The U5 and U6 Small Nuclear RNAs as Active Site Components of the Spliceosome

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Five small nuclear RNAs (U1, U2, U4, U5, and U6) participate in precursor messenger RNA (pre-mRNA) splicing. To probe their interactions within the active center of the mammalian spliceosome, substrates containing a single photoactivatable 4-thiouridine residue adjacent to either splice site were synthesized, and crosslinks were induced during the course of in vitro splicing. An invariant loop sequence in U5 small nuclear RNA contacts exon 1 before and after the first step of splicing because a crosslink between U5 and the last residue of exon 1 appeared in the pre-mRNA and then in the cutoff exon 1 intermediate. Both of these crosslinked species could undergo subsequent splicing, indicating that the crosslinks reflect a functional interaction that is maintained through both reaction steps. The same U5 loop aligns the two exons for ligation since the first residue of exon 2 also became crosslinked to U5 in the lariat intermediate. An invariant sequence in U6 RNA became crosslinked to the conserved second position of the intron within both the lariat intermediate and the lariat intron product. On the basis of these results, several conformational arrangements of small nuclear RNAs within the spliceosomal active center can be distinguished, and additional mechanistic parallels between the spliceosome and self-splicing introns can be drawn.

During precursor messenger RNA (pre-mRNA) splicing, intervening sequences (IVSs or introns) are precisely excised from primary transcripts, and the exons are joined to generate mature mRNA (*1*). A large complex called the spliceosome carries out the two sequential transesterification reactions involved in splicing: (i) A 2′ hydroxyl group at the branch site attacks the 5′ splice site to generate exon 1 (E1) and lariat intron-exon 2 (lariat IVS-E2) intermediates, and (ii) the 3′ hydroxyl of the free E1 intermediate attacks the 3′ splice site to generate spliced exons (E1-E2) and the excised lariat product (lariat IVS). The identification of five small nuclear RNAs (snRNAs) as essential components of the splicing machinery has led to the proposal that the transesterification reactions may be fundamentally RNA-based. The self-splicing of group II introns proceeds through an identical pathway (*2*).

The snRNAs have been suggested to function in splice site recognition, spliceosome assembly, and catalysis on the basis of characterization of their interactions with each other and with the splicing substrate. It has long been known that U1 base pairs to the 5′ splice site, U2 base pairs with the branch site, and U4 and U6 base pair to each other (*1*). Genetic suppression and biochemical crosslinking experiments (*3*) have more recently demonstrated U1-3′ splice site base pairing (in at least some introns) (*4*), U5 interactions with exon sequences at both the 5′ and 3′ splice sites (*5*, *6*), contacts of both U5 (*7*) and U6 (*7–9*) with intron sequences near the 5′ splice site, two distinct base pairing interactions between U2 and U6 (*10–12*), and a non-Watson-Crick interaction between the two conserved guanosine residues at the 5′ and 3′ ends of the intron (*13*). The temporal order of some of these interactions has been established (*6–9*).

Little is known, however, about the contribution of snRNAs to catalysis in the spliceosome. Both splicing steps involve direct in-line nucleophilic attack (*14*), and changes in the active site are likely to occur between steps (*14*, *15*), but the active site components have not been identified. We do not yet know what activates the nucleophiles, what stabilizes the transition states and leaving groups, or what holds and aligns the intermediates for the second step. A model for RNA-directed catalysis by two Mg^{2+} ions in the spliceosome has been proposed (*15*) but not proved. Our lack of understanding is especially pronounced for the second reaction step. The only interaction of an snRNA with a splicing intermediate that has been demonstrated biochemically is between U6 and the lariat IVS-E2 (*9*) although genetic interactions between U5 and exon 2 (*5*) and between the terminal intron residues (*13*) also seem likely to involve IVS-E2. In particular, no factors (either RNA or protein) that hold and orient the E1 intermediate for the second step have yet been identified.

We previously described site-specific crosslinking studies (*6*) with splicing substrates containing a single photoactivatable 4-thiouridine (4-thioU) residue located two nucleotides upstream of the 5′ splice site (*16*), for which we characterized contacts with the U5 small nuclear ribonucleoprotein (snRNP) (*6*). As an intrinsic photoaffinity probe, 4-thioU has advantages (*17*) over solvent-based crosslinking reagents such as psoralen, which are likely to be excluded from the spliceosomal active site, just as hydroxyl radical cleavage reagents are excluded from the P site of the ribosome (*18*) or the catalytic core of group I introns (*19*).

By placing 4-thioU at several positions in the immediate vicinity of the 5′ and 3′ splice sites, we have now been able to identify and characterize new substrate-snRNA crosslinks. Since these interactions occur within several angstroms of the scissile phosphates at the 5′ and 3′ splice sites, they provide strong evidence for snRNA participation in the chemistry of nuclear pre-mRNA splicing.

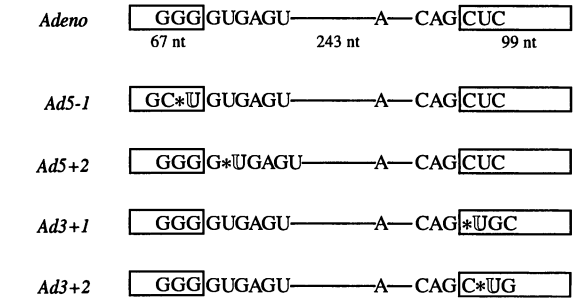

Fig. 1. Site-specific crosslinking substrates. The standard adenovirus splicing substrate (*20*), marked *Adeno* on the left, is shown at the top; exons are boxed, and the intron is shown as a single line. Intron and exon sequences at the splice sites (including any changes) are shown, along with the branch-point adenosine. Sizes (in nucleotides) of the intron and both exons are given underneath. Site-specific crosslinking substrates are shown below (*46*) with their names given in italics on the left. In each case, the U shown as an open letter is 4-thioU; it is preceded by a single $^{32}PO_4$ residue (designated by *). In addition to the 4-thioU replacements, the G at position minus 2 in *Ad5-1* was replaced with C (to avoid creation of a GU dinucleotide, which could be recognized as a cryptic 5′ splice site); in *Ad3+1* and *Ad3+2*, the second or third position of E2 was replaced with G (to allow in vitro transcription of the 3′ half RNAs).

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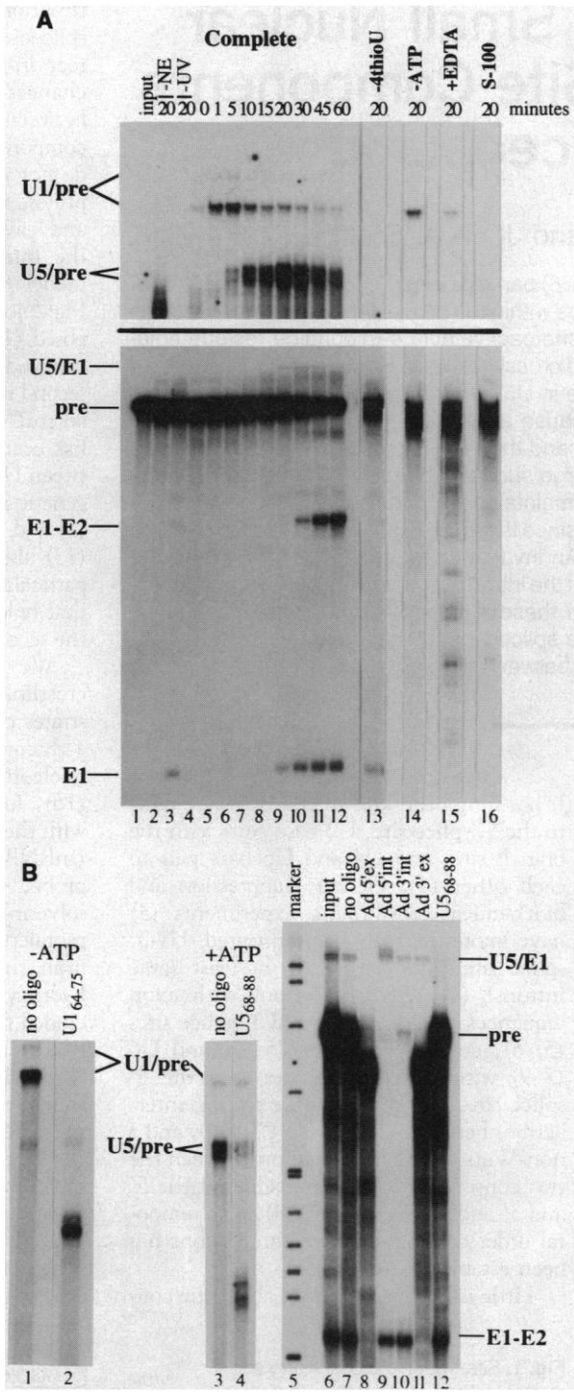

Fig. 2. In vitro splicing and crosslinking of adenovirus pre-mRNA containing 4-thioU in the last position of exon 1. (**A**) Ad5-1 pre-mRNA (*46*) was incubated in HeLa nuclear extract (*47*) at 30°C under standard splicing conditions (*6*), except that the final concentration of $MgCl_2$ was 2.2 mM (lanes 4 to 12). Samples removed at various times (indicated at the top) were irradiated on a Parafilm-covered aluminum block in ice for 10 minutes with 365 nm light (*7*). Control reactions, incubated at 30°C for 20 minutes prior to irradiation, were as above, except that (i) nuclear extract was replaced by buffer D (*47*) (lane 2), (ii) ultraviolet irradiation was omitted (lane 3), (iii) 4-thioU was omitted from the Ad5-1 substrate (*46*) (lane 13), (iv) ATP and creatine phosphate were omitted (lane 14), (v) 2.2 mM $MgCl_2$ was replaced by 4 mM EDTA (lane 15), and (vi) nuclear extract was replaced by S-100 extract (*23, 47*) (lane 16). All samples were incubated with proteinase K at 1.5 mg/ml and 0.1 percent SDS at 37°C for 30 minutes; RNAs were extracted with a mixture of phenol, chloroform, and isoamyl alcohol (50:49:1) and precipitated with ethanol. Half of each sample was fractionated on a denaturing 5 percent polyacrylamide gel (upper panel) to visualize snRNA/pre-mRNA crosslinked species (U1/pre and U5/pre), and the other half was fractionated on a denaturing 8 percent polyacrylamide gel (lower panel) to visualize the U5/E1 crosslinked species and uncrosslinked pre-mRNA (pre), E1 intermediate, and E1-E2 product. The doublet appearance of U5-crosslinked species is probably due to heterogeneity of the U5 snRNA population (*48*). Quantitation revealed that 2.2 percent of pre-mRNA and 24 percent of E1 became crosslinked to U5 after 20 minutes of incubation. Input (lane 1) is unspliced Ad5-1 pre-mRNA. The upper and lower panels are separated by a line. (**B**) Oligonucleotide-directed RNase H digestion of Ad5-1 crosslinked RNAs. (Lanes 1 and 2) Ad5-1 pre-mRNA was incubated for 15 minutes in a splicing reaction lacking ATP and creatine phosphate. The sample was irradiated, and extracted RNAs were incubated with RNase H in the presence (lane 2) or absence (lane 1) of the $U1_{64-75}$ oligonucleotide (*49*) and then fractionated on a 5 percent polyacrylamide gel. (Lanes 3 and 4) Ad5-1 pre-mRNA was incubated for 20 minutes in an active splicing reaction. The sample was irradiated, and extracted RNAs were then incubated with RNase H in the presence (lane 4) or absence (lane 3) of the $U5A_{68-88}$ oligonucleotide (*49*) and fractionated on a 5 percent polyacrylamide gel. (Lanes 6 to 12) Ad5-1 pre-mRNA was incubated for 30 minutes in an active splicing reaction. The sample was irradiated, and extracted RNAs were incubated with RNase H without oligonucleotide (lane 7), or in the presence of oligonucleotide Ad 5′ex (lane 8), Ad 5′int (lane 9), Ad 3′int (lane 10), Ad 3′ex (lane 11), or $U5A_{68-88}$ (lane 12) (*49*). Input RNA (lane 6) was not subjected to RNase H incubation. Markers (lane 5), ^{32}P-labeled pBR322 Msp I-digested DNA. The samples were fractionated on an 8 percent polyacrylamide gel. The various undigested RNA species are labeled as in (A).

Crosslinking of U5 to the last nucleotide of exon 1 before and after 5′ splice site cleavage. A splicing substrate derived from the adenovirus major late transcript (*20*) was synthesized containing 4-thioU in place of guanosine directly upstream of the 5′ splice site (Ad5-1) (Fig. 1). This is the nucleotide that acts as the leaving group during the first step of splicing and as the nucleophile during the second step. Splicing of Ad5-1 in HeLa nuclear extract yielded the correct E1 intermediate and spliced product with normal kinetics (Fig. 2A, lower, lanes 4 to 12), as expected on the basis of previous observations that exon mutations do not impair splicing (6, *21*). Lariat IVS-E2 intermediate and lariat IVS product are not visualized since the single [^{32}P]phosphate is 5′ to the 4-thioU in E1 (Fig. 1). Samples were removed at intervals, irradiated with ultraviolet (365 nm) light for 10 minutes, and digested with proteinase K; the RNA was then analyzed by gel electrophoresis. Several slowly migrating bands, whose formation required nuclear extract, ultraviolet (UV) light, and 4-thioU in the substrate (Fig. 2, compare lanes 4 to 12 with lanes 2, 3, and 13, upper), may represent crosslinked species. The two slowest migrating bands (U1/pre) appeared even without incubation at 30°C, whereas the doublet (U5/pre) appeared weakly after 5 minutes of incubation, peaked at 20 minutes, and then diminished. [Throughout this article, crosslinks are denoted by a slash (/).] Since these crosslinked species appeared before either the E1 intermediate or E1-E2 product became visible (20 minutes) (Fig. 2, lane 9, lower), they are likely to contain unspliced pre-mRNA. A fourth crosslinked band, migrating just above the pre-mRNA, appeared after incubation for 20 minutes, then decreased (Fig. 2, lanes 9 to 12, lower), and could contain either pre-mRNA or E1 intermediate.

We used ribonuclease (RNase) H digestion directed by oligonucleotides complementary to snRNAs or portions of the substrate to identify the crosslinked species. The two slowest migrating pre-mRNA crosslinked bands were targeted by the $U1_{64-75}$ oligonucleotide (Fig. 2B, lane 2); thus, two U1/5′ splice site crosslinks form early in the splicing reaction, as has been observed with a substrate having 4-thioU at position minus 2 instead of minus 1 upstream of the 5′ splice site (6). The faster migrating doublet is U5/pre-mRNA, as shown by its specific digestion in the presence of an oligonucleotide complementary to U5 snRNA (Fig. 2B, lane 4). In other experiments digestion was complete. Although U5 has been observed to crosslink to pre-mRNA containing 4-thioU at position minus 2 relative to the 5′ splice site

(6), the interaction detected with Ad5-1 was distinct in its kinetics (reaching a peak at 20 minutes rather than at 5 minutes of incubation) (6), its divalent metal ion requirement (Fig. 2A, lane 15), and its site of contact in U5 snRNA, as shown below.

The crosslinked species that appeared only after 20 minutes of incubation (Fig. 2A, lower) was digested by RNase H in the presence of oligonucleotides complementary to U5 snRNA and exon 1, but not with oligonucleotides complementary to intron or exon 2 sequences (Fig. 2B, lanes 6 to 12). This band is therefore a U5/E1 crosslinked species, and represents the unequivocal identification of an snRNA contact with the E1 intermediate. Formation of the U5/E1 crosslink is extremely efficient; at the 20-minute time point, 24 percent of the free E1 intermediate was obtained in crosslinked form (22), as compared with the 2 to 4 percent efficiencies of other crosslinks described below.

Omission of individual reaction components blocks splicing complex assembly at specific stages (23). At all time points, Ad5-1 formed the two U1 crosslinks in the absence of adenosine triphosphate (ATP) or Mg^{2+} and very weakly in whole-cell (S-100) extract (Fig. 2A, lanes 14 to 16) (24). In contrast, the U5/pre-mRNA and U5/E1 crosslinked species did not appear under any of these conditions. Because the U5 crosslinks previously characterized with a substrate containing 4-thioU two nucleotides upstream of the 5′ splice site accumulated in EDTA-treated extracts (6), the U5/pre-mRNA crosslinks at positions minus 1 and minus 2 of E1 probably reflect two functionally distinct interactions.

Interaction of U6 with the conserved GU dinucleotide at the 5′ splice site after the first step of splicing. Most eukaryotic introns contain a 5′ splice site GU dinucleotide (1) whose importance for splicing has been confirmed by mutational (21) and chemical modification studies (25). We synthesized an adenovirus splicing substrate (Ad5+2) (Fig. 1) containing 4-thioU in place of uridine two nucleotides downstream of the 5′ splice site. Its splicing in HeLa nuclear extract yielded a correct lariat IVS-E2 intermediate and lariat IVS product with normal kinetics (Fig. 3A) (24). The E1 intermediate and E1-E2 spliced product were not visualized because the single [^{32}P]phosphate resides within the intron (Fig. 1). Crosslinking generated several UV- and 4-thioU-dependent bands that appeared and peaked at various times during the splicing reaction (Fig. 3A, compare lanes 4 to 12 with lanes 2, 3, and 13). None of these appeared without ATP or Mg^{2+} (Fig. 3A, lanes 14 and 15), but a new doublet (U1/pre), sometimes seen in the complete reaction (24), was observed. No discrete intermolecular crosslinked bands were generated in an S-100 extract (Fig. 3A, lane 16).

Digestion with RNase H in the presence of specific oligonucleotides revealed that the crosslink involving position plus 2 that forms in the absence of ATP and in the presence of EDTA contains U1 RNA (Fig. 3B, lane 8) and pre-mRNA (24). The slowest migrating band formed in the presence of ATP is U6 crosslinked to lariat IVS-E2, and the fastest migrating species is U6 crosslinked to lariat IVS (Fig. 3B, lanes 2 to 4 and 6). The bands of intermediate mobility are both U2 crosslinked to lariat IVS (Fig. 3B, lanes 2 to 5).

Crosslinking of U5 to the first nucleotide of the 3′ exon within the lariat IVS-E2 intermediate. To examine snRNA interactions with exon sequences at the 3′ splice site, we constructed two adenovirus splicing substrate derivatives (Ad3+1 and Ad3+2) (Fig. 1) that contained 4-thioU one or two nucleotides downstream of the 3′ splice site, respectively. The 4-thioU in Ad3+1 was directly linked to the 3′ splice site phosphate; its crosslinking therefore should identify snRNA residues within the active site of the spliceosome during the second reaction step. As anticipated from the relative lack of conservation of exon 2 sequences (26), alteration of the C in the first position of E2 did not interfere with splicing (Fig. 4A, lower, lanes 4 to 12); incubation of Ad3+1 pre-mRNA in HeLa

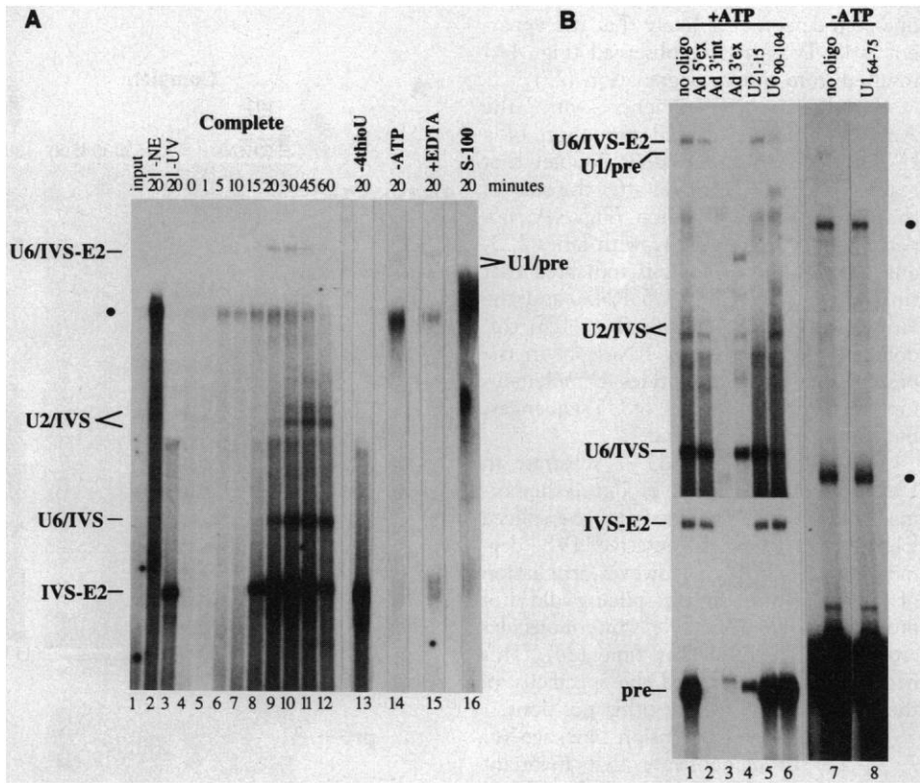

Fig. 3. In vitro splicing and crosslinking of adenovirus pre-mRNA containing 4-thioU in the second position of the intron. (**A**) Ad5+2 pre-mRNA (46) was subjected to in vitro splicing and crosslinking as described in Fig. 2A and fractionated on a 5 percent polyacrylamide gel. U6/lariat IVS-E2 intermediate (U6/IVS-E2), U2/lariat IVS product (U2/IVS), U6/lariat IVS product (U6/IVS), and U1/pre-mRNA (U1/pre, visible only in lanes 14 and 15) crosslinked species and uncrosslinked lariat IVS-E2 intermediate (IVS-E2) are indicated. Quantitation of these species revealed that, at 20 minutes of incubation, 3.1 percent of the lariat IVS-E2 intermediate became crosslinked to U6, while 2.5 and 4.1 percent of the excised lariat IVS became crosslinked to U2 and U6, respectively. A band corresponding to an intramolecular crosslink is indicated by a dot. (**B**) Oligonucleotide-directed RNase H digestion of Ad5+2 crosslinked RNAs. (Lanes 1 to 6) Ad5+2 pre-mRNA was incubated in a splicing reaction for 20 minutes, and irradiated. Extracted RNAs were incubated with RNase H in the presence (lanes 2 to 6) or absence (lane 1) of oligonucleotides complementary to substrate (lanes 2 to 4), U2 snRNA (lane 5), or U6 snRNA (lane 6) (49), and fractionated on a 5 percent polyacrylamide gel. The upper and lower portions of lanes 1 to 6 are different exposures of different regions of the same gel; the lower portion shows uncrosslinked lariat IVS-E2 intermediate and pre-mRNA. (Lanes 7 and 8): Ad5+2 pre-mRNA was incubated for 15 minutes in a splicing reaction lacking ATP and creatine phosphate. The sample was irradiated; RNAs were extracted and the extract was incubated with RNase H in the presence (lane 8) or absence (lane 7) of the $U1_{64-75}$ oligonucleotide (49) and fractionated on a 5 percent polyacrylamide gel. Bands corresponding to intramolecular crosslinks are indicated on the right by dots.

nuclear extract generated the correct lariat IVS-E2 intermediate and E1-E2 product with normal kinetics. Since the single [^{32}P]phosphate in Ad3+1 was at the 3′ splice site within E2 (Fig. 1), the E1 intermediate was not visible. Although we would expect the lariat IVS also not to be visible, a band with the mobility of this product appeared ("IVS") (Fig. 4A, lower, lanes 9 to 12). Moore and Sharp (*16*) had observed a similar unexpected lariat product when they used RNAs that were inefficient substrates for the second step of splicing because of 2′ modifications at the splice sites; they demonstrated that this species arose from cleavage of lariat IVS-E2 just downstream of the 3′ splice site, and that this breakdown occurred even with unmodified substrates that did not display a second-step defect. It is likely that the apparent lariat IVS that we observed (Fig. 4A) resulted from similar degradation (*27*).

Crosslinking experiments with the Ad3+1 substrate showed formation of a UV- and 4-thioU-dependent doublet that reached a peak 30 minutes after the start of the active splicing reaction (Fig. 4A, upper, compare lanes 4 to 12 with lanes 2, 3, and 13). RNase H analysis indicated that this doublet contained U5 RNA and the lariat IVS-E2 intermediate (Fig. 4B); this doublet was degraded by RNase H in the presence of oligonucleotides complementary to the intron, exon 2, or U5 sequences, but not to exon 1 sequences.

Incubation of the Ad3+2 substrate in HeLa nuclear extract also produced the normal lariat IVS-E2 intermediate, the spliced E1-E2 product, and the putative "IVS" degradation product (*24*). However, irradiation of this substrate during splicing did not produce any detectable intermolecular crosslinked species at any time (*24*). This negative result confirmed the specificity of the crosslinks observed at other positions.

We used primer extension blockage (*6*, *7*, *9*, *28*) to demonstrate that invariant nucleotides in U5 and U6 crosslink to the 5′ and 3′ splice sites. Crosslinked RNAs were gel-purified to provide templates for reverse transcription primed with end-labeled oligonucleotides complementary to U5 or U6; reverse transcription terminated at the base preceding the crosslink (*29*). Since U2 became crosslinked only to the lariat IVS product of the Ad5+2 substrate, these bands seemed unlikely to provide information regarding the chemical steps of splicing and were not analyzed further. In each mapping experiment, three control templates were used for comparison; these were (i) uncrosslinked pre-mRNA, (ii) RNAs that comigrated with the crosslinked species from an irradiated reaction mixture that lacked substrate to ensure that terminated transcripts were not copied from unrelated RNAs, and (iii) total RNA from this same mixture to ensure that termination was not the result of nonspecific UV damage.

The U5/pre-mRNA and U5/E1 crosslinks formed with the Ad5-1 substrate both mapped to the same two nucleotides (U40 and U41) within the invariant U5 loop sequence GCCUUUUAC (nucleotides 37 to 45) (Fig. 5, A and B, lanes 6), indicating that the U5 loop interacts with the last residue of E1 before and after the first reaction step. This crosslinking profile was different from that characterized previously where 4-thioU was at position minus 2 upstream of the 5′ splice site in the pre-mRNA; in that case, U41 and U43 were the predominant sites of crosslinking (*6*). The U6 crosslink to the Ad5+2 lariat IVS-E2 intermediate mapped to A45, which is the fifth base within the conserved essential hexanucleotide sequence ACAGAG (nt 41 to 46) (Fig. 5C, lane 6) (*30*–*32*). The U6 crosslink to the Ad5+2 excised lariat IVS mapped to three nearby bases (Ψ40, A43, and G44), as well as to A45 (Fig. 5C, lane 8). The crosslink to the Ad3+1 lariat IVS-E2 intermediate mapped to U5 residues C39 and U40 (Fig. 5D, lane 1), within the conserved loop.

Functionality of the U5/pre-mRNA crosslinked species in both splicing steps. Since crosslinks involving the last residue of E1 (substrate Ad5-1) mapped to the same positions of the U5 conserved loop in both the pre-mRNA and cutoff E1, it seemed possible that contacts established prior to catalysis are maintained through the first step of splicing. To find out whether the

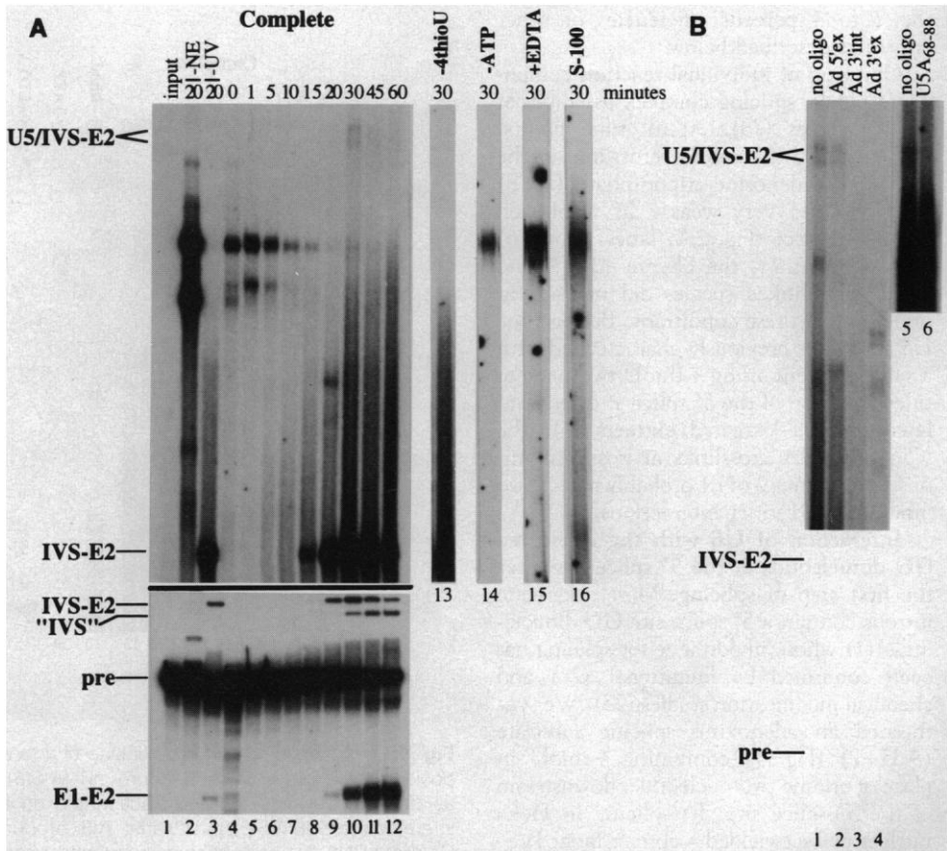

Fig. 4. In vitro splicing and crosslinking of adenovirus pre-mRNA containing 4-thioU in the first position of exon 2. (**A**) Ad3+1 pre-mRNA (*46*) was subjected to in vitro splicing and crosslinking as described in Fig. 2A, except that control samples (lanes 13 to 16) were incubated for 30 minutes and fractionated on a 5 percent polyacrylamide gel. The upper and lower panels are separated by a line. The U5/lariat IVS-E2 intermediate crosslinked species (U5/IVS-E2), and uncrosslinked pre-mRNA (pre), lariat IVS-E2 intermediate (IVS-E2), and E1-E2 spliced product (E1-E2) are indicated. Quantitation revealed that 4 percent of the IVS-E2 became crosslinked to U5 after 30 minutes of incubation. Bands corresponding to intramolecular crosslinks are indicated by dots. The band marked "IVS" in lanes 9 to 12 (lower panel) is apparently a specific degradation product. (**B**) Oligonucleotide-directed RNase H digestion of Ad3+1 crosslinked RNAs. Ad3+1 pre-mRNA was incubated in a splicing reaction for 30 minutes, irradiated, and extracted RNAs were incubated with RNase H in the presence (lanes 2 to 4 and 6) or absence (lanes 1 and 5) of oligonucleotides complementary to either the substrate (lanes 2 to 4) or U5 snRNA (lane 6) (*49*), and fractionated on 5 percent polyacrylamide gels. The upper and lower portions of lanes 1 to 4 are different exposures of different regions of the same gel.

U5/pre-mRNA crosslinked species could undergo splicing, we irradiated after 15 minutes of incubation because this time allows significant U5/pre-mRNA crosslinking without 5′ splice site cleavage (Fig. 2, lane 8). After incubation of Ad5-1 RNA in nuclear extract for 15 minutes under splicing conditions, the mixture was irradiated for 5 minutes on ice, then restored to 30°C to allow further processing. Samples were removed at intervals, digested with proteinase K, and analyzed by gel electrophoresis (Fig. 6A, lanes 11 to 18). A control sample was treated identically, except that UV irradiation was omitted during the 5 minutes on ice (lanes 19 to 25). For comparison, a standard splicing-crosslinking experiment (in which there was no further incubation after each UV irradiation step) was performed in parallel (lanes 3 to 10).

In the "pre-crosslinked" panel (Fig. 6A), no E1 intermediate or E1-E2 spliced product was generated after the initial 15-minute incubation (lane 11, lower). After UV irradiation, the U1/pre-mRNA and U5/pre-mRNA crosslinked species appeared (lane 12, upper) but no U5/E1 crosslinked species was detected (lane 12, lower). Subsequent incubation at 30°C generated a UV-dependent band of the same mobility as the U5/E1 crosslinked RNA [compare lanes 14 to 18 with lanes 5 to 10 and 19 to 25 (lower)]. Its appearance was accompanied by a decrease in the U5/pre-mRNA crosslinked band (Fig. 6A, upper, lanes 12 to 18). In addition, a band of slower mobility (designated U5/E1-E2 in Fig. 6A) appeared exclusively in lanes 14 to 18, with kinetics identical to the appearance of E1-E2 spliced product (Fig. 6A, lower). RNase H analysis of affinity-selected U5-crosslinked RNAs (Fig. 6B) confirmed the identity of the U5/E1 band and indicated that the novel species represents a U5/E1-E2 crosslink; the latter was digested in the presence of oligonucleotides complementary to exons 1 and 2, but not to the intron (Fig. 6B). Approximately half of the U5/E1 crosslinked species underwent the second step of the reaction and was converted into crosslinked product (Fig. 6A, lower, lane 18).

These results show that the U5/pre-mRNA and U5/E1 crosslinked species can each function in the subsequent splicing step, and therefore do not represent dead-end products. Most strikingly, we detected a crosslinked species (U5/E1-E2) that is not observed when crosslinking is performed during a standard splicing time course (Fig. 6A, lower, lanes 3 to 10). The appearance of this species suggests that a U5-product interaction occurs in the spliceosome, but is not normally captured by crosslinking because E1-E2 is rapidly released (*33*).

Dynamic changes in the architecture of the spliceosomal active center. The substrate nucleotides that we have replaced with 4-thioU are within several angstroms of the 5′ and 3′ splice site phosphates, and so must lie within or extremely close to the catalytic center of the spliceosome. Their relatively efficient cross-linking to conserved snRNA residues, combined with kinetic analyses of crosslink formation, allow

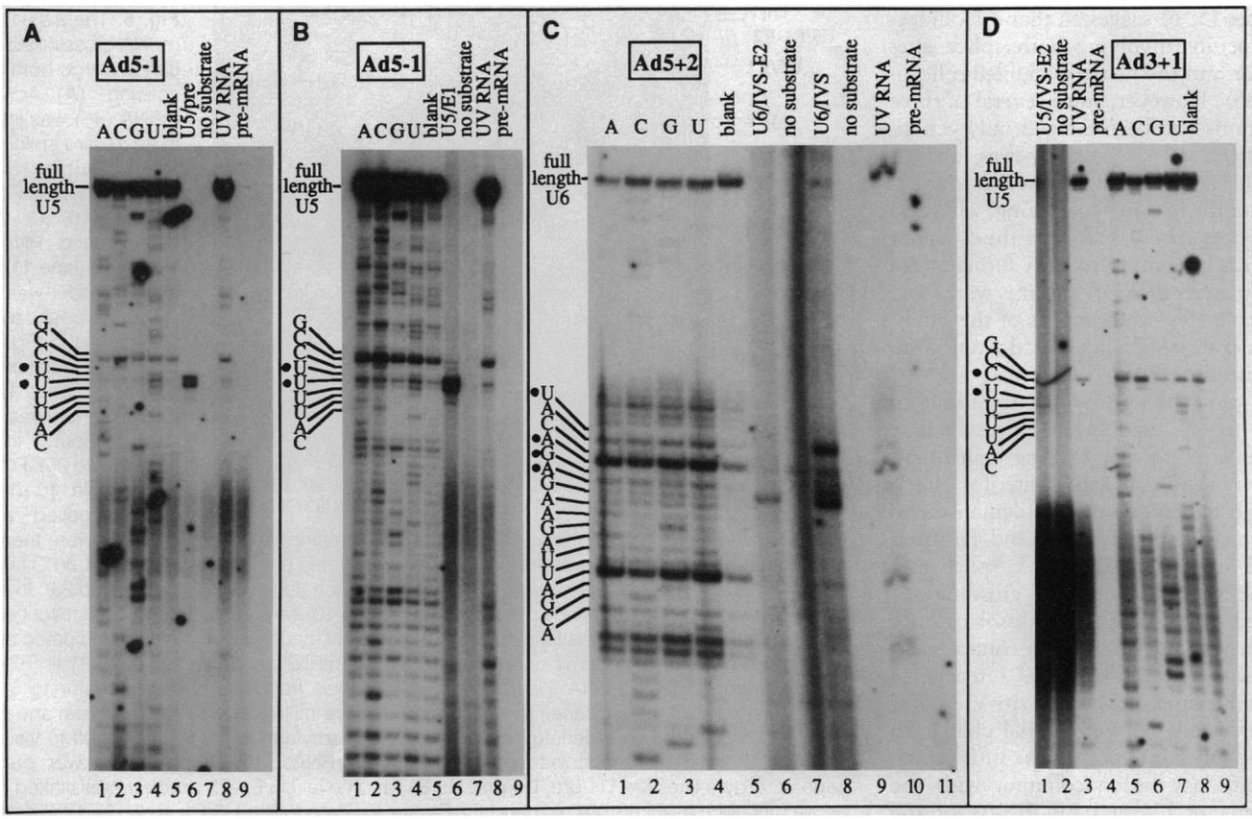

Fig. 5. Primer extension mapping of crosslinked sites in U5 and U6 snRNAs. An in vitro splicing reaction was incubated with or without 4-thioU–containing pre-mRNA, and extracted RNAs were fractionated on 5 percent or 8 percent polyacrylamide gels. Specific crosslinked species [Ad5-1 U5/pre-mRNA (U5/pre) (**A**), Ad5-1 U5/exon 1 intermediate (U5/E1) (**B**), Ad5+2 U6/lariat IVS-E2 intermediate (U6/IVS-E2) and U6/lariat IVS (U6/IVS) (**C**), and Ad3+1 U5/lariat IVS-E2 intermediate (U5/IVS-E2) (**D**)] were excised from the gel, along with the equivalent region of a lane containing RNA from extract cross-linked in the absence of substrate (no substrate), for use as templates. Uncrosslinked substrate (pre-mRNA) and total unfractionated RNA from extract irradiated in the absence of substrate (UV RNA) were also prepared as control templates. Dideoxynucleotide sequencing ladders (A, C, G, U, and blank) were generated for comparison. In (A), (B), and (D), primer extension reactions were performed with end-labeled $U5_{84-104}$ oligonucleotide primer as described (*6*); in (C), reactions were performed with end-labeled $U6_{90-104}$ oligonucleotide primer as described (*7*). Products of the reactions were fractionated on denaturing 12.5 percent polyacrylamide gels. Positions of conserved snRNA regions are given to the left of each panel; sites of crosslinking (one nt 5′ to the positions of primer extension blockage) are indicated by dots next to the sequence.

formulation of explicit models for the contributions of these snRNA sequences to the chemistry of nuclear pre-mRNA splicing. The ability of the U5/pre-mRNA crosslink to undergo both steps of the splicing reaction demonstrates that the crosslinking captures functional, rather than dead-end, intermediates. Evidence also emerges for specific conformational rearrangements within the active center as splicing proceeds.

The earliest snRNA-substrate interaction detected with the Ad5-1 construct involved U1 snRNA and was ATP-independent, as expected (*1*). Yet, the appearance of two distinct U1/pre-mRNA species [both of which were also observed with 4-thioU at position minus 2 (*6*)] was unexpected, suggesting that two different regions of U1 may contact exon 1 at early stages of spliceosome assembly. One of the crosslinks involving position minus 2 has been mapped to the 5′ end of U1 (*34*); the identities of the other crosslink at position minus 2 and both of the Ad5-1/U1 crosslinks remain to be determined.

The combined interactions of U1 with intron sequences (*1*, *4*) and U5 with exon sequences (*5*, *6*) suggested that a Holliday-like structure involving both splice sites might form in the newly assembled spliceosome (*35*). However, since several of these interactions had been defined only genetically, their relative timing was unclear (*35*). Our detection of U5/exon 2 contacts only after the first step of splicing, when U1 is no longer base-paired with the 5′ splice site (*1*), is in contrast to early formation of U5/exon 1 crosslinks (6 and this work) and argues that the two branches of the Holliday structure involving U5 do not form simultaneously (*36*).

The crosslinking of the last residue of exon 1 to U40 and U41 in the U5 loop identifies a previously missing component of the spliceosomal active site (or sites). Although genetic results (*5*) demonstrated an interaction between U5 and positions minus 2 and minus 3 at the 5′ splice site, the kinetics of the minus 2 crosslink (*6*) indicated that that contact dissolves prior to the first step of splicing. In contrast, our crosslink with position minus 1 reaches a peak at the time of 5′ splice site cleavage, implying that a conformational change in the U5-exon 1 interaction has intervened. This shift may be concomitant with the unwinding of U4 and U6 RNAs or the establishment of U6-5′ splice site pairing (or both) (*7*–*9*, *37*). A shift in U5 interactions was deduced previously on the basis of psoralen crosslinking (*7*), but the base pairing scheme hypothesized with intron sequences is not compatible with our data. Rather, the crosslinking of U41 of U5 to both positions minus 1 (in this article) and with intron sequences beyond plus 5 (*7*) suggests a contorted conformation of the 5′ splice site within the active center of the spliceosome (*38*). Perhaps such conformational flexibility also underlies the capacity of the conserved loop in human U5 snRNA, when mutated, to pair in several different registers with the 5′ splice site (*39*). Since the U5 conserved loop maintains contact with position minus 1 after 5′ splice site cleavage, it is implicated as the factor that holds the free E1 intermediate in the spliceosome, aligning it with the 3′ splice site for the second reaction step. This role is supported by the appearance of a crosslinked species containing U5 and the spliced E1-E2 product, detected only when the U5/pre-mRNA crosslink is formed early and then followed through the reaction.

The ability of U5 residues C39 and U40 to cross-link to the Ad3+1 substrate in the lariat IVS-E2 splicing intermediate indicates that the conserved loop also contacts

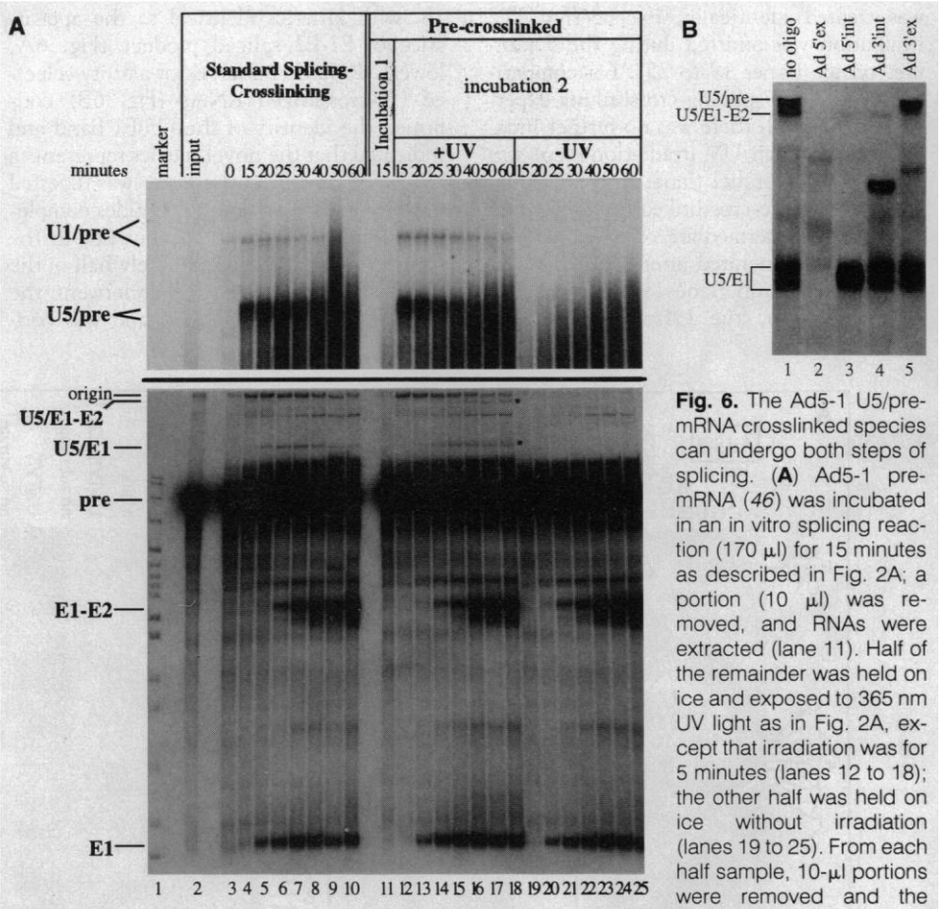

Fig. 6. The Ad5-1 U5/pre-mRNA crosslinked species can undergo both steps of splicing. (**A**) Ad5-1 pre-mRNA (*46*) was incubated in an in vitro splicing reaction (170 μl) for 15 minutes as described in Fig. 2A; a portion (10 μl) was removed, and RNAs were extracted (lane 11). Half of the remainder was held on ice and exposed to 365 nm UV light as in Fig. 2A, except that irradiation was for 5 minutes (lanes 12 to 18); the other half was held on ice without irradiation (lanes 19 to 25). From each half sample, 10-μl portions were removed and the RNAs were again extracted (lanes 12 and 19). The remainders of both samples were then placed at 30°C, and 10-μl portions were removed at various times (the total incubation at 30°C is given at the top of each lane), and RNAs were extracted from each (lanes 13 to 18 and 20 to 25). In addition, a standard splicing-crosslinking time course (lanes 3 to 10) series was obtained as described in Fig. 2A, except that irradiation was for 5 minutes; the times of incubation at 30°C are indicated at the top of each lane. Input (lane 2) is unspliced Ad5-1 pre-mRNA, and marker (lane 1) is ^{32}P-labeled pBR322 Msp I-digested DNA. Half of each sample was fractionated on a denaturing 5 percent polyacrylamide gel (upper panel) to visualize snRNA/pre-mRNA crosslinked species, and the other half was fractionated on a denaturing 10 percent polyacrylamide gel (lower panel) to visualize E1 and E1-E2 crosslinked species and uncrosslinked RNAs. The upper and lower panels are separated by a line. The U1/pre, U5/pre, U5/E1, and novel U5/E1-E2 product crosslinked species, as well as uncrosslinked pre, E1, and E1-E2 are indicated. The U5/E1 and U5/E1-E2 crosslinked bands are also indicated by black dots to the right of lane 18 (lower panel). (**B**) Ad5-1 pre-mRNA was incubated in a splicing reaction for 15 minutes, irradiated on ice, and further incubated at 30°C for 45 minutes as described in (A). Extracted RNAs were subjected to affinity selection with a biotinylated 2′-*O*-methyl oligonucleotide, B.U5Ae (complementary to nucleotides 69 to 87 of U5 RNA), as described (*7*). Selected RNAs were incubated with RNase H without oligonucleotide (lane 1), or in the presence of oligonucleotide Ad 5′ex (lane 2), Ad 5′int (lane 3), Ad 3′int (lane 4), or Ad 3′ex (lane 5) (*49*), and fractionated on a denaturing 10 percent polyacrylamide gel. Undigested U5/pre, U5/E1-E2, and U5/E1 crosslinked species are indicated. Although targeting of E2 yields the expected truncated pre-mRNA product, targeting of E1 in the pre-mRNA reproducibly leads to additional degradation, which is not understood.

the 3′ splice site during the second step of the reaction. This result is in accord with the genetic suppression data of Newman and Norman (5) that demonstrated base-pairing of the *Saccharomyces cerevisiae* equivalent of U40 with position plus 1 of the 3′ exon in substrates carrying 5′ or 3′ splice site mutations. Our data extend their observations to show that the U5-3′ exon interaction occurs in the mammalian spliceosome, after the first step of splicing, in the absence of mutations in conserved intron residues, and without a requirement for Watson-Crick complementarity.

The U5 conserved loop can now be viewed as the spliceosomal counterpart of the internal guide sequence (IGS) of group I self-splicing introns (*40*) and the exon binding site 1 (EBS1) of group II introns (*5, 41*) (Fig. 7). In all cases, specific intron or snRNA sequences maintain contact with E1 throughout the reaction, and adjacent nucleotides bring E2 into close proximity for the second step (*40, 41*). Base pairing is involved in the autocatalytic RNAs, whereas in the spliceosome the relative lack of exon sequence conservation (*26*) indicates that U5 often fulfills this function by way of non-Watson-Crick interactions, perhaps stabilized by U5 proteins (6). Group I and group II introns and the spliceosome were recently modeled as having two-metal-ion catalytic sites in which RNA functional groups form three binding sites (1, 2, and 3) that orient bases flanking the scissile phosphates (*15*). We propose that U40 and U41 of U5 comprise site 1 of the postulated two-metal-ion active site of the mammalian spliceosome; site 1 holds the last nucleotide of the 5′ exon, which acts as the leaving group during the first chemical step and as the nucleophile during the second step. U5 residues C39 and U40 would then contribute to site 3, which holds the end of the 3′ exon for nucleophilic attack, for the second step only.

The crosslinking of U6 residue A45 to the conserved GU dinucleotide at the 5′ splice site in both the lariat IVS-E2 intermediate and lariat IVS product firmly places the invariant hexanucleotide of U6 in the active center of the spliceosome for the second reaction step since the first and last nucleotides of the intron interact at this stage (*13*). We did not detect crosslinks between U6 and the GU dinucleotide within the pre-mRNA, even though U6 has been shown to contact the 5′ splice site prior to step 1 (*7–9*). Hence, our U6 crosslink with the lariat IVS-E2 intermediate hints that U6's mode of interaction with the 5′ splice site may change between the first and second steps (*36*), consistent with proposed remodeling of the spliceosomal active site (*14, 15*). Crosslinking of a single U6 residue to the lariat IVS-E2 intermediate and of multiple U6 (as well as U2) residues to the lariat IVS product also indicates that interactions with the 5′ splice site are rigidly constrained while the chemical steps of splicing are in progress, but then relax. U6 residues adjacent to the hexanucleotide had been shown to crosslink to 5′ splice site nucleotides further downstream in the intron in both the pre-mRNA (*7*) and lariat IVS-E2 intermediate (*9*), suggesting that part of the hexanucleotide engages in Watson-Crick pairing with the 5′ splice site in the pre-mRNA (*7*). Genetic suppression experiments in *S. cerevisiae* have confirmed some of these base pairs (*37*). The importance of U6 in 5′ splice site use has received support from in vitro reconstitution studies which demonstrate that mutation of certain U6 residues upstream of the ACAGAG sequence results in aberrant attack of the branch site on U6 snRNA instead of the 5′ splice site in the pre-mRNA (*42*).

A45 is particularly critical for U6 function. Mutation of the analogous A residue (A51) in *S. cerevisiae* is lethal in vivo (*31*) and causes a dramatic defect in the second step of splicing in vitro (*30*); also, replacement of A51 with 2′-deoxyadenosine results in complete inhibition of the second step of splicing (*43*). Similar splicing defects are observed in vivo and in vitro when A45 of human U6 is mutated (*32*). The first step of splicing is impaired when the phosphate group 5′ to either of the two G residues flanking *S. cerevisiae* A51 is substituted with thiophosphate, perhaps indicating that this region of U6 contributes to a specific Mg^{2+} binding site (or sites) (*44*). Finally, this A residue is the site of intron insertion in the fungus *Rhodosporidium dacryoidum* (*45*). Our results add to this growing list, and further suggest that A45 of U6 may contribute to site 2 for step 2 (*15*) by functioning analogously to ε, a sequence that pairs with intron nucleotides near the 5′ splice site for both steps of group II self-splicing (*41*). As more RNA-RNA contacts involved in pre-mRNA splicing are identified, further parallels between the spliceosome and self-splicing introns are likely to emerge.

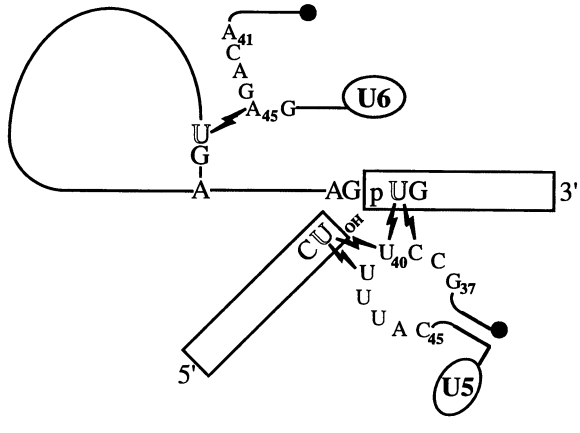

Fig. 7. U5 and U6 snRNA interactions with the E1 and lariat IVS-E2 intermediates during the second step of splicing. Designations for the Adeno substrate are as in Fig. 1; the 3′ hydroxyl group of the E1 intermediate (OH) and the 3′ splice site phosphate in the lariat IVS-E2 intermediate (p) are shown. Invariant U5 and U6 nucleotides are given; the filled circles are 5′ cap structures. Lightning bolts connecting snRNA and substrate residues represent site-specific crosslinks characterized in this article. Other known interactions that are likely to occur during the second step include 5′ splice site (intron) contacts with other residues in U6 (*9*), U2 base pairing with the branch site (*50*), two U2-U6 base-pairing interactions (*10, 11*) and a non-Watson-Crick interaction between the G residues at the extreme 5′ and 3′ ends of the intron (*13*).

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17. Some advantages of 4-thioU are (i) it is a commercially available non-bulky uridine analog that can be specifically and efficiently photoactivated by short (2 to 10 minutes) exposure to long UV wavelengths; (ii) it forms crosslinks with either RNA or protein; (iii) crosslinking occurs only at very close range; and (iv) RNA-RNA crosslinks are favored in regions of non-Watson-Crick inter-

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14 September 1993; accepted 24 November 1993