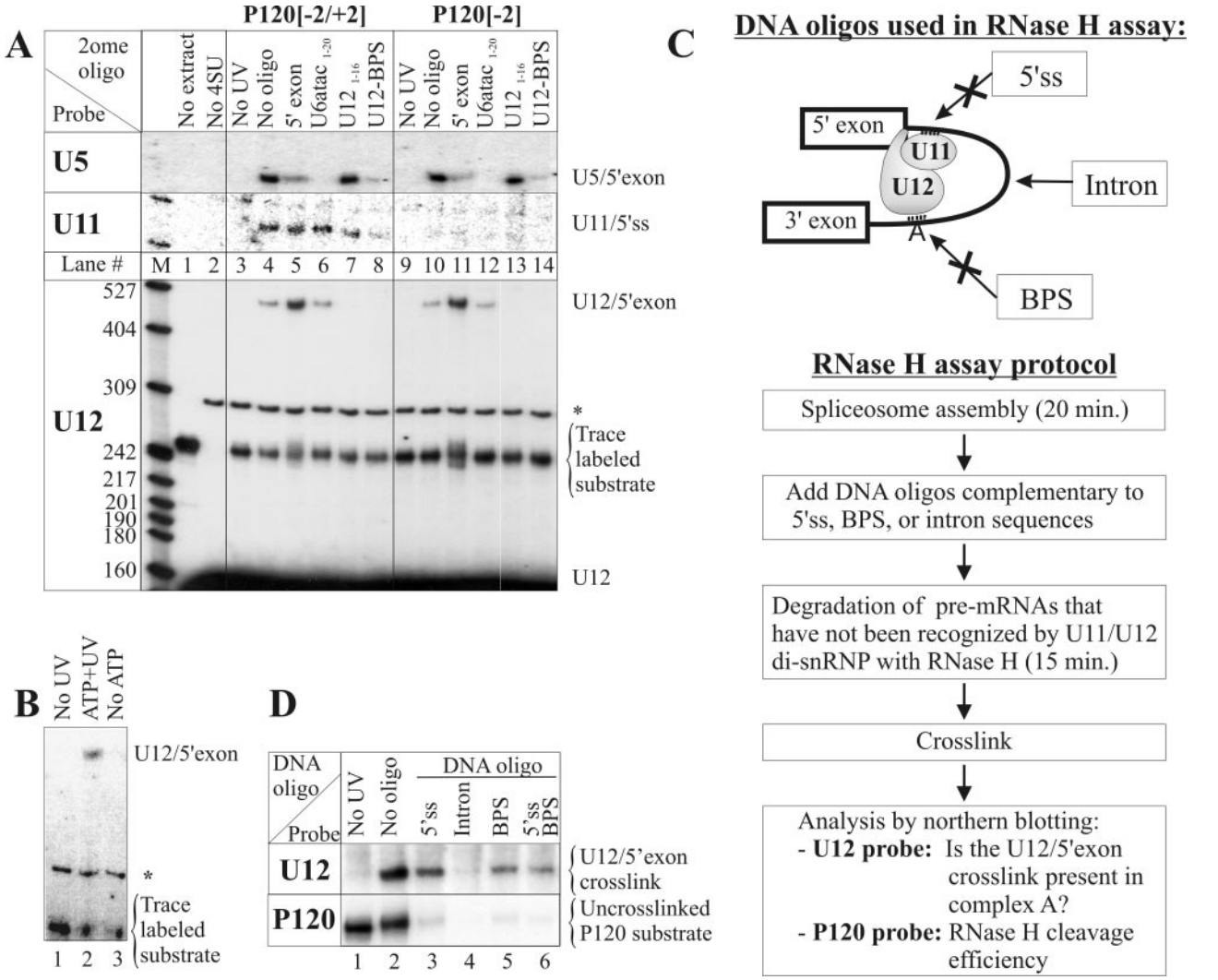

FIG. 2. (A) Localization of the ^{4S}U cross-linked position in the P120 pre-mRNA. P120 splicing substrates containing either two (P120[−2/+2], lanes 1 and 3 to 8) or one ^{4S}U residue (P120[−2], lanes 9 to 14) were cross-linked after incubation at 30°C for 40 min (as in Fig. 1C) and analyzed by Northern blotting using probes specific for U5, U11, or U12 as indicated. The specific 2ome oligonucleotide included in each reaction is indicated above each lane. The identity of each specific cross-link is indicated on the right, and the sizes of molecular weight marker (M, in nucleotides), a ^{32}P-labeled pBR322 MspI digest, are shown on the left. The asterisk denotes a nonspecific cross-reacting band. (B) ATP dependence of the U12 cross-link formation. P120[−2] was incubated in HeLa nuclear extract under normal splicing conditions (lane 2) or in conditions without exogenously added ATP or creatine phosphate (lane 3) and which has also been preincubated at 30°C for 20 min to hydrolyze endogenous ATP. The asterisk denotes a nonspecific cross-reacting band. (C) Protocol for the RNase H assay. (Top) The location and effect of DNA oligonucleotides used in the RNase H assay. In the spliceosome A complex only the intron-specific DNA oligonucleotide is able to bind P120 pre-mRNA and support RNase H hydrolysis, while the U11/U12 di-snRNP binds 5′ss and BPS sequences and protects those sites from DNA oligonucleotide-directed RNase H cleavage as indicated in the figure. (Bottom) Experimental protocol for the RNase H assay. (D) Protection of the U12/5′exon cross-link from the DNA oligonucleotide directed RNase H cleavage. The experimental protocol and the DNA oligonucleotides used have been described for panel C.

both the 5′ss and BPS have been simultaneously recognized by the spliceosomal components. We have previously used DNA oligonucleotide-directed RNase H hydrolysis to show that the 5′ss and BPS are recognized by U11/U12 di-snRNP (10), which renders those sites resistant to RNase H hydrolysis. Here we used a similar method to ask if the U12/5′ exon cross-link is resistant to a prior RNase H hydrolysis at the 5′ss and BPS. Short DNA oligonucleotides directed to 5′ss, intron, or BPS were added to the splicing reactions prior to the UV irradiation step, and the cross-link formation was subsequently investigated by Northern blotting (see Fig. 2C). We found that the U12/5′ exon cross-link is resistant to the RNase H hydrolysis at the 5′ss and BPS, even when both sites were targeted simultaneously (Fig. 2D, upper panel, lanes 2, 3, 5, and 6). In contrast, the DNA oligonucleotide targeting the intron sequence resulted in a loss of the cross-link signal (lane 4). The reduced cross-linking intensity in the presence of DNA oligonucleotides targeting the 5′ss or BPS in comparison to the control reaction (compare lane 2 to lanes 3, 5, and 6) is most likely due to competition between oligonucleotide versus U11/U12 com-

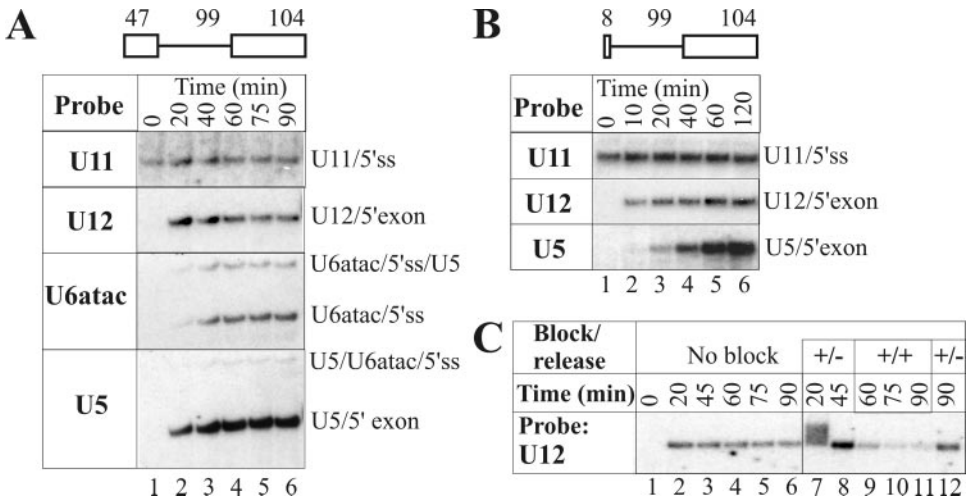

FIG. 3. (A) Time course of cross-link formation. The P120[−2/+2] splicing substrate containing a 47-nucleotide 5′ exon was cross-linked as in Fig. 1C at time points indicated above each lane and subsequently analyzed by Northern blotting. The filters were probed for U11, U12, U6atac, or U5 snRNAs, as indicated. The identity of each cross-linked band is indicated on the right. (B) Time course analysis with P120[−2/+2] substrate containing an 8-nt 5′ exon. The reactions were analyzed as in panel A. The strong U11/5′ss signals in lane 1 are the results of saturating concentrations of the substrate used in this experiment (see Materials and Methods). (C) Reversal of the U12-5′ exon interaction. Lanes 1 to 6, time course of U12 cross-link formation under standard reaction conditions. Lanes 7 to 12, a block/release reaction in which a tailed version of the 5′ exon 2ome oligonucleotide (9) was used to arrest the U12-specific cross-link for 45 min (lanes 7 and 8) followed by the addition of a complementary release oligonucleotide (lanes 9 to 11) as indicated above each lane. Lane 12 shows a control reaction without release oligonucleotide. The reactions were analyzed by Northern blotting as in Fig. 1C.

plex binding to the pre-mRNA, since we can detect a similar reduction in the A complex intensity in native gel analyses following the RNase H treatment with 5′ss- and BPS-specific oligonucleotides (data not shown).

The efficiency of the RNase H hydrolysis with each oligonucleotide was confirmed by probing the same blot for P120 splicing substrate (Fig. 2D, lower panel). The level of RNase H resistant P120 signal (maximum, ~5% of the input) in lanes 3, 5, and 6 agrees with our earlier study (10), and suggests that while only a minor population of the splicing substrates have been assembled into RNase H resistant spliceosomal complexes, the majority, if not all, of the U12/5′ exon interactions are taking place in that population. We conclude that the U12/5′ exon cross-link takes place in spliceosomal complexes in which both the 5′ss and BPS have been recognized by the components of the spliceosome, most likely by U11 and U12 snRNPs, respectively.

The U12 snRNA-5′ exon interaction occurs in the prespliceosome and is transient. To investigate the timing of the U12-5′ exon interaction, we compared the kinetics of the U12-specific cross-link formation with the other snRNA–pre-mRNA interactions occurring in the vicinity of the 5′ss. The time course of various snRNA/pre-mRNA cross-links was followed using the P120[−2/+2] substrate by hybridizing the same Northern blot with probes complementary to the individual snRNA components of the U12-dependent spliceosome. Maximal U12/5′ exon cross-linking was detected after 20 min of incubation at 30°C (Fig. 3A, lane 2), after which the cross-link slowly decreased. Comparison of U12/5′exon cross-link formation with the tri-snRNP-specific U6atac/5′ss and U5/5′ exon cross-links suggests that the U12-5′ exon interaction takes place prior to the formation of the tri-snRNP-specific interactions, which level off after 60 min. More detailed kinetic data were obtained by repeating the above experiment with a P120[−2/+2] substrate containing only an 8-nucleotide (nt) 5′ exon (Fig. 3B). This substrate does not interact with U6atac, and the appearance of the U5/5′ss cross-link is delayed and its formation is less efficient (9). Using this truncated splicing substrate, the U12-specific cross-link was readily detected at the 10-min time point (Fig. 3B, lane 2). In contrast, only a faint U5/5′ exon cross-link was visible after 20 min (lane 3), indicating that the U12/5′ exon interaction occurs prior to the U5- or U6atac-specific interactions with pre-mRNA. Interestingly, the U12/5′ exon cross-link intensity was not reduced with the truncated substrate at the later time points, suggesting that the reduction of the U12-5′ exon interaction at the later time points may be linked to the entry of the tri-snRNP.

In contrast, the U11-5′ss interaction was observed with both splicing substrates incubated on ice (Fig. 3A and B, lane 1). The lack of a U12-specific cross-link on ice along with the ATP requirements for the U12/5′ exon cross-link formation (Fig. 2B) suggest that the U12-5′ exon interaction requires prior spliceosome assembly.

The kinetics of the U12-5′ exon interaction were further investigated using the oligonucleotide block-release method (9), which synchronizes the spliceosome assembly, thereby increasing the precision with the kinetic measurements. Since the U12/5′ exon cross-link intensity was enhanced with the 5′ exon oligonucleotide, we tested the outcome after the release from the blockage. Compared with the control time course (Fig. 3C, lanes 1 to 6), the presence of the blocking oligonucleotide enhanced cross-link formation (lanes 7 and 8; note that the long, tailed oligonucleotides sometimes smear the bands containing cross-links). When the blocking oligonucleotide was released, the U12/5′ exon cross-link signal disappeared almost completely (lanes 9 to 11). These data, together

with the time course experiment above (Fig. 3A and B), suggest that the U12-5′ exon interaction not only occurs early but also is transient.

5′ exon oligonucleotide increases the stability of the complex A. Since the U12-5′ exon interaction appears to take place early during spliceosome assembly, we next tested whether the 5′ exon 2ome oligonucleotide affects spliceosomal complex formation. To distinguish the various complexes in a native gel, we performed the analysis using unlabeled P120 substrate followed by Northern blotting. Interestingly, similar to the U12/5′ exon cross-link formation, the 5′ exon oligonucleotide increased the intensity of the complex A band (containing both U11 and U12 snRNAs) by three- to fivefold as compared with the control reaction (Fig. 4A, compare lanes 2 and 3 and 5 and 6). In contrast, the intensity of the complex B band was reduced approximately twofold (lanes 8 and 9 and 11 and 12).

The increased intensity of the complex A suggests that more spliceosomal complexes form in the presence of the 5′ exon oligonucleotide. This result contradicts our cross-linking data, especially the U11/5′ss and U12/BPS cross-links, both of which should have also been stimulated with the 5′ exon oligonucleotide if the number of prespliceosomes had increased (Fig. 2A; see also Fig. 2 and 4 in reference 9). To resolve this apparent inconsistency, we asked if the stability of prespliceosomes instead of their number was increased under the native gel conditions. To test this possibility, we took advantage of our earlier observation that increased heparin concentration in the native gel loading buffer dissociates the U11 snRNP from the complex A (10). We titrated the reactions with heparin in the presence of either the 5′ exon oligonucleotide or an unrelated 2ome oligonucleotide of equal length. The assay was performed with both a ^{32}P-labeled P120 substrate and an unlabeled substrate followed by Northern blotting to confirm the loss of the U11 snRNP (Fig. 4B). We found that the presence of the 5′ exon oligonucleotide increased the stability of the complex A, resulting in approximately twofold higher tolerance to heparin concentration (Fig. 4C). We conclude that the native gel analyses with 5′ exon oligonucleotide support the cross-linking time course analyses which indicated that the U12-5′exon interaction takes place at the prespliceosome stage.

Mapping the cross-link within U12 snRNA. The relatively fast gel mobility of the U12-specific cross-link (compare the migration of U12/BPS and U12/5′exon cross-links in Fig. 1B, lane 4) and the sensitivity to the $U12_{1\text{-}16}$ 2ome oligonucleotide suggest that the 5′ end of the U12 snRNA may interact with the −2 position of the substrate. To map the location of the specific cross-link within U12 snRNA, the P120[−2] substrate was used together with the 5′ exon 2ome oligonucleotide to maximize the yield of cross-linked species. A large-scale reaction was performed, followed by specific 3′ end labeling (see Materials and Methods) and gel purification of the U12-specific cross-linked products. A 3′-end-labeled un-cross-linked U12 snRNA was also gel purified for control reactions.

Standard primer extension blockage analysis did not reveal any clear intermediate stops within the cross-linked U12 snRNA (not shown). Therefore, we localized the cross-link site by partially hydrolyzing the 3′-end-labeled cross-linked RNAs using ribonucleases T_1 and PhyM, which hydrolyze RNA after G residues or after C and U residues, respectively. Since the cross-linked U12 snRNA was 3′ end labeled, the partial hydrolysis was expected to reproduce the hydrolysis pattern of an un-cross-linked U12 snRNA as long as linear RNA fragments were produced. When hydrolysis occurs on the 5′ side of the cross-linked site, similarity with the control reaction should disappear because of the decreased gel mobility of the resulting branched fragments.

Partial hydrolysis with RNase T_1 yielded the most detailed results. The partial hydrolysis pattern of the cross-linked RNA (Fig. 5A, lane 3) was virtually identical to that of the control U12 snRNA (lane 1); even the cleavage at G3 near the very 5′ end of the U12 snRNA was recognized, and only the full-length U12 snRNA band was missing or greatly reduced. These data suggest that the cross-link must be located upstream of the G3 residue, within the first two 5′-most nucleotides of the U12 snRNA. Supporting results were also obtained with RNase PhyM, but due to the reduced activity of this RNase in the reaction buffer the resulting bands were very weak. This method was validated with control reactions containing previously mapped psoralen cross-links (U12/BPS and U12/U6atac Ia), which were found to produce the expected results (not shown).

Alignment of the 5′ exon and the U12 sequences revealed a potential base-pairing interaction between the U12 snRNA and the P120 substrate that places the ^{4S}U residue (−2 position) immediately adjacent to the first 5′ nucleotide of U12 snRNA. This scenario is consistent with the observed cross-link positions within U12 snRNA. Furthermore, in this base-pairing model the 5′ exon oligonucleotide aligns immediately adjacent to the proposed U12/5′ exon helix, thus forming a long double-stranded region upstream of the 5′ss (Fig. 5B). Such structure could stabilize the U12/5′ exon helix either by excluding splicing factors from the 5′ss and/or by stabilizing the short U12/5′exon helix by coaxial stacking, which could lead to the observed stimulation of the U12/5′ exon cross-link (Fig. 2A) in the presence of 5′ exon oligonucleotide. The effect of the 5′ exon oligonucleotide is further supported by our mutational analysis and the use of different 5′ exon oligonucleotides, which indicate that the alignment of the oligonucleotide with the U12/5′exon helix is important for the stabilization of the A complex and also inhibition of splicing that we have described previously (see Fig. S1 in the supplemental material and reference 9).

Detection of the U12-5′ exon interaction requires base pairing. To test the contribution of the proposed U12/5′exon base pairing for the cross-link formation, we constructed a P120[−2] cross-linking substrate in which the proposed base pairing was weakened by inverting the GC dinucleotide located upstream of the ^{4S}U residue (P120-CG [Fig. 6A]). When such a substrate was used in cross-linking reactions, the U12/5′ exon cross-link was lost and not observed even in the presence of the 5′ exon oligonucleotide (Fig. 6B), suggesting that the base-pairing interaction is indeed needed for the detection of the U12/5′exon cross-link. Thus, the results with P120 splicing substrate support the view that the 5′ end of U12 snRNA could be stabilized near the 5′ss by base-pairing interactions which in turn allowed us to capture the two RNAs in proximity of each other via cross-linking.

We further tested this possibility by constructing two additional cross-linking substrates, both containing a ^{4S}U residue at

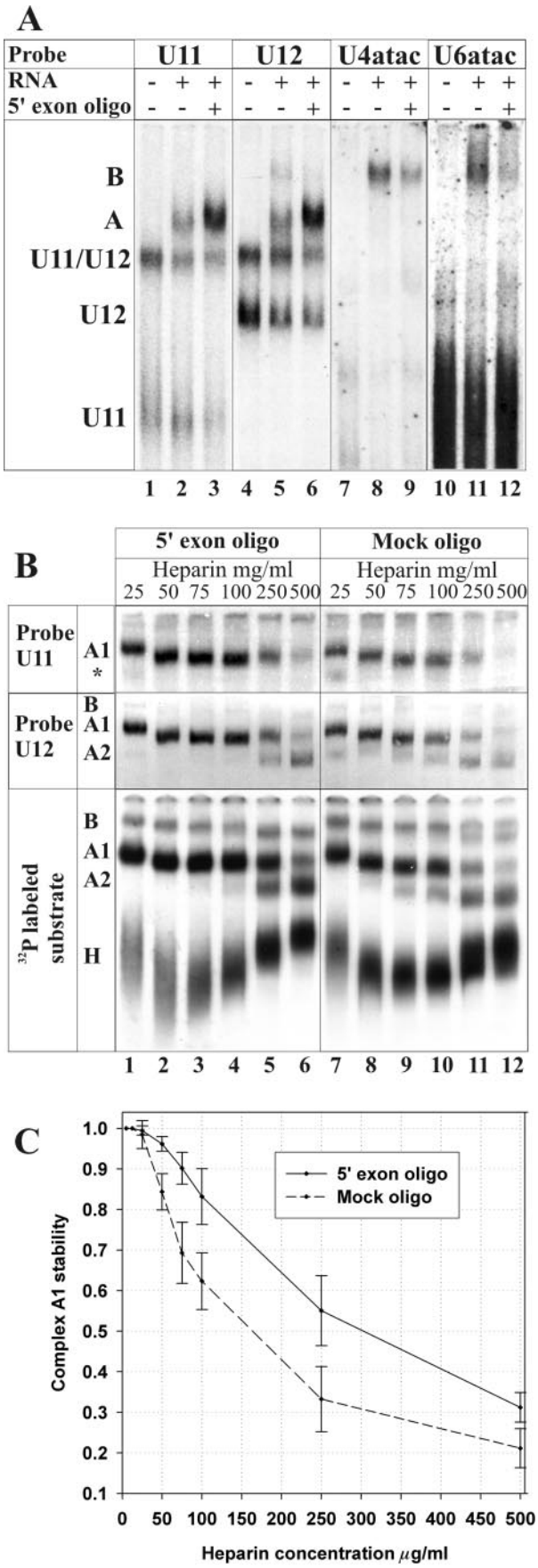

FIG. 4. The effect of a 5′ exon oligonucleotide on spliceosomal complex formation. (A) Spliceosome assembly performed in the absence or presence of the 5′ exon 2ome oligonucleotide using unlabeled P120 splicing substrate. The reactions were subsequently separated on native gels and analyzed by Northern blotting with probes indicated above each panel. The migration of spliceosomal complexes A and B and the U11- and U12-specific snRNA complexes (on lanes 1 to 6) is shown on the left. (B and C) Stability of complex A in the presence of the 5′ exon 2ome oligonucleotide. P120 splicing substrate was incubated under splicing conditions for 30 min in the presence of 1 μM 5′ exon 2ome oligonucleotide (lanes 1 to 6) or a mock oligonucleotide of the same length (lanes 7 to 12). The samples were subsequently transferred to ice, and heparin was added to each sample to the final concentration shown above each lane; the samples were then incubated on ice for an additional 2 min. The samples were then loaded onto a 4% native gel. (B) In top and middle panels, unlabeled P120 splicing substrate was used and the gels were analyzed by Northern blotting as in panel A using U11- and U12-specific riboprobes as indicated. Reactions in the bottom panel contained ^{32}P-labeled P120 splicing substrate. The positions of spliceosomal complexes B, A1 (contains both U11 and U12 snRNPs), A2 (contains only U12 snRNP), and the unspecific complex H are indicated. (C) Complex A1 stability as a function of heparin concentration. Complexes A1 and A2 were quantified with a phosphoimager in six independent experiments with the ^{32}P-labeled P120 substrate. The relative stability of A1 was calculated using the formula $A1/(A1 + A2)$.

the −2 position (Fig. 6A) and derived from a well-characterized SCN4a-ENH1 splicing substrate (66). To facilitate the U12/5′ exon base pairing the SCN4a[−2] cross-linking substrate was mutated immediately upstream of the ^{4S}U residue to include the same three-nucleotide sequence that forms the putative U12/5′ exon base-pairing interaction in the P120[−2] substrate, while the other substrate (SCN4a-CG) resembles the P120-CG and has the GC dinucleotide upstream of the ^{4S}U residue inverted, thus weakening the possible U12-5′exon interaction (Fig. 6A). Furthermore, the length of the 5′ exon in the SCN4a cross-linking substrates was shortened so that the migration of the SCN4a and P120-specific cross-links would be similar in denaturing polyacrylamide gel electrophoresis (PAGE) gels.

Each SCN4a cross-linking substrate described above was incubated under splicing conditions and cross-linked, and the resulting cross-links were separated in 5% and 8% gels (Fig. 6C and D, respectively) followed by detection by Northern blotting with U12-specific probes. The P120[−2] cross-linking substrate was used as a control (Fig. 6C and D, lane 7).

A slowly migrating band which has very similar gel mobility as the U12/5′exon cross-link produced with P120[−2] substrate was detected in both 5% and 8% PAGE gels in reactions containing SCN4a[−2] (Fig. 6C and D, lane 2) but not in those containing SCN4a-CG substrate (Fig. 6C and D, lane 5; note the similar nonlinear migration of the SCN4a and P120 specific cross-links in 5% and 8% PAGEs in panels C and D, respectively). The SCN4a-specific cross-link bands were missing from the reactions that were not UV irradiated (Fig. 6C and D, lane 1) or which contained a 2ome oligonucleotide complementary to the 5′ end of the U12 snRNA ($U12_{1\text{-}16}$ in Fig. 6C and D, lane 3), further suggesting that an authentic U12/5′exon cross-link was indeed detected with the SCN4a[−2] cross-linking substrate. Due to the lower cross-linking efficiency it was not possible to map the SCN4a cross-link location on U12 snRNA

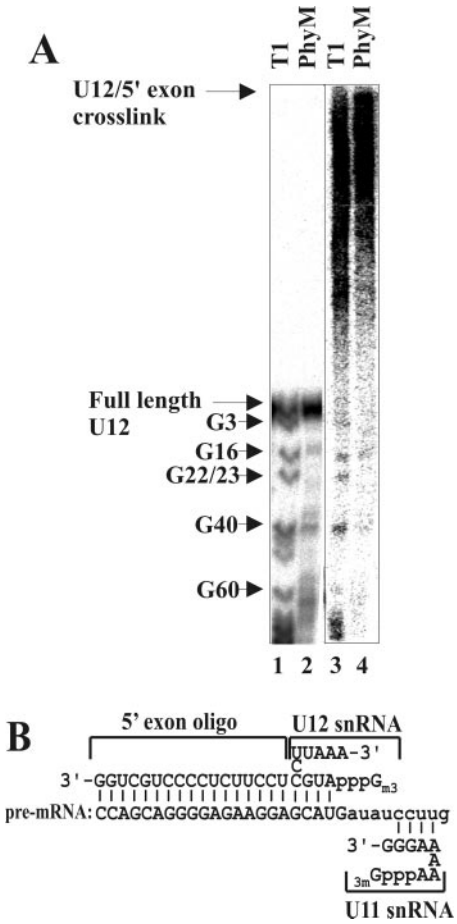

FIG. 5. Mapping the ^{4S}U cross-link position on U12 snRNA. (A) 3′-End-labeled un-cross-linked and cross-linked U12 snRNAs (see Materials and Methods) were partially hydrolyzed with RNase T_1 or PhyM and analyzed on a 5% polyacrylamide gel. Lanes 1 and 2, partial hydrolysis of the uncross-linked U12 snRNA. Lanes 3 and 4, partial hydrolysis of the cross-linked U12 species. The positions of the detected G residues are indicated on the left. (B) Proposed base-pairing interaction of the U12 snRNA with the 5′ exon sequences of the cross-linked P120 substrate (uppercase, 5′ exon sequence; lowercase, intron sequence). The position of the 5′ exon oligonucleotide and the U11 snRNA base paired with the 5′ss is also indicated.

similarly as with the P120[−2] substrate. However, the similar gel mobility of the SCN4a- and P120-specific cross-link bands in both 5% and 8% gels and the sensitivity of the cross-link formation to a mutation that reduces the extent of the 5′ exon/U12 base-pairing potential further suggests that the location of the cross-link within the U12 snRNA is similar with both P120 and SCN4a cross-linking substrates. We conclude that the proximity of the U12 snRNA with the 5′ss can be detected with other splicing substrates besides P120, but efficient detection by cross-linking requires stabilization via base-pairing interaction between 5′ exon and U12 snRNAs.

The effect of U12/5′exon base pairing on splicing activity. To evaluate if the U12-5′ exon interaction could affect splicing activity in vitro, we mutated the nucleotides on P120 substrate that are involved in U12/5′ exon base pairing. We did not detect any major changes in splicing activity in our in vitro splicing experiments with P120 substrates containing mutations in the 5′ exon region binding to U12 snRNA (see Fig. S1B in the supplemental material; Frilander and Meng, unpublished data). Therefore, to ensure an accurate comparison with the wild-type substrate containing wild-type sequences near the 5′ss, the effects of mutations were studied using modified P120 splicing substrates which contained two functional 5′ss that were separated by 20 nucleotides from each other (Fig. 7A). Each construct contained mutations either in the intron-proximal or in the intron-distal 5′ss that prevent the U12-5′ exon base-pairing interactions, while the other site contained a wild-type sequence. We used two different types of mutations to confirm that the possible effects would be specific to the disruption of the U12/5′exon base pairing (Fig. 7A).

We found that with the "WT" substrate (Fig. 7A) both the proximal and the distal site were used with near equal efficiency. Mutations in the proximal sites suggest that splicing to the distal site is more sensitive to the mutations that disturb the U12/5′ exon base pairing. In particular mutations in the proximal site (substrates 1 and 3) increased the splicing at the distal site approximately 1.5-fold compared to the control reaction (Fig. 7B, compare substrates WT, 1, and 3), causing a shift in the relative splice site usage in favor to the distal site (Fig. 7C, compare substrates WT, 1, and 3). Mutations in the distal site (substrates 2 and 4) led to a decrease of splicing at the distal site to about 75% of the wild-type level (Fig. 7B, compare substrates WT, 2, and 4), but unexpectedly these mutations have also a negative effect on the splicing of the proximal site (Fig. 7B, compare substrates WT, 2, and 4) and did not change the relative splicing efficiencies between the two sites (Fig. 7C). Therefore, we conclude that the effects of U12/5′ exon base pairing are relatively mild and may be dependent on the sequence context. Thus, such an interaction does not have any major effect on splice site usage.

DISCUSSION

Using site-specific cross-linking we have captured a novel interaction between U12 snRNA and the −2 position of 5′ exon immediately upstream of the 5′ss. The U12-5′exon interaction was captured using two unrelated cross-linking substrates derived from P120 and SCN4a genes. This interaction is dependent on ATP and a functional U12-BPS interaction and is present in splicing complexes in which both the 5′ss and BPS have been recognized, presumably by the U11/U12 di-snRNP (Fig. 2). Time course of the cross-link formation and native gel analyses further indicate that the U12-5′ exon interaction takes place during or immediately after the formation of the prespliceosome (complex A) but prior to the entry of the U4atac/U6atac.U5 tri-snRNP. Consistently, we found that the U12-5′ exon interaction is not sensitive to the 2ome oligonucleotides which block the formation of U5- or U6atac-specific cross-links during spliceosome assembly (Fig. 2). Thus, our data suggest the unexpected conclusion that the U12 snRNA is in proximity to both the 5′ss and BPS of the pre-mRNA during the early stages of spliceosome assembly.

Mapping of the cross-link on U12 snRNA to the very end of the molecule suggests that the U12 snRNA forms a base-pairing interaction with the 5′ exon sequences of the P120 splicing substrate which could promote the observed cross-link formation by aligning the two molecules (Fig. 5). This is sup-

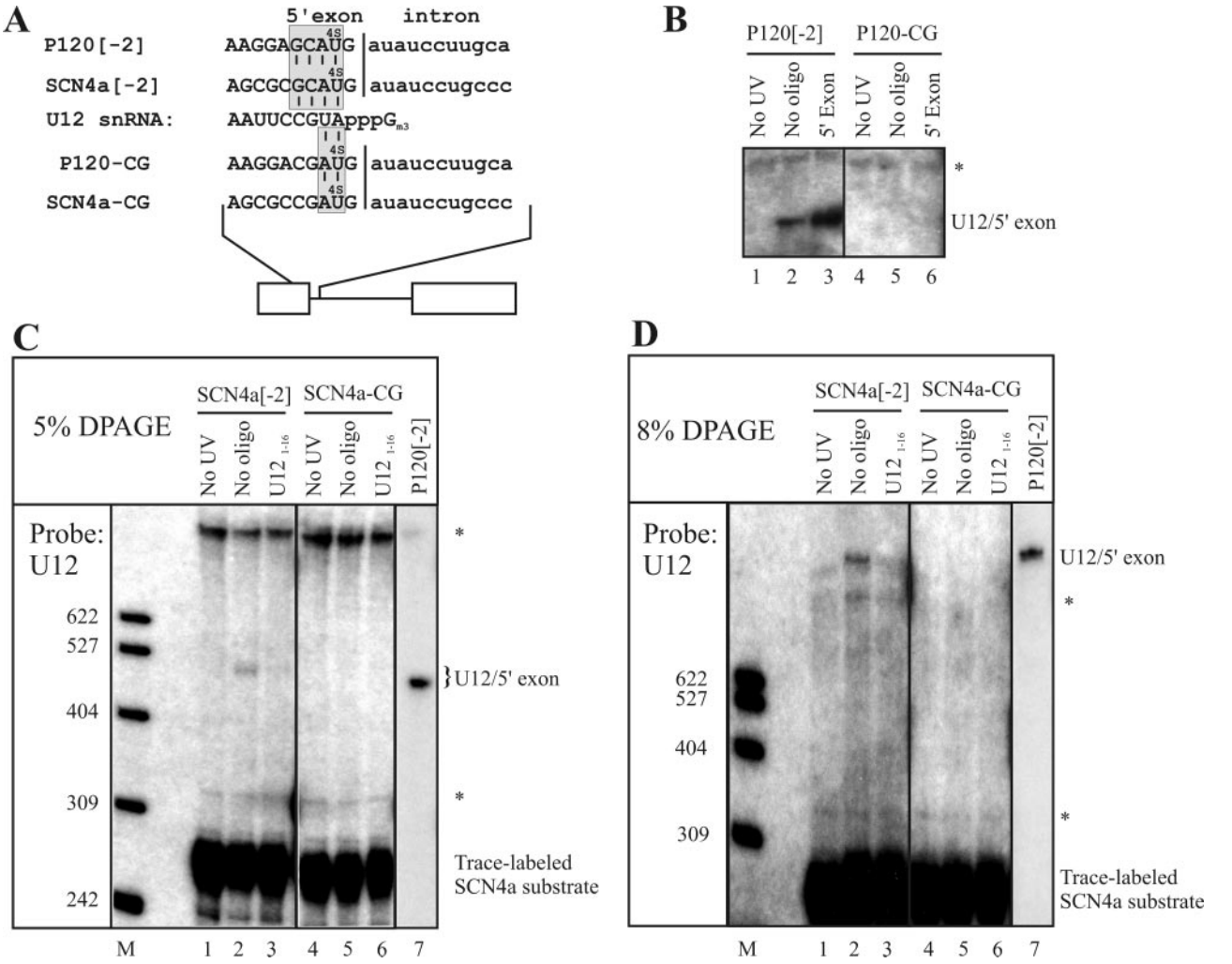

FIG. 6. The significance of U12/5′exon base pairing. (A) Sequences of the cross-linking substrates used in panels B to D. The total length of the P120 cross-linking substrate is 250 nt, and that of SCN4a substrates is 275 nt. (B) U12/5′ exon cross-link formation using P120[−2] and P120-CG substrates. The cross-linking substrates and conditions or oligonucleotides used are indicated above each lane. Otherwise the reactions were carried out as in Fig. 2A and probed with a U12-specific probe. (C) U12/5′exon cross-link formation using SCN4a[−2] and SCN4a-CG substrates. The reactions were performed in SCN4a splicing conditions (see Materials and Methods), except for lane 7, which was performed as in panel B. Following the cross-linking the reactions were separated on 5% denaturing PAGE gel, and the U12-specific cross-links were detected by Northern blotting using U12-specific probes. The labels are as in Fig. 2A. (D) The same as in panel C, but the reactions were run on 8% denaturing page gel.

ported by experiments using additional P120 and SCN4a cross-linking substrates in which the U12/5′exon cross-link was observed only when a sufficient base-pairing potential upstream of the ^{4S}U residue was present while the mutations that weakened the base pairing resulted in the loss of cross-link formation (Fig. 6). Since the proposed U12/5′ exon helix has a melting temperature of approximately 10 to 12°C, it should not be stable under the experimental conditions unless stabilized by protein factors and/or oriented near the 5′ss by the architecture of the U12 snRNA in the complex A. We have not found evidence for specific stabilizing protein factors by ^{4S}U-cross-linking (J. Turunen and M. Frilander, unpublished data), suggesting that the U12 snRNA may be positioned near the 5′ss solely by the favorable architecture within complex A. Consistently, we observed no U12/5′ exon cross-links when the spliceosome assembly was blocked in conditions that lack ATP or contain a 2ome oligonucleotide that blocks the U12-BPS interaction or in reactions that contain a cross-linking substrate lacking a functional BPS, even though in each case the 5′ end of the U12 snRNA should be free to interact with the 5′ exon sequences. These results, and the observed resistance of the cross-link to DNA oligonucleotide-directed RNase H degradation using a BPS-specific DNA oligonucleotide (Fig. 2D), indicate that the U12-5′exon interaction takes place in functional spliceosomal complexes which probably position the 5′ end of the U12 near the 5′ss. On the other hand, the loss of cross-linking signal when the base-pairing interaction was weakened using both P120 and SCN4a cross-linking substrates suggests a proximity, instead of rigid alignment, of the U12 snRNA and 5′ss in complex A. Detection of cross-link signal using two unrelated substrates suggests that such a proximity may be a general property of the system, but base pairing with

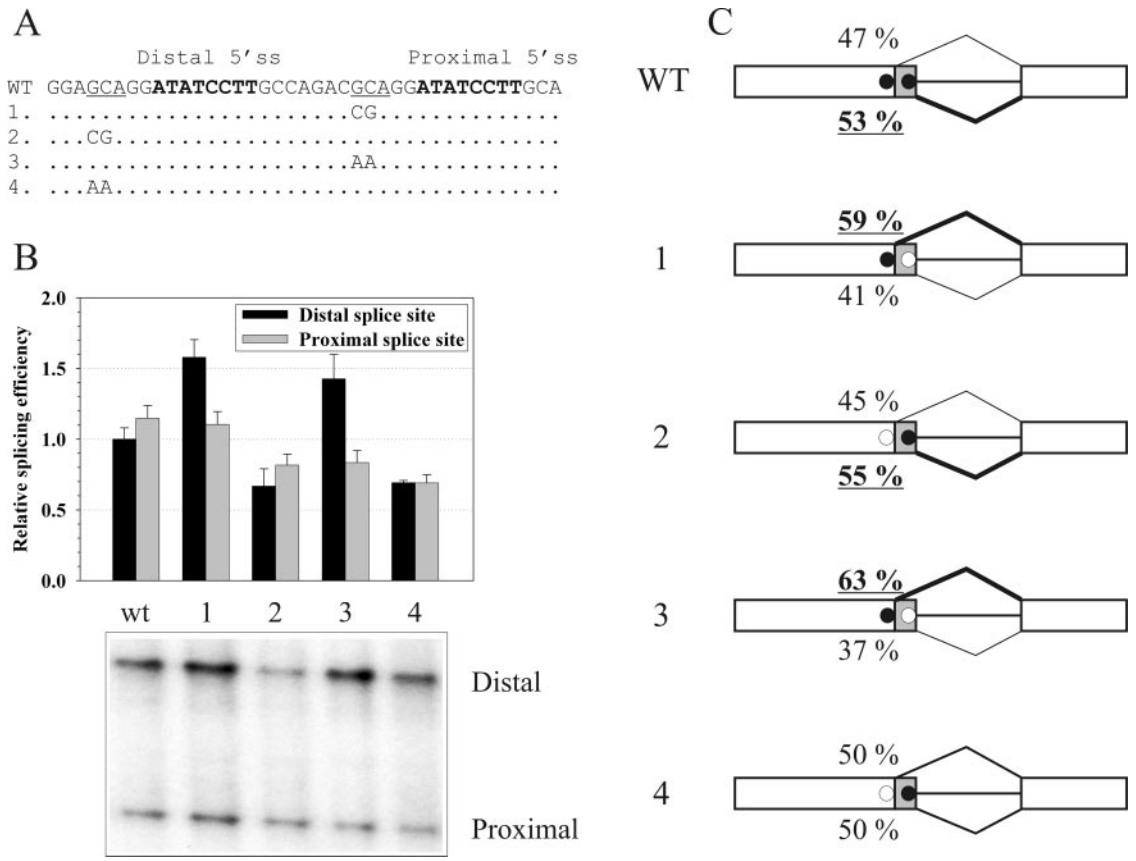

FIG. 7. The effect of U12/5′exon binding on splicing activity as measured using a substrate competition assay in which competing 5′ss have been positioned in tandem on a single splicing substrate. (A) 5′ss sequences of the modified substrates used in the experiment. In WT substrate the 5′ss has been duplicated (indicated by bolded letters), and both sites contain a sequence element complementary to the U12 snRNA helix Ib region (underlined). In the following four mutant substrates one of the complementary sequences (either upstream of the proximal or distal site) has been mutated by inversion (substrates 1 and 2) or by substitution (substrates 3 and 4) as indicated in the figure. (B) Quantification of the splicing activities at proximal and distal sites. Equal amounts of each splicing substrate were incubated under splicing conditions for 3 h and processed as described in Materials and Methods. The splicing activities at proximal and distal sites were quantified by measuring the lariat product formation representing the proximal or distal splice site usage using a phosphoimager. The bar chart shows mean values from three to six individual experiments, which were normalized to WT distal activity, which was set at 1.00. Additionally, the proximal splice site values were multiplied by 1.190 to take account the difference in the number of the ^{32}P-labeled U-residues on the lariat products resulting from splicing at proximal versus distal sites (21 versus 25, respectively). Standard deviation is indicated by error bars. The autoradiograph of a denaturing polyacrylamide gel under the bar chart shows a lariat formation from an individual experiment. (C) Relative splice site usage of the different mutant substrates described in panel A. Black circles represent wild-type 5′ exon sequence upstream of the 5′ss and hollow circles represent mutated sites. The preferred 5′ splice sites are indicated on bold. The values were obtained from the same data set as in panel B.

the substrate would transpire only if suitable and sufficiently long complementary sequences are present within the physical range of the U12 snRNA.

The U12-5′ exon base-pairing interaction suggests a model for the previously observed reversible inhibitory effect of the 5′ exon 2ome oligonucleotide (9). The 5′ exon oligonucleotide forms, together with the 5′ end of the U12 snRNA, a continuous double-stranded structure which can prevent binding of necessary splicing factors (such as Prp8, EJC complex proteins, or specific helicases [28, 30, 58]) near the 5′ss and thus prevent the exchange of U11 to U6atac at the 5′ss and results in the weaker U5/5′ exon and U12/U6atac Ib cross-link signal (9). The rapid recovery of splicing activity after the removal of the 5′ exon oligonucleotide suggested that this arrested complex containing U12-5′ exon interaction represented an on-pathway assembly intermediate (9).

The sequences upstream of the U12-type 5′ss in human, *Arabidopsis*, or *Drosophila* genes are not conserved (29, 46, 73), suggesting that cross-link formation between U12 and the P120 5′ exon was facilitated in part by fortuitous sequence complementarity, which does not necessarily serve any essential role in intron recognition or splicing. This is supported by our in vitro splicing experiments in which splicing substrates containing mutations in the region binding to U12 snRNA did not show any significant differences in splicing activity as compared to control reactions (Frilander and Meng, unpublished; see Fig. S1B in the supplemental material). In a competition situation with splicing substrates containing two tandem 5′ss, we found that mutations which disrupt the U12/5′exon base pairing do change the relative splicing efficiency but only at the distal site (Fig. 7). We found reproducible 1.5-fold stimulation of distal splice site usage with proximal site mutations (Fig. 7, substrates 1 and 3) and similarly approximately 30% inhibition of distal splice site usage with mutations at the distal site (Fig.

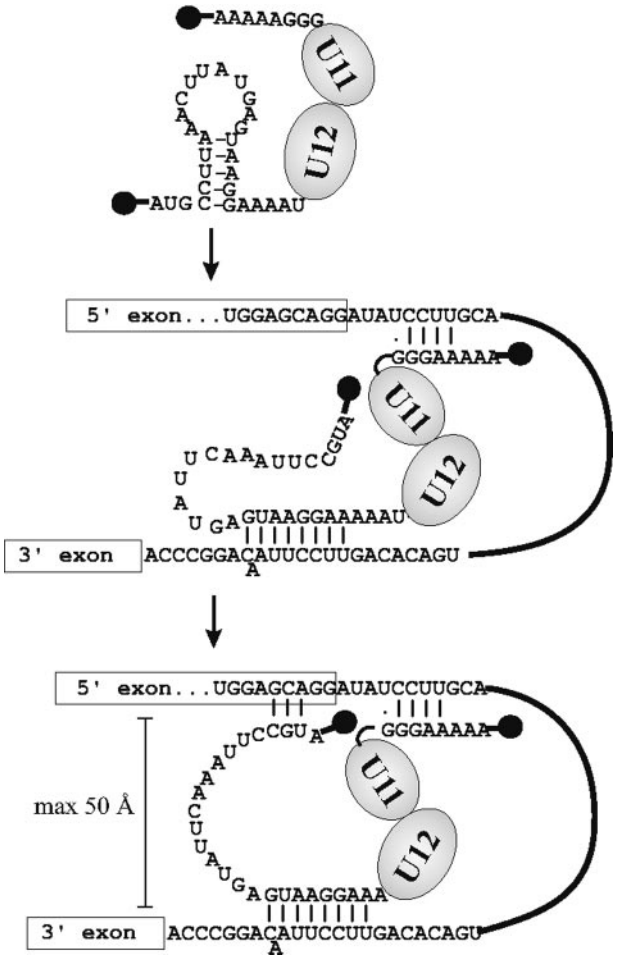

FIG. 8. A model for U12-specific interactions with the P120 splicing substrate during the early stages of spliceosome assembly. Following the recognition of the BPS in the spliceosome A complex, the helix Ib region of the U12 snRNA is released from the stem-loop structure and positioned near the 5′ss. Transient base-pairing interaction with the 5′ exon sequences can take place if a suitable complementary sequence is present in the 5′ exon near the 5′ss. The trimethyl cap is depicted as a black circle.

7, substrates 2 and 4). In contrast, the effects at the proximal site were either neutral or slightly negative, possibly due to an interference with the two closely spaced 5′ss as reported earlier with the U2-dependent spliceosome (5, 7). As the effects of U12-5′ splice site interaction on splicing are relatively small, we conclude that this interaction reflects mostly the molecular architecture of the early spliceosomal complexes instead of playing any major, if any, regulatory role.

Protection of the U12/5′ exon cross-link in the RNase H experiment with BPS-specific DNA oligonucleotides (Fig. 2D) indicates a simultaneous interactions of U12 with both the BPS (10) and the 5′ exon sequences. This simultaneous interaction allows to estimate the distance between the 5′ss and BPS in the complex A, given that there are 13 nucleotides between the base-paired sites in U12 snRNA (Fig. 8). Assuming that the RNA is extended and has a 3.1-Å base-to-base spacing, the distance between the 5′ss and the BPS sequences should not exceed 40 to 50 Å. This indicates that, at least in the complex A, the 5′ss and BPS binding elements of the U11/U12 di-snRNP are close in space, perhaps forming a discrete structure for the recognition of the two signals in the pre-mRNA. Electron microscopy studies of the U2-dependent spliceosome provide support to this interpretation as the distance measured above is similar to the diameter of a single protuberance in U1 snRNP (51), while the diameters of individual U1 and U2 snRNPs range from 80 to 120 Å (26, 51) and the diameter of a U2-type complex A is ~250 Å (12).

The 40- to 50-Å maximal distance between the 5′ss and BPS calculated above also agrees with similar measurements for the U2-dependent spliceosome. Kent and McMillan showed recently (22) that the 5′ss and the elements at the 3′ end of the intron are within 10 to 20 Å in the complex E, most likely bridged by the SR proteins that interact with U1-70K and U2AF65. The short distance between the 5′ss and BPS in the U2-dependent spliceosome is presumably maintained in the complex A, although this may require additional components as suggested by the recent identification of the Prp5 protein as an essential component mediating interactions between the U1 and U2 snRNPs (71). In comparison, in the U11/U12 complex similar interactions are probably mediated by an integral di-snRNP protein(s) (62, 63).

Localization of the site of cross-link on U12 snRNA to the very 5′ end of the molecule (Fig. 5) provides insight to the possible significance of the U12-5′ exon interaction. In the catalytic core of the U12-dependent spliceosome, the very 5′ end of the U12 snRNA forms helix Ib together with U6atac snRNA (48, 54). This and the other RNA-RNA interactions found from the catalytic core of the U12-dependent spliceosome are similar to the interactions described in the U2-dependent spliceosome, with U2 and U6 snRNA base paired together as originally proposed by Madhani and Guthrie (32) based on yeast mutation data. The role of helix Ib has been investigated primarily on the U2-dependent spliceosome in which it has been shown to be essential for the catalysis of splicing (18, 32, 64; but see reference 8), possibly functioning in the second step while the U2 and U6 snRNA would adopt an alternative ribozyme-like conformation for the first step (45, 53). Consistent with the catalytic role of the U2/U6 helix Ib, genetic interactions that link the 5′ss and helix Ib, either directly (Ib/5′ss interaction [31]) or indirectly (Ib/U5 interaction [70]) have been described in yeast. Several studies have identified cross-bridging interactions that link 5′ss and BPS and can thus bring the U2 in the vicinity of the 5′ss for the formation of the catalytic core structure. These include SR proteins and other factors bridging 5′ss and BPS at the E-complex stage (1, 65), the Prp5 protein directly bridging U1 and U2 snRNPs (71), the U2-U6 helix II interaction that can be observed in HeLa nuclear extracts by psoralen cross-linking even in the absence of pre-mRNA (17, 60), and most recently, a preformed penta-snRNP structure that could serve as a physical link between the components involved in intron recognition (34, 52). However, even though the presence of bridging interactions between 5′ss and BPS at the time of intron recognition has been well documented in the U2-dependent spliceosome, the orientation and positioning of the individual components, including U2 snRNA, in these structures is mostly unknown. Our detection of U12 snRNA in the proximity of the 5′ss during the early phase of the spliceosome assembly argues

that at least in the U12-dependent spliceosome the helix Ib region of the U12 snRNA may be positioned near the 5′ss already at the time of the prespliceosome formation, thus resulting in the bridging of the 5′ss and BPS by a single spliceosomal component and further suggesting that the molecular architecture present in the spliceosome catalytic core may start forming at the earliest stages of the spliceosome assembly.

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