Association of U6 snRNA with the 5'-splice site region of pre-mRNA in the spliceosome

Hitoshi Sawa and Yoshiro Shimura¹

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

U6 snRNA is one of the five RNA species required for splicing of nuclear pre-mRNAs. High conservation of its sequence has led to the hypothesis that U6 snRNA plays a catalytic role in splicing. If this is the case, U6 snRNA should be localized close to sites where the splicing reaction occurs. However, this has never been demonstrated. Here, we have shown that U6 snRNA is cross-linked to the 5'-splice site region of pre-mRNA by UV irradiation during the in vitro splicing reaction. We have also detected the cross-link of U6 snRNA and the region around the branchpoint of the intron lariat. The results show that U6 snRNA is present near the splice sites in the splicing reaction and support the idea that U6 snRNA is a catalytic element in the spliceosome.

[**Key Words:** Pre-mRNA splicing; U6 snRNA; spliceosome; UV cross-linking; catalytic RNA]

Received September 6, 1991; revised version accepted December 6, 1991.

Nuclear pre-mRNA splicing requires four kinds of small nuclear ribonucleoprotein (snRNP) complexes, U1, U2, U5, and U4/U6, which contain five distinct snRNAs (Guthrie and Patterson 1988). Among these snRNPs, U1 and U2 bind to the 5'-splice site and the branchpoint, respectively. The binding of these snRNPs to pre-mRNA is the result of base-pairing between the small nuclear RNAs (snRNAs) and the two pre-mRNA regions, because mutations in the 5'-splice site and branchpoint regions are suppressed by compensatory base changes of U1 and U2 snRNAs, respectively (Zhuang and Weiner 1986, 1989; Wu and Manley 1989). U5 snRNP has also been suggested to associate with the 3'-splice site region (Chabot et al. 1985). However, direct association of U4/U6 snRNP with specific regions of pre-mRNA has not been demonstrated.

Splicing of nuclear pre-mRNA proceeds via two-step reactions that occur in the spliceosome (Krainer and Maniatis 1988). The first step includes cleavage at the 5'-splice site, generating the first exon and the intron/second-exon lariat RNA (lariat intermediate), whereas the second step involves cleavage at the 3'-splice site and ligation of the two exons. These reactions share some fundamental properties with those of self-splicing of group II introns that occur by the catalytic activity of introns themselves (Peebles et al. 1986; van der Veen et al. 1986). Therefore, it has been hypothesized that nuclear pre-mRNA splicing is catalyzed by snRNAs rather than by protein factors (Sharp 1985; Cech 1986). U6

snRNA has been proposed to be a candidate for the catalytic element of splicing for several reasons (Brow and Guthrie 1989). First, U6 snRNA is the most conserved snRNA, especially in the middle of the snRNA sequences (Guthrie and Patterson 1988). Second, mutational analyses of U6 snRNA revealed importance of the highly conserved region for both steps of the splicing reaction (Bindereif et al. 1990; Fabrizio and Abelson 1990; Madhani et al. 1990; Vankan et al. 1990). Third, the presence of introns in the U6 snRNA genes of some organisms (Tani and Ohshima 1989, 1991) has been interpreted to be the result of evolutionary mishaps during pre-mRNA splicing, in which the introns excised from pre-mRNA were inserted accidentally by reverse reaction into specific sites of U6 snRNA, thereby reflecting the proximity of the sites to the catalytic center of the spliceosome (Brow and Guthrie 1989). Fourth, structural similarity of U6 snRNA to the catalytic center of the satellite RNA of tobacco ring spot virus, which has self-catalyzed cleavage activity, has been suggested (Tani and Ohshima 1991). If this hypothesis is correct, we can expect that U6 snRNA is localized close to the sites of pre-mRNA where the cleavage–ligation reactions occur and this snRNA interacts directly with these sites. However, the association of U6 snRNA with pre-mRNA has never been demonstrated.

We have analyzed the association of snRNAs with pre-mRNA by a UV cross-linking technique. Two cross-linked products of U6 snRNA and the 5'-splice site region of pre-mRNA were observed, in addition to those between pre-mRNA and U1 snRNA or U2 snRNA. Furthermore, we have observed a cross-linked product of U6

¹Corresponding author.

snRNA and the intron lariat. Its cross-linking site was mapped near the branchpoint of the intron lariat. The results indicate that U6 snRNA is localized very close to the 5'-splice site before the first-step reaction and near the 3'-splice site after the second-step reaction.

Results

Cross-linking of snRNAs to pre-mRNA during the splicing reaction

We prepared pre-mRNA (δ EX14-15 RNA) by *in vitro* transcription of the plasmid that contains exons 14 and 15 and the interrupting intron of the δ -crystalline gene as described previously (Sawa et al. 1988). This pre-mRNA was shown to be spliced precisely when incubated with a nuclear extract of HeLa cells. After incubation of uniformly labeled δ EX14-15 pre-mRNA with the nuclear extract, the reaction mixture was diluted with buffer and irradiated with UV light. RNAs were then recovered after proteinase treatment and analyzed by denaturing polyacrylamide gel electrophoresis. Several UV-dependent bands, which migrated more slowly than pre-mRNA, were observed (Fig. 1, lanes 2, 9, 16). It is likely that these bands were produced by the cross-linking of pre-mRNA to RNAs that were present in the nuclear extract and associated directly with pre-mRNA during incubation. To examine whether some of the bands represented the cross-linked products between snRNAs and pre-mRNA, irradiated RNAs were incubated with RNase H and oligodeoxynucleotides complementary to the five snRNAs prior to electrophoresis. The oligonucleotides employed were essentially the same as those used previously to disrupt the five snRNPs (Kramer et al. 1984; Black et al. 1985; Black and Steitz 1986), although such disruption experiments were not performed with naked snRNAs. With this treatment, we can expect that snRNAs cross-linked to pre-mRNA are digested specifically by RNase H, if the oligonucleotides hybridize with the cross-linked RNAs. These experiments would be

misleading if the oligonucleotides fortuitously target the pre-mRNA substrate. Fortunately, however, unexpected cleavage of pre-mRNA due to such fortuitous targeting was not observed with the digestion products that we analyzed.

When the irradiated RNAs, which had been incubated with the nuclear extract for 5 min, were analyzed (Fig. 1, lanes 2–7), the slowest-migrating band among four major cross-linked products almost disappeared upon incubation with RNase H and a U2-specific oligonucleotide. This indicates that this band was a cross-linked product of U2 snRNA and pre-mRNA (designated U2-pre). Similarly, two bands that migrated below the U2-pre band were identified as cross-linked products of U1 snRNA and pre-mRNA (designated U1-preI and U1-preII). The band migrating below U1-preII, tentatively designated X RNA, could not be characterized, because this band was not cleaved by RNase H with any oligonucleotides used here. When incubation with the nuclear extract was carried out for 20 min before UV irradiation (Fig. 1, lanes 9–14), a cross-linked product whose electrophoretic mobility was similar to that of X RNA was observed in addition to U2-pre. This product was identified as a cross-linked product of U6 snRNA and pre-mRNA (designated U6-preI), because this product almost disappeared by RNase H digestion with a U6-specific oligonucleotide. In all cases, none of the cross-linked bands disappeared totally after RNase H digestion. This might be the result of contaminating RNAs. However, such uncleaved bands were also observed even with free pre-mRNA (not UV-irradiated) hybridized with oligonucleotides, depending on the conditions employed (data not shown). Moreover, the amounts of the undigested bands definitely increased with the cross-linked RNAs. Thus, the incomplete digestion of the cross-links is probably the result of some damage of the cross-linked RNAs caused by UV irradiation and/or low hybridization efficiency of oligonucleotides. On the basis of these results, we think that the presence of the undigested bands cannot be attributed primarily to contaminating RNAs.

Figure 1. Identification of cross-linked products of snRNAs and pre-mRNA. 32 P-labeled δ EX14-15 pre-mRNA was incubated with a HeLa nuclear extract in 5- μ l reaction mixture under the standard splicing conditions for 5 min (lanes 1–7), 20 min (lanes 8–14), or 30 min in the presence of 2.5 mM EDTA (lanes 15–21). Each reaction mixture was then irradiated and RNAs were recovered as described in Materials and methods. The recovered RNAs were treated with RNase H and oligonucleotide (oligo) complementary to a specific snRNA (indicated at the top of each lane) and analyzed as described in Materials and methods. (ori) The origin of the electrophoresis. The bands of cross-linked products are indicated, and the positions of the lariat intermediate, intron lariat, and pre-mRNA are shown schematically.

When pre-mRNA was incubated with the nuclear extract in the presence of EDTA before irradiation (lanes 16–21), an additional product cross-linked to U6 snRNA was detected below U6-preI (designated U6-preII). The formation of the spliceosome occurred in the presence of EDTA, but the first-step reaction was blocked (data not shown), as reported previously (Abmayr et al. 1988). A cross-linked product similar to U6-preII was also present in a small amount in the irradiated reaction mixtures that had been incubated for 20 min in the absence of EDTA. In all of the experiments, some other minor cross-linked products were observed in addition to those described above, but those minor products were not characterized well here. We could not detect any pre-mRNA molecules cross-linked simultaneously to more than two snRNAs. This was probably the result of low efficiency of the cross-linking under the conditions employed. Less than 0.1% of pre-mRNA was converted to each cross-linked product. Using another pre-mRNA (δ EX13-14 RNA) that was also derived from the δ -crystalline gene but contained exons 13 and 14 and the interrupting intron sequence (Ohno et al. 1987), a cross-linked product similar to U6-preI was detected (data not shown). Also in the case of β -globin pre-mRNA (H β Δ 6 RNA; Krainer et al. 1984), a similar cross-linked product of U6 snRNA was detected (data not shown) only when the product was selected by affinity with a biotinylated oligonucleotide complementary to U6 snRNA (see below).

To investigate the relationship between the formation of the cross-links and the splicing of pre-mRNA, δ EX14-15 pre-mRNA was incubated with the nuclear extract for different times prior to the irradiation (Fig. 2). These analyses were possible because all of the five cross-linked products that we characterized were sufficiently pure and very little cross-contamination between the cross-links was present. Although it is not clear in Figure 2 (lanes 1, 7, 13), the presence of small amounts of the two cross-linked products with U1 snRNA (U1-preI and U1-preII) were detected even without incubation or in the absence of ATP. This is consistent with the previous observation that U1 snRNP binds to the 5'-splice site region independently of ATP and incubation (Black et al. 1985; Bindereif and Green 1987). The cross-linked products with U1 snRNA were increased significantly after 5 min incubation and decreased gradually after 10 min incubation (Fig. 2, lanes 2–6). It appears that the formation of U1-preI and U1-preII is stabilized by incubation with ATP but subsequently destabilized in the course of the splicing reaction. The appearance of the U2 and two U6 cross-linked products was dependent on ATP and incubation. Moreover, these cross-linked products could not be detected when incubation was carried out with a cytoplasmic S-100 fraction from HeLa cells (Fig. 2, lane 14). It has been shown that this fraction contains all sets of snRNPs required for splicing but is inactive in the splicing reaction by itself (Krainer and Maniatis 1985) and even in spliceosome formation (Krainer et al. 1990). These results show that the formation of U2-pre and two U6 cross-linked products is dependent on the forma-

Figure 2. UV-cross-linking analyses during the splicing reactions. 32 P-labeled δ EX14-15 pre-mRNA was incubated in 5- μ l reaction mixture with a HeLa nuclear extract (NE) or an S-100 fraction (lane 14) for the times indicated at the top of each lane under standard splicing conditions (lanes 1–6, 14), in the presence of 2.5 mM EDTA (lanes 7–12), or in the absence of ATP and creatine phosphate (lane 13). Each reaction mixture was then UV-irradiated, and RNAs were recovered and analyzed by electrophoresis on a denaturing 4.5% polyacrylamide gel.

tion of splicing complexes. Thus, although we have not characterized all of the cross-linked RNAs, we conclude that the time course of the appearance and disappearance of the five cross-linked products we examined is essentially consistent with the previous reports on the behavior of U1, U2, and U6 snRNPs during the splicing reaction (Grabowski and Sharp 1986; Konarska and Sharp 1986; Bindereif and Green 1987).

Determination of cross-linking sites

To map the cross-linking sites, each of the five cross-linked products was purified by gel electrophoresis and cleaved by RNase H in the presence of oligonucleotides complementary to pre-mRNA or snRNAs. It should be noted that purified cross-linked products were not always cleaved completely as described above and that the uncleaved material remaining in most of the samples in Figure 3 was not be cleaved upon longer incubation.

The cross-linking sites on pre-mRNA were analyzed using oligonucleotides complementary to various portions of pre-mRNA (Fig. 3B). The cleaved RNAs were electrophoresed using the cleavage products of intact pre-mRNA as markers. The rationale behind this experiment is illustrated in Figure 3A. If a cross-linking site is located upstream of the region complementary to an oligonucleotide, a linear RNA fragment corresponding to

Figure 3. Mapping of cross-linking sites on pre-mRNA. (A) The experimental strategy described in the text is shown schematically. The thick small bars represent complementary oligodeoxynucleotides. (PAGE) Denaturing polyacrylamide gel electrophoresis. (B) The nucleotide sequences of the 5'-splice site (5'SS) region and the branchpoint (BP)-3'-splice site (3'SS) region of 8EX14-15 pre-mRNA. Sequences complementary to respective oligonucleotides used in this study are underlined. (C-E) The cross-linking sites of U1-preI and U1-preII (C), U2-pre (D), or U6-preI and U6-preII (E) are mapped. Cross-linked products were purified by electrophoresis of irradiated RNAs that had been incubated under standard splicing conditions for 5 min (U1-preI and U1-preII), 20 min (U2-pre and U6-preI), or 30 min in the presence of 2.5 mM EDTA (U6-preII). The purified cross-linked RNAs and uncross-linked pre-mRNA were incubated with RNase H in the absence (no oligo) or in the presence of the oligonucleotide (indicated at the top of each lane), and the digestion products were analyzed by electrophoresis on a denaturing 4.5% polyacrylamide gel. Brackets labeled 5' and 3' indicate the positions of 5'-side and 3'-side fragments of pre-mRNA, respectively.

the 3' portion of cleavage products of pre-mRNA (3'-side fragment of pre-mRNA) is produced and an RNA fragment corresponding to the 5' portion of cleavage products of pre-mRNA (5'-side fragment of pre-mRNA) is still cross-linked to snRNA. The latter fragment can be identified as a slowly migrating band. In contrast, if the site is located downstream, a linear 5'-side fragment of pre-mRNA is generated.

As shown in Figure 3C, when U1-preI and U1-preII were digested with oligonucleotide 5'E, the small 5'-side fragment of pre-mRNA and its 3'-side cross-linked counterparts were detected, indicating that cross-linking sites of these RNAs are located downstream of the region complementary to oligonucleotide 5'E (5'E region). On the other hand, when oligonucleotide 5'SII was used, the 3'-side fragment of pre-mRNA was produced from both U1-preI and U1-preII. In the case of U1-preI, the band migrating immediately below the 3'-side fragment represents the 5'-side cross-linked counterpart. The band migrating immediately above the 3'-side fragment originated from U1-preII by digestion with 5'SII is not intact pre-mRNA, although the electrophoretic mobilities of the two bands are similar, but represents the 5'-side fragment cross-linked with U1 snRNA. This conclusion is based on the following results. First, there was no sub-

stantial contamination of intact pre-mRNA in the U1-preII preparation (Fig. 3C, lane labeled no oligo in U1-preII). In addition, these two bands never comigrated upon close examination (data not shown). Furthermore, the 5'-side fragment seen in the digests of U1-preII with 5'E disappeared in the 5'SII digests and a new band appeared above the 3'-side fragment. These results led us to conclude that the cross-linking sites of both U1-preI and U1-preII must be present in the region spanning from the 5'E region to the 5'SII region. Although the difference between U1-preI and U1-preII was not clear in this experiment, their cross-linking sites may be different from each other within this region of pre-mRNA and/or their cross-linking sites on U1 snRNA could differ from each other. Similarly, the cross-linking site of U2-pre was mapped in the region spanning from the BP region to the 3'-S region (Fig. 3D). In Figure 3D, the two bands produced by cutting U2-pre with oligonucleotide BP are the 5'-side fragment and the 3'-side cross-linked counterpart. The latter fragment migrated immediately above the former fragment. On the other hand, when U2-pre was digested with 3'S, the 3'-side fragment and its cross-linked 5'-side counterpart were observed. These results confirm the previous reports that U1 and U2 snRNAs interact by base-pairing with the 5'-splice site and the

branchpoint, respectively (Zhuang and Weiner 1986, 1989; Wu and Manley 1989).

To analyze the cross-linking sites of U6-preI and U6-preII, these RNAs were digested by RNase H with oligonucleotides 5'SI, 5'SII, and IntI (Fig. 3E). When both RNAs were digested with oligonucleotide 5'SI, the small 5'-side fragment of pre-mRNA was generated. Thus, the cross-linking sites of U6-preI and U6-preII must be downstream of the 5'SI region. Although faint bands corresponding to the 3'-side fragment of pre-mRNA were also detected, it is likely that these fragments were derived from contaminating uncross-linked pre-mRNA (Fig. 3E, lanes labeled no oligo). When U6-preI was digested with oligonucleotide 5'SII, the small 5'-side fragment of pre-mRNA and its cross-linked 3'-side counterpart were detected, indicating that the cross-linking site is downstream of the 5'SII region. In contrast, when U6-preII was cleaved with oligonucleotide 5'SII, the 3'-side fragment of pre-mRNA was generated efficiently. Unexpectedly, however, in addition to the 3'-side fragment, the 5'-side fragment of pre-mRNA was also produced from U6-preII. This seems to suggest that the cross-linking site of U6-preII is, in all likelihood, located within the 5'SII region, and that both sides of this site were cleaved by RNase H. In any case, we conclude that the site is located within the 5'SI and 5'SII regions. When U6-preI and U6-preII were digested by RNase H with oligonucleotide IntI, the 3'-side fragment of pre-mRNA was generated efficiently. Therefore, the cross-linking of U6-preI occurred in the region spanning from the 5'SII region to the IntI region. It is worth noting that when similar RNase H digestion experiments were carried out with various oligonucleotides downstream of IntI, the cross-linking sites were mapped upstream of these oligonucleotide regions with both U6-preI and U6-preII (data not shown). These results also show that all of the cross-linked molecules of snRNAs characterized here were produced with intact pre-mRNA but not with the intermediates or products of the splicing reaction, because RNase H digestion of the cross-linked products at either side of the cross-linking sites generated linear RNA fragments that were the same as those obtained by RNase H digestion of intact uncross-linked pre-mRNA with corresponding oligonucleotides.

Cross-linked product of U6 snRNA and intron lariat

The results described above show that U6 snRNA is localized near the 5'-splice site prior to the first cleavage-ligation reaction as consistent with the hypothesis that U6 snRNA is a catalytic element of the splicing reaction (Brow and Guthrie 1989). To further examine this hypothesis, it is important to know the location of U6 snRNA during the later stages of the splicing reaction. Although cross-links of U6 snRNA with the intermediates or products of the splicing reaction were not observed in the experiment presented in Figure 1, it is likely that they were present below the detectable level owing to the nonspecific cross-linked products present throughout each lane. To detect such minor components

among the poorly resolved nonspecific products, we performed affinity selection of the cross-linked products using a biotinylated oligonucleotide that was complementary to U6 snRNA. After irradiation of the reaction mixture, RNAs were recovered and incubated with the biotinylated U6 oligonucleotide, followed by incubation with streptavidin-agarose. After the washing of the beads, bound RNAs were eluted and analyzed by electrophoresis.

As shown in Figure 4A, when the splicing reaction mixture was incubated for 10 min and, thus, the reaction was allowed to proceed to stages just before the second-step reaction, only U6-preI and U6-preII were observed after the affinity-selection procedure (lane 2). About 30% of these RNAs were selected by this procedure. In contrast, when the reaction was allowed to proceed for 20 min, an additional cross-linked RNA (tentatively designated Y RNA) was selected (lane 5). That the splicing reaction proceeded faster in the affinity-selection experiments than in the initial cross-linking experiments, appears to be the result of the difference of the splicing activities between the nuclear extracts (e.g., cf. Fig. 2 with Fig. 4A). However, no qualitative difference was present in the two experiments and essentially the same results were obtained with any extract preparations. Y RNA was cleaved with RNase H and a U6-specific oligonucleotide that was different from that used for affinity selection (lane 6), confirming that this is the cross-linked product of U6 snRNA. Y RNA was not observed when the second-step reaction was blocked by using a preheated HeLa nuclear extract (lane 8; Krainer and Maniatis 1985), indicating that Y RNA represents a cross-link of U6 snRNA with the intermediates or the products of the splicing reaction.

To determine whether Y RNA contained the lariat structure, pre-mRNA, the intron lariat, and Y RNA were recovered from the gel and then digested completely with RNase T2. The digests were fractionated by two-dimensional thin-layer chromatography (Fig. 4B–D). When the intron lariat was analyzed (Fig. 4C), a nuclease-resistant spot (indicated by the arrowhead) was observed in addition to the four canonical nucleotide-3'-phosphates (Ap, Gp, Cp, and Up). Similar nuclease-resistant spots were reported previously to be generated from lariat RNAs (Padgett et al. 1984; Ruskin et al. 1984; Newman et al. 1985; Sakamoto et al. 1987) and shown to be a branched trinucleotide (Padgett et al. 1984). A similar oligonucleotide spot was also produced from Y RNA (Fig. 4D). This suggests that Y RNA contains the lariat structure. When Y RNA was subjected to the debranching assay using the nuclear extract or S-100 fraction (Ruskin and Green 1985), the mobility of the molecule was increased, suggesting the occurrence of debranching (data not shown). Moreover, Y RNA was cleaved by RNase H with oligonucleotides complementary to intron (see below) but not with those complementary to 5' or 3' exon (data not shown). On the basis of these results, we conclude that Y RNA is the cross-linked product of U6 snRNA and intron lariat (designated hereafter as U6-lariat).

Figure 4. Affinity selection of cross-linked products of U6 snRNA. (A) The reaction mixture incubated for 10 min (lanes 1–3), 20 min (lanes 4–6) with the nuclear extract (NE), or 1 hr with the nuclear extract that had been preincubated at 45°C for 15 min (lanes 7,8) were irradiated, and the RNAs were recovered. One-tenth volume of the RNA preparation was directly electrophoresed (lanes 2,4,7), and the rest of the RNA preparation was used for affinity selection by a biotinylated U6 oligonucleotide (U6a in Fig. 6) as described in Materials and methods. Half of the selected RNA was electrophoresed before (lanes 2,5,8) or after (lanes 3,6) treatment with RNase H and U6 oligonucleotide (U6c in Fig. 6). The bands of cross-linked products are indicated, and the positions of the lariat intermediate, intron lariat, and pre-mRNA are shown schematically. The bands of RNase H cleavage products of U6–preI and U6–preII are indicated by the star, and that of Y RNA by the asterisk. Pre-mRNA (B) and intron lariat (C) purified by electrophoresis, and Y RNA (D) purified by affinity selection and electrophoresis, were digested completely by RNase T2, and the digests were fractionated by two-dimensional thin-layer chromatography as described previously (Sakamoto et al. 1987). The first dimension is from *bottom to top*; the second dimension is from *left to right*, as indicated by arrows. The four canonical nucleotides (Ap, Gp, Cp, and Up), origin (o), and the nuclease-resistant spots (arrowheads) are indicated.

To determine the location of the cross-linking site of U6–lariat, the RNA was cleaved with RNase H and oligonucleotides complementary to specific regions of the intron, and electrophoresed using the digestion products of the uncross-linked intron lariat as size markers (Fig. 5). When oligonucleotide PPY was used, the cleavage product migrated slightly faster than the intact U6–lariat (lane 5), indicating that this cleavage product still contained U6 snRNA. Therefore, the cross-linking site is located upstream of the PPY region. When oligonucleotides IntI and IntII were used simultaneously, an RNA fragment derived from the large loop region of the lariat

structure was produced from U6–lariat (lane 6) as well as from the intron lariat (lane 3), indicating that the cross-linking site is not in this RNA fragment. Consequently, the site of U6–lariat was mapped in the region very close to the branchpoint of the intron lariat, located upstream of the IntI region, downstream of the IntII region, and upstream of the PPY region. In these RNase H cleavage experiments, the smaller counterparts of the larger cleavage products were probably not detected because they had little radioactivities.

Cross-linking sites on U6 snRNA

To examine where the cross-linking occurred on U6 snRNA, U6–preI, U6–preII, and U6–lariat were digested by RNase H with four oligonucleotides complementary to different portions of U6 snRNA (Fig. 6A). Because U6 snRNA was not radioactively labeled, only cross-linked cleavage products containing pre-mRNA were detectable. As shown in Figure 6B, when these cross-linked

Figure 5. Mapping of the cross-linking site of U6–lariat. (A) Schematic representation of the intron lariat and the regions complementary to respective oligonucleotides used in this experiment are indicated (for details, see Fig. 3B). (B) Uncross-linked intron lariat (lanes 1–3) and U6–lariat (lanes 4–6), purified as described in Fig. 4, were treated with RNase H in the absence (no oligo) or presence of the oligonucleotides (indicated at the *top* of each lane), and the digestion products were analyzed by electrophoresis on a denaturing 4.5% polyacrylamide gel.

Figure 6. Mapping of the cross-linking sites of U6-preI, U6-preII and U6-lariat on U6 snRNA. (A) Nucleotide sequence of that entire U6 snRNA. The two regions that base-pair with U4 snRNA are indicated as stems I and II (Bindereif et al. 1990). The sites equivalent to the insertion sites of the U6 snRNA introns of some organisms (Tani and Ohshima 1989, 1991) are indicated by arrows. Sequences complementary to oligonucleotides used in this experiment are underlined. (B) Electrophoretic gel patterns of RNase H digests of U6-preI, U6-preII, and U6-lariat. U6-preI and U6-preII were purified as described in Fig. 3, and U6-lariat was purified as described in Fig. 4. RNase H treatments of these RNAs in the absence (no oligo) or presence of the oligonucleotide (indicated at the top of each lane) were performed, and the digestion products were analyzed by electrophoresis on a denaturing 4.5% polyacrylamide gel. The arrowhead, star, and asterisk indicate the positions of the free pre-mRNA, lariat intermediate, and intron lariat, respectively.

products were digested with oligonucleotides U6a–U6c, the electrophoretic mobilities of the cleavage products became gradually faster, indicating that the cross-linking sites were present upstream of the U6c region. When U6-preII and U6-lariat were digested with oligonucleotide U6d, the cleavage products migrated much more slowly than those generated with oligonucleotide U6c (Fig. 6B). These results show that the cross-linking sites of U6-preII and U6-lariat are in the region spanning from U6d to U6c. This region is known to contain the essential sequence for the function of U6 snRNA (Bindereif et al. 1990; Fabrizio and Abelson 1990; Madhani et al. 1990; Vankan et al. 1990). It is not clear why the cleavage products with oligonucleotides U6a and U6d from U6-preII migrated differently, despite the fact that U6 snRNA fragments of similar length were expected to be generated in both cases. It would be possible to assume that this is the result of the difference in length of the remaining portions of U6 snRNA relative to the cross-linking site for each cleavage product. In the case of U6-preI, we could not define the cross-linking site precisely, because this RNA was not cleaved by RNase H with oligonucleotide U6d. We could only say that the site is upstream of the U6c region.

Association of U6 snRNA in the spliceosome

In view of the fact that the splicing reaction occurs in the spliceosome, it is important to examine whether the association of snRNAs with pre-mRNA takes place in this

complex. The UV cross-linking experiments were performed after fractionation of the splicing reaction mixture by glycerol density gradient sedimentation. The *in vitro* splicing reaction was allowed to occur for 20 min under the standard conditions, and the reaction mixture was then fractionated by glycerol density gradient sedimentation. As reported previously (Frederewey and Keller 1985; Grabowski et al. 1985; Bindereif and Green 1986; Sawa et al. 1988), three radioactive peaks were observed after fractionation (Fig. 7, top). The fastest sedimenting peak represented the 65S spliceosome, as judged by the accumulation of the lariat intermediate. The other peaks were the 45S precursor form of the spliceosome (prespliceosome) and the 20S nonspecific complexes. The fractions of even numbers were irradiated and analyzed by electrophoresis.

As shown in the bottom panel of Figure 7, U2-pre was observed only in the spliceosome fractions. This suggests that direct association of U2 snRNA with pre-mRNA occurs after spliceosome formation, although U2 snRNP is incorporated into the prespliceosome (Konarska and Sharp 1986). This is consistent with the previous observation that mutations in the branchpoint sequence that inhibit splicing do not prevent spliceosome assembly (Reed and Maniatis 1988). Both U1-preI and U1-preII were observed in the prespliceosome and also in the nonspecific complexes, but not in the spliceosome. This is consistent with previous reports that association of U1 snRNP with the spliceosome is unstable (Grabowski and Sharp 1986; Konarska and Sharp 1986; Bindereif and

Figure 7. UV cross-linking analyses after fractionation of splicing complexes. After the standard splicing reaction for 20 min, the reaction mixture was sedimented through a 10–30% glycerol density gradient as described previously (Sawa et al. 1988). Nine-drop fractions were collected from the bottom of the tube and the Cerenkov count of each fraction was measured (top). The positions of the 65S, 45S, and 20S complexes (see text) are indicated by arrows. The fractions of even numbers were irradiated without dilution and analyzed by electrophoresis (bottom). (Lane T) The UV cross-linking analysis of the reaction mixture without fractionation.

Green 1987). U6-preI was detected only in the spliceosome fractions, which is consistent with the previous finding that U6 snRNP is present in the spliceosome but not in the prespliceosome (Bindereif and Green 1987; Konarska and Sharp 1987). A cross-linked product similar to U6-preI observed from fractions 10 to 20 was probably uncharacterized X RNA (see Fig. 1), because its electrophoretic mobility was slightly faster than U6-preI. In this connection, only the band corresponding to U6-preI in the spliceosome fractions disappeared by treatment with RNase H and the U6 oligonucleotide but not X RNA band in fractions 10–20 (data not shown). Essentially similar results were obtained with the reaction mixture that was incubated for 5 min (data not shown). As was the case with U6-preI, U6-preII was observed only in the spliceosome fractions when the reaction mixture that had been incubated in the presence of EDTA was analyzed (data not shown).

To determine the complexes from which U6-lariat was generated, each peak fraction obtained by the density gradient sedimentation was irradiated, and RNAs were then recovered and affinity selected using the biotinylated U6 oligonucleotide (Fig. 8). U6-preI and U6-preII were selected only from the 65S spliceosome fraction (lane 6), which is consistent with the results described in Figure 7. U6-lariat was observed in the 65S and 45S fractions (lanes 6,7). It has been shown that the excised intron lariat was released from the spliceosome to form a smaller complex (postsplicing complex) containing U2, U5, and U6 snRNAs (Bindereif and Green 1986; Konarska and Sharp 1987; Company et al. 1991). Our results show that U6 snRNA associates with the intron lariat in the spliceosome as well as in the postsplicing complex. Although the intron lariat was present in the 20S fraction (Fig. 8, lane 4), U6-lariat was not observed in this fraction (lane 8). This is consistent with the previous report that the intron lariat in such small complexes does not assemble with snRNPs (Bindereif and Green 1986).

Figure 8. Affinity-selection experiments using fractionated splicing complexes. The 65S, 45S, and 20S fractions were obtained as described in Fig. 7. Affinity-selection experiments were then performed after irradiation of each fraction and unfractionated reaction mixture (T). The selected RNA preparations (lanes 5–8) and the RNAs recovered from 1% of each fraction and unfractionated reaction mixture without irradiation (lanes 1–4) were electrophoresed. The bands of cross-linked products are indicated, and the positions of lariat intermediate, intron lariat, and pre-mRNA are shown schematically.

Discussion

The UV cross-linking procedures have been used widely to probe specific intermolecular and intramolecular contacts of RNAs (Branch et al. 1989). Using this technique, we have shown the association of U1, U2, and U6 snRNAs with pre-mRNA during the splicing reaction. Although we could not detect any cross-linked products with U4 and U5 snRNAs in the present experiments, this does not imply that these snRNAs do not associate with pre-mRNA. The association of U1 and U2 snRNAs with pre-mRNA observed in the present study is consistent with the previous findings of their associated regions in pre-mRNA and also the kinetics of their association, thereby ensuring the validity of our experimental methodology for the analysis of the interaction between snRNAs and pre-mRNA during the *in vitro* splicing reaction. In addition to U1 and U2 snRNAs, we have shown that U6 snRNA associates with pre-mRNA and the intron lariat in an ATP-dependent manner and in the spliceosome. In the case of U6-preI, the cross-linking site was mapped in an unconserved region of pre-mRNA downstream of the 5'-splice site. The association of U6 snRNA in U6-preII is interesting because the cross-linking occurred in the neighborhood of the 5'-splice site or just on this site. The association of U6 snRNA with this region of pre-mRNA was observed clearly in the presence of EDTA but detected weakly under the standard splicing conditions. This probably suggests that this association is transient in the splicing reaction. From these results and also considering the previous report that in the yeast splicing system the spliceosome assembled in the presence of EDTA did not contain U4 snRNA (Cheng and Abelson 1987), it is tempting to speculate that U6 snRNA is localized downstream of the 5'-splice site after spliceosome formation and then comes closer to the splice site after destabilization of U4-U6 base-pairing. However, the correlation between the cross-linked products and the U4-U6 interaction has never been shown. In any case, we conclude that U6 snRNA is localized near the 5'-splice site of pre-mRNA in the spliceosome before the first-step reaction.

The cross-linking site of U6-lariat was mapped in the region around the branchpoint of the intron lariat. This region includes the cross-linking site of U6-preI and U6-preII. Therefore, it would be possible to think that association of U6 snRNA in U6-preI or U6-preII continues after the first-step reaction, although no cross-linked product of U6 snRNA and lariat intermediate was detectable. U6-lariat was observed in the spliceosome in which the splicing reaction had already taken place, suggesting that this association reflects the location of U6 snRNA in the spliceosome at the time of the second cleavage-ligation reaction. Because the branchpoint is present only 29 nucleotides upstream of the 3'-splice site (Sakamoto et al. 1987), it is likely that U6 snRNA is localized near the 3'-splice site during the second-step reaction. We could not identify any sequences in U6 snRNA that could potentially interact by base-pairing with the 5'-splice site region or the branchpoint region.

Presumably, the association between the two RNA species would be mediated by a protein in the spliceosome.

As described earlier, if U6 snRNA represents the catalytic element of splicing, we can anticipate that this snRNA should be localized near the sites of pre-mRNA where the splicing reactions occur. In this respect, the results obtained in this study support this notion. However, if this is the case, U6 snRNA should also be close to the branchpoint in addition to the 5'-splice site during the first-step reaction, because the first-step reaction is known to occur concomitantly at both the 5'-splice site and the branchpoint. On the basis of the fact that U2 snRNA interacts with the branchpoint region in the spliceosome and that base-pairing between U2 and U6 snRNAs is required for the splicing reaction to occur (Hausner et al. 1990; Datta and Weiner 1991; Wu and Manley 1991), it seems likely that U6 snRNA is localized near the branchpoint as well as the 5'-splice site via the interaction between U2 and U6 snRNAs.

Materials and methods

Oligonucleotides

Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer A380 and purified by electrophoresis on a 10% denaturing polyacrylamide gel. The oligonucleotides used in Figure 1 were complementary to positions 64–75 of U1 snRNA, positions 28–42 of U2 snRNA, positions 1–15 of U4 snRNA, positions 33–51 of U5 snRNA, and positions 78–95 of U6 snRNA (U6a in Fig. 6).

UV-cross-linking analyses

³²P-Labeled ΔEX14-15 pre-mRNA and a HeLa nuclear extract were prepared as described previously (Dignam et al. 1983; Sawa et al. 1988). Standard splicing reaction was performed as described previously (Sakamoto et al. 1987). The reaction mixture contained 60% of the nuclear extract and 2.5 fmole/μl of pre-mRNA. After incubation, the reaction mixture was diluted 20-fold with buffer E (12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 1.5 mM MgCl₂, 0.12 mM EDTA, 12% glycerol) to minimize non-specific cross-linking. The diluted reaction mixtures were then irradiated with UV light (wavelength 254 nm) in Stratilinker (Stratagene) at 250,000 μJ/cm² on a microtiter plate on ice at a distance of 10 cm from UV light. The irradiated samples were deproteinized with proteinase K (Merck) and pronase (Calbiochem) in proteinase buffer [100 mM Tris-HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, 1% SDS] followed by phenol extraction and ethanol precipitation, and analyzed by electrophoresis on a 4.5% polyacrylamide gel containing 8 M urea.

RNase H digestion experiments

RNA preparations were annealed with 10 μg/ml of oligonucleotides complementary to snRNAs or pre-mRNA in solution containing 60% buffer D (Dignam et al. 1983) and 1 mg/ml of yeast tRNAs by heating at 70°C for 3 min and standing at room temperature for 10 min. After annealing, *Escherichia coli* RNase H (Takara Shuzo Co.) and MgCl₂ were added to 50 U/ml and 1.5 mM, respectively, and each reaction mixture was incubated at 30°C for 10 min.

Affinity selection of cross-linked products

A U6-specific oligonucleotide was biotinylated in a reaction mixture containing 20 μ M oligonucleotide, 150 μ M biotin-21-dUTP (Clontech), 100 mM potassium cacodylate (pH 7.2), 0.2 mM DTT, 2 mM CoCl_2 , and 1 U/ μ l of terminal deoxynucleotidyl transferase (Takara Shuzo Co.). The oligonucleotide was then purified by gel-filtration chromatography (nick column, Pharmacia). In affinity-selection experiments, RNAs were recovered after the irradiation and annealed with 20 μ g/ml of the biotinylated oligonucleotide as described in RNase H digestion experiments. About one-fifth volume of streptavidin-agarose was then added and incubated for 30 min with gentle vortexing. After the agarose beads were washed extensively with buffer D, bound RNAs were eluted by incubation with buffer D at 70°C for 3 min.

Acknowledgments

We thank H. Sakamoto and K. Inoue for critical comments and very considerate help on the manuscript. This work was supported by a Grant-in-Aid for Special Project Research (62065009) from the Ministry of Education, Science, and Culture of Japan.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Abmayr, S.M., R. Reed, and T. Maniatis. 1988. Identification of a functional mammalian spliceosome containing unspliced pre-mRNA. *Proc. Natl. Acad. Sci.* **85**: 7216–7220.
- Bindereif, A. and M.R. Green. 1986. Ribonucleoprotein complex formation during pre-mRNA splicing *in vitro*. *Mol. Cell Biol.* **6**: 2582–2592.
- . 1987. An ordered pathway of snRNP binding during mammalian pre-mRNA splicing complex assembly. *EMBO J.* **6**: 2415–2424.
- Bindereif, A., T. Wolff, and M.R. Green. 1990. Discrete domains of human U6 snRNA required for the assembly of U4/U6 snRNP and splicing complexes. *EMBO J.* **9**: 251–255.
- Black, D.L. and J.A. Steitz. 1986. Pre-mRNA splicing *in vitro* requires intact U4/U6 small nuclear ribonucleoprotein. *Cell* **46**: 697–704.
- Black, D.L., B. Chabot, and J.A. Steitz. 1985. U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. *Cell* **42**: 737–750.
- Branch, A.D., B.J. Benenfeld, C.P. Paul, and H.D. Robertson. 1989. Analysis of ultraviolet-induced RNA-RNA cross-links: A means for probing RNA structure-function relationships. *Methods Enzymol.* **180**: 418–442.
- Brow, D.A. and C. Guthrie. 1989. Splicing a spliceosomal RNA. *Nature* **337**: 14–15.
- Cech, T.R. 1986. The generality of self-splicing RNA: Relationship to nuclear mRNA splicing. *Cell* **44**: 207–210.
- Chabot, B., D.L. Black, D.M. La Master, and J.A. Steitz. 1985. The 3'-splice site of pre-messenger RNA is recognized by a small nuclear ribonucleoprotein. *Science* **230**: 1344–1349.
- Cheng, S.-C. and J. Abelson. 1987. Spliceosome assembly in yeast. *Genes & Dev.* **1**: 1014–1027.
- Company, M., J. Arenas, and J. Abelson. 1991. Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. *Nature* **349**: 487–493.
- Datta, B. and A.M. Weiner. 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. *Nature* **352**: 821–824.
- Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**: 1475–1489.
- Fabrizio, P. and J. Abelson. 1990. Two domains of yeast U6 small nuclear RNA required for both steps of nuclear precursor messenger RNA splicing. *Science* **250**: 404–409.
- Fredericewey, D. and W. Keller. 1985. Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences. *Cell* **42**: 355–367.
- Grabowski, P.J. and P.A. Sharp. 1986. Affinity chromatography of splicing complexes: U2, U5, and U4 + U6 small nuclear ribonucleoprotein particles in the spliceosome. *Science* **233**: 1294–1299.
- Grabowski, P.J., S.R. Seiler, and P.A. Sharp. 1985. A multicomponent complex is involved in the splicing of messenger RNA precursors. *Cell* **42**: 345–353.
- Guthrie, C. and B. Patterson. 1988. Spliceosomal snRNAs. *Annu. Rev. Genet.* **22**: 387–419.
- Hausner, T.-P., L.M. Giglio, and A.M. Weiner. 1990. Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles. *Genes & Dev.* **4**: 2146–2156.
- Konarska, M.M. and P.A. Sharp. 1986. Electrophoretic separation of complexes in the splicing of precursors to mRNAs. *Cell* **46**: 845–855.
- . 1987. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. *Cell* **49**: 763–774.
- Krainer, A.R. and T. Maniatis. 1985. Multiple factors including the small nuclear ribonucleoproteins U1 and U2 are necessary for pre-mRNA splicing *in vitro*. *Cell* **42**: 725–736.
- . 1988. RNA splicing. In *Frontiers in molecular biology: Transcription and splicing* (ed. B.D. Hames and D.M. Glover), pp. 131–206. IRL Press, Oxford/Washington D.C.
- Krainer, A.R., T. Maniatis, B. Ruskin, and M.R. Green. 1984. Normal and mutant human β -globin pre-mRNAs are faithfully and efficiently spliced *in vitro*. *Cell* **36**: 993–1005.
- Krainer, A.R., G.C. Conway, and D. Kozak. 1990. Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. *Genes & Dev.* **4**: 1158–1171.
- Kramer, A., W. Keller, B. Appel, and R. Luhrmann. 1984. The 5' terminus of the RNA moiety of U1 small nuclear ribonucleoprotein particles is required for the splicing of messenger RNA precursors. *Cell* **38**: 299–307.
- Madhani, H.D., R. Bordonne, and C. Guthrie. 1990. Multiple roles for U6 snRNA in the splicing pathway. *Genes & Dev.* **4**: 2264–2277.
- Newman, A.J., R.-J. Lin, S.-C. Cheng, and J. Abelson. 1985. Molecular consequences of specific intron mutations on yeast mRNA splicing *in vivo* and *in vitro*. *Cell* **42**: 335–344.
- Ohno, M., H. Sakamoto, and Y. Shimura. 1987. Preferential excision of the 5' proximal intron from mRNA precursors with two introns as mediated by the cap structure. *Proc. Natl. Acad. Sci.* **84**: 5187–5191.
- Padgett, R.A., M.M. Konarska, P.I. Grabowski, S.F. Hardy, and P.A. Sharp. 1984. Lariat RNA's as intermediates and products in the splicing of messenger RNA precursors. *Science* **225**: 898–903.
- Peebles, C.L., P.S. Perlman, K.L. Mecklenburg, M.L. Petrillo, J.H. Tabor, K.A. Jarrell, and H.-L. Cheng. 1986. A self-splicing RNA excises an intron lariat. *Cell* **44**: 213–223.
- Reed, R. and T. Maniatis. 1988. The role of the mammalian in

Sawa and Shimura

- branchpoint sequence pre-mRNA splicing *Genes & Dev.* **2**: 1268–1276.
- Ruskin, B. and M.R. Green. 1985. An RNA processing activity that debranches RNA lariats. *Science* **229**: 135–140.
- Ruskin, B., A.R. Krainer, T. Maniatis, and M.R. Green. 1984. Excision of an intact introns as a novel lariat structure during pre-mRNA splicing *in vitro*. *Cell* **38**: 317–331.
- Sakamoto, H., M. Ohno, K. Yasuda, K. Mizumoto, and Y. Shimura. 1987. *In vitro* splicing of a chicken Δ -crystallin pre-mRNA in a mammalian extract. *J. Biochem.* **102**: 1289–1301.
- Sawa, H., M. Ohno, H. Sakamoto, and Y. Shimura. 1988. Requirement of ATP in the second step of the splicing reaction. *Nucleic Acids Res.* **16**: 3157–3164.
- Sharp, P.A. 1985. On the origin of RNA splicing and introns. *Cell* **42**: 397–400.
- Tani, T. and Y. Ohshima. 1989. The gene for the U6 small nuclear RNA in fission yeast has an intron. *Nature* **337**: 87–90.
- . 1991. mRNA-type introns in U6 small nuclear RNA genes: Implications for the catalysis in pre-mRNA splicing. *Genes & Dev.* **5**: 1022–1031.
- van der Veen, R., A.C. Arnberg, G. van der Horst, L. Bonen, H.F. Tabak, and L.A. Grivell. 1986. Excised Group II introns in yeast mitochondria are lariats and can be formed by self-splicing *in vitro*. *Cell* **44**: 225–234.
- Vankan, P., C. McGuigan, and I.W. Mattaj. 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. *EMBO J.* **9**: 3397–3404.
- Wu, J. and J.L. Manley. 1989. Mammalian pre-mRNA branch site selection by U1 snRNP involves base pairing. *Genes & Dev.* **3**: 1553–1561.
- . 1991. Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA. *Nature* **352**: 818–821.
- Zhuang, Y. and A.M. Weiner. 1986. A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell* **46**: 827–835.
- . 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes & Dev.* **3**: 1545–1552.

Association of U6 snRNA with the 5'-splice site region of pre-mRNA in the spliceosome.

H Sawa and Y Shimura

Genes Dev. 1992, **6**:

Access the most recent version at doi:[10.1101/gad.6.2.244](https://doi.org/10.1101/gad.6.2.244)

References

This article cites 45 articles, 16 of which can be accessed free at:
<http://genesdev.cshlp.org/content/6/2/244.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).