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ATP-dependent interaction of yeast U5 snRNA loop 1 with the 5′ splice site

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ABSTRACT

Pre-messenger RNA splicing is a two-step process by which introns are removed and exons joined together. In yeast, the U5 snRNA loop 1 interacts with the 5′ exon before the first step of splicing and with the 5′ and 3′ exons before the second step. In vitro studies revealed that yeast U5 loop 1 is not required for the first step of splicing but is essential for holding the 5′ and 3′ exons for ligation during the second step. It is critical, therefore, that loop 1 contacts the 5′ exon before the first step of splicing to hold this exon following cleavage from the pre-mRNA. At present it is not known how U5 loop 1 is positioned on the 5′ exon prior to the first step of splicing. To address this question, we have used site-specific photoactivated crosslinking in yeast spliceosomes to investigate the interaction of U5 loop 1 with the pre-mRNA prior to the first step of splicing. We have found that the highly conserved uridines in loop 1 make ATP-dependent contacts with an approximately 8-nt region at the 5′ splice site that includes the invariant GU. These interactions are dependent on functional U2 and U6 snRNAs. Our results support a model where U5 snRNA loop 1 interacts with the 5′ exon in two steps during its targeting to the 5′ splice site.

Keywords: 5′ splice site; pre-mRNA splicing; spliceosome; U5 snRNA; yeast

INTRODUCTION

The splicing of intron regions from pre-messenger RNA (pre-mRNA) takes place in a large multicomponent complex known as the spliceosome. It is within the spliceosome that the two catalytic steps key to intron removal occur. In the first step, an adenosine residue, found within the intron as part of a conserved branchpoint sequence, attacks the phosphodiester bond at the 5′ splice site, resulting in a 5′ exon and an intron-3′ exon intermediate. In the second step, the 5′ exon attacks the phosphodiester bond at the 3′ splice site, producing ligated exons and removed intron. Conserved sequences at the 5′ and 3′ splice sites, along with the branchpoint, are recognized in multiple steps by the snRNPs and non-snRNP protein components of the spliceosome bringing about the fidelity required for accurate intron removal (reviewed in Will & Lührmann, 1997; Staley & Guthrie, 1998; Burge et al., 1999; Reed, 2000).

The spliceosome assembly pathway begins with interaction of the U1 snRNP with the 5′ splice site region. Specifically, the 5′ end of the U1 snRNA base pairs at the 5′ splice site with protein components of the U1 snRNP stabilizing this interaction (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988; Puig et al., 1999; Zhang & Rosbash, 1999). The conserved sequences at the 3′ end of the intron, the branchpoint region and 3′ splice site, are recognized by a number of protein factors (reviewed in Reed, 2000). Following initial recognition of the 5′ and 3′ ends of the intron, the U2 snRNP binds to the branchpoint region with the U2 snRNA base pairing with the conserved branchpoint sequence (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989; Query et al., 1994). Once the U1 and U2 snRNPs interact with the pre-mRNA, a pre-assembled U4/U6.U5 particle interacts with the pre-mRNA and snRNPs already bound to the pre-mRNA. This results in a number of dynamic and specific RNA and protein rearrangements to form the active spliceosome (Nilsen, 1998; Staley & Guthrie, 1998). The RNA rearrangements include U1 base pairing at the 5′ splice site being replaced by base pairing of U6 with the conserved intronic sequences and interaction of U5 with exon sequences at the 5′ splice site. In addition, base pairing between U4 and U6 in the U4/U6.U5 particle is dissolved, allowing U6 to form specific base pairing interactions with U2, which re-

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mains base paired to the branchpoint. These rearrangements lead to activation of the spliceosome and rapid execution of the two catalytic steps of splicing.

One snRNP that plays a critical role in pre-mRNA splicing is the U5 snRNP. This is highlighted by U5 being the only snRNP that is a component of both the major (U2) and minor AT-AC (U12) spliceosomes (Tarn & Steitz, 1997). Furthermore, the U5 snRNP contains the most evolutionarily conserved splicing factor, Prp8 (Hodges et al., 1995). Prp8 is known to interact with the 5′ splice site, 3′ splice site, and branchpoint during splicing, as well as with the U5 and U6 snRNAs (Wyatt et al., 1992; MacMillan et al., 1994; Teigelkamp et al., 1995; Umen & Guthrie, 1995; Chiara et al., 1996, 1997; Reyes et al., 1996; Dix et al., 1998; Vidal et al., 1999). Alleles of Prp8 can also suppress 5′ and 3′ splice site mutations, leading to a model where Prp8 stabilizes RNA tertiary structures within the spliceosomal active site and facilitates formation of the catalytic core (reviewed in Collins & Guthrie, 2000).

The RNA component of the U5 snRNP, the U5 snRNA, contains a conserved stem loop sequence that includes an invariant 9-nt loop 1 that interacts with the 5′ and 3′ exons during splicing (Newman, 1997). This loop sequence makes contact with the 5′ exon in the pre-mRNA adjacent to the 5′ splice site before the first step of splicing (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Cortes et al., 1993; Sontheimer & Steitz, 1993; Newman et al., 1995). Following the first step, loop 1 continues to interact with the 5′ exon and also forms contacts with the 3′ exon next to the 3′ splice site. These interactions were proposed to tether the exons in the correct orientation for the second step of splicing (Newman & Norman, 1992; Sontheimer & Steitz, 1993). This was proven utilizing a yeast in vitro system where U5 loop 1 mutations affect the alignment of exons blocking the second step of splicing (O'Keefe et al., 1996; O'Keefe & Newman, 1998). In a mammalian in vitro system, mutation of the U5 loop 1 still allows both steps of splicing, and it has been proposed that the protein components of the U5 snRNP hold the exons in the correct orientation for the second step of splicing (Ségault et al., 1999).

In yeast, it is essential that U5 loop 1 interacts with the 5′ exon before the first step of splicing. This interaction, though not required for the first step, must be in place before the first step to tether the 5′ exon intermediate in the correct orientation for the second step of splicing (O'Keefe et al., 1996; O'Keefe & Newman, 1998). It is still unclear by what process loop 1 is positioned on the 5′ exon before the first step of splicing. To determine the mechanism of loop 1 positioning on exon sequences at the 5′ splice site, it is important to know how loop 1 first interacts with the pre-mRNA. Specifically, is loop 1 directly positioned at the 5′ splice site or does loop 1 interact generally with the 5′ exon with subsequent precise arrangement with the 3′ end of the 5′ exon? To answer this question, we replaced four conserved uridines in loop 1 with the site-specific crosslinking group 4-thio-uridine (4-thioU). By depletion of endogenous U5 in yeast splicing extract and reconstitution of functional U5 snRNPs with 4-thio-U containing U5 snRNA, we assessed the interaction of loop 1 with the pre-mRNA prior to the first step of splicing. We found that each of the four conserved uridines in loop 1 interact with the pre-mRNA prior to the first step of splicing. These loop 1 residues primarily interact with an 8-nt region not only within the 5′ exon but extending into the intron to the conserved GU residue. These interactions are ATP dependent and are reduced considerably by targeted degradation of U2 or U6 snRNAs. This suggests that the conserved uridines in U5 loop 1 are specifically targeted to the 5′ splice site and may be positioned by the U2 and U6 snRNAs.

RESULTS

Incorporation of modified bases into U5 snRNA loop 1 for site-specific crosslinking

To determine the contacts U5 loop 1 makes with pre-mRNA prior to the first step of splicing, U5 snRNAs containing 4-thioU were produced. An 11-nt RNA, comprising part of U5 loop 1 (nt 93–103; see Fig. 1A), was chemically synthesized with 4-thioU incorporated at specific locations. The four uridines in loop 1 (U96 to U99) were either all or individually replaced with 4-thioU. These synthetic RNAs were ligated to in vitro-transcribed 5′ and 3′ RNAs comprising the remaining portions of U5 (Moore & Sharp, 1992). The resulting full-length U5 was purified from ligation intermediates by electrophoresis (Fig. 1B) and employed for in vitro crosslinking. The uridine residues in loop 1 (U96 to U99 in yeast and U40 to U43 in mammals) are the sites of crosslinking to pre-mRNA containing 4-thioU at positions (−1) and (−2) in the 5′ exon (Wyatt et al., 1992; Newman et al., 1995). The first two uridines in loop 1 (U96, U97 in yeast and U40, U41 in mammals) are also the sites of crosslinking to the 5′ exon intermediate containing 4-thioU at the (−1) position of the 5′ exon (Sontheimer & Steitz, 1993; Newman et al., 1995). In yeast, pre-mRNA and the 5′ exon intermediate with 4-thioU at positions (−2) and (−3) can be crosslinked to U5 loop 1 (R. O'Keefe, unpubl. data).

U5 snRNAs containing 4-thioU in loop 1 are functional and interact with the pre-mRNA and exon 1 intermediate

Functional yeast U5 snRNPs that carry out both steps of splicing can be reconstituted by addition of in vitro-transcribed U5 to extract depleted of endogenous U5 (O'Keefe et al., 1996; O'Keefe & Newman, 1998). Reconstitution with U5 snRNAs containing 4-thioU in loop

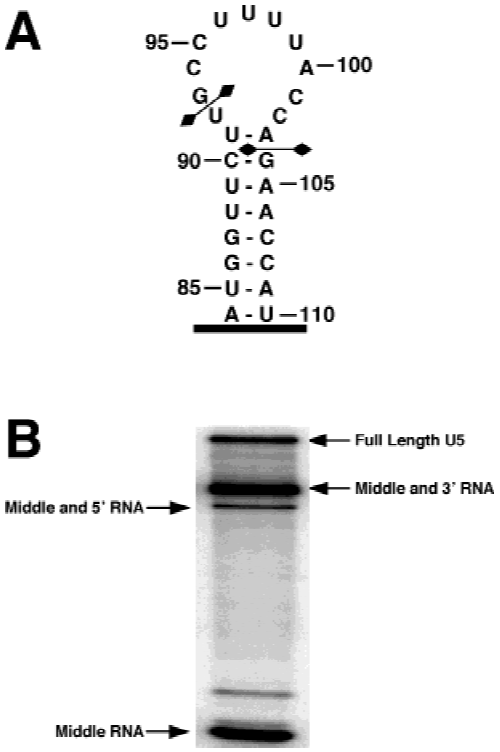

FIGURE 1. Ligation strategy for introduction of 4-thioU into loop 1 of the *S. cerevisiae* U5 snRNA. **A:** Diagram depicting nt 84–110 of the U5 snRNA stem-loop 1. Junctions utilized for RNA ligation are indicated with bars. **B:** Visualization of three-way RNA ligation reaction for introduction of 4-thioU into U5 loop 1. End labeled, chemically synthesized, middle RNA encompassing nt 93–103 of U5 loop 1 was ligated to 5′ (nt 1–92) and 3′ (nt 104–214) portions of U5 to produce full-length U5 snRNA. The middle RNA contained 4-thioU at positions U96, U97, U98, U99, or U96–U99 of U5 loop 1.

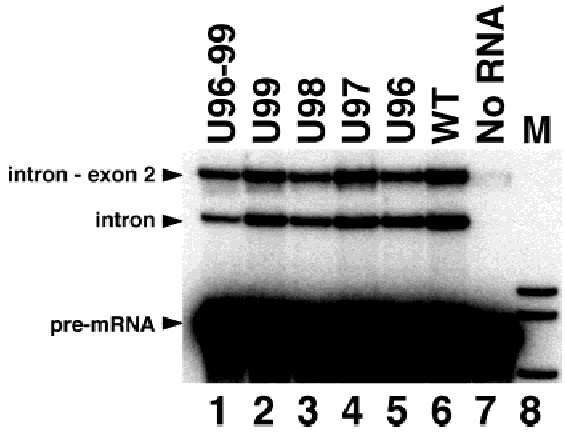

FIGURE 2. Splicing activity of U5 snRNAs containing 4-thioU in loop 1. Extract depleted of U5 was reconstituted with U5 snRNAs containing 4-thioU incorporated at indicated positions in loop 1. In vitro-transcribed *CYH2* pre-mRNA was added to assay for splicing activity. Splicing was reconstituted with each U5 containing 4-thioU (lanes 1–5) or in vitro-transcribed wild-type U5 (lane 6). Reconstitution with no RNA (lane 7). pBR322 *Msp*I end-labeled size markers (lane 8). The positions of the in vitro-transcribed *CYH2* pre-mRNA, the intron-exon 2 splicing intermediate, and the lariat intron splicing product are indicated on the left of the panel. The exon 1 intermediate and mRNA products are not shown.

1 and incubation with uniformly labeled *CYH2* or actin pre-mRNA allows splicing to occur. Splicing activity obtained with *CYH2* pre-mRNA for each of the 4-thioU-containing U5 snRNAs (U96, U97, U98, U99, and U96–99; Fig. 2, lanes 1–5) and actin pre-mRNA (data not shown) was equivalent to in vitro-transcribed wild-type U5 (Fig. 2, lane 6; data not shown). In the absence of added RNA, splicing activity is not reconstituted in U5-depleted extract (Fig. 2, lane 7).

To determine how U5 interacts with pre-mRNA before the first step of splicing, extract reconstituted with 4-thioU-containing U5 was UV irradiated in the presence of *CYH2* pre-mRNA containing a single ^{32}P label in exon 1 between the (−1) and (−2) positions. This pre-mRNA was utilized for the reason that with a single label in the 5′ exon only the pre-mRNA, exon 1 intermediate and mRNA product can be visualized. The intron–exon 2 intermediate and the intron product, which usually run above the pre-mRNA in our gel system, are not visible. This allowed us to be certain that bands observed above the pre-mRNA, following crosslinking with 4-thioU-containing U5, were likely crosslinks between U5 and pre-mRNA.

Yeast extract depleted of U5 was reconstituted with each U5 containing 4-thioU in loop 1. Splicing was initiated by addition of single labeled *CYH2* pre-mRNA. Each 4-thioU-containing U5 (U96, U97, U98, U99, and U96–99; Fig. 3A, lanes 3–7) produced a specific crosslink that migrated slower than the pre-mRNA and arose with different efficiencies. Predictably, the U5 with all uridines in loop 1 replaced by 4-thioU (U96–99, Fig. 3A, lane 7) gave the most intense crosslink. The other single substitutions in loop 1 gave varying crosslinking efficiencies, with U97 and U99 (Fig. 3A, lanes 4 and 6) generating more efficient crosslinks than U96 and U98 (Fig. 3A, lanes 3 and 5). All crosslinks were dependent on 4-thioU, as a wild-type in vitro-transcribed U5 did not produce crosslinks following UV irradiation (Fig. 3A, lane 2). Furthermore, all crosslinks were dependent on UV irradiation (data not shown). Finally, formation of each crosslink was dependent on the presence of ATP in the splicing reaction (Fig. 3A, lane 8 and data not shown) indicating that spliceosome assembly was required for these interactions to occur.

The identity of each individual crosslink was determined next by ribonuclease H (RNase H) analysis employing oligonucleotides targeted to U1 and U5, as well as to the pre-mRNA. In Figure 3B a representative RNase H analysis is shown for the crosslink produced with 4-thioU at U99. This crosslink was degraded by oligonucleotides against U5 (Fig. 3B, lane 3) and the pre-mRNA (Fig. 3B, lanes 5–7), but not U1 (Fig. 3B, lane 4). This crosslink, therefore, is U5 with 4-thioU at position U99 crosslinked to the pre-mRNA (U5/pre-mRNA; crosslinks are denoted by a slash [/]). Similar

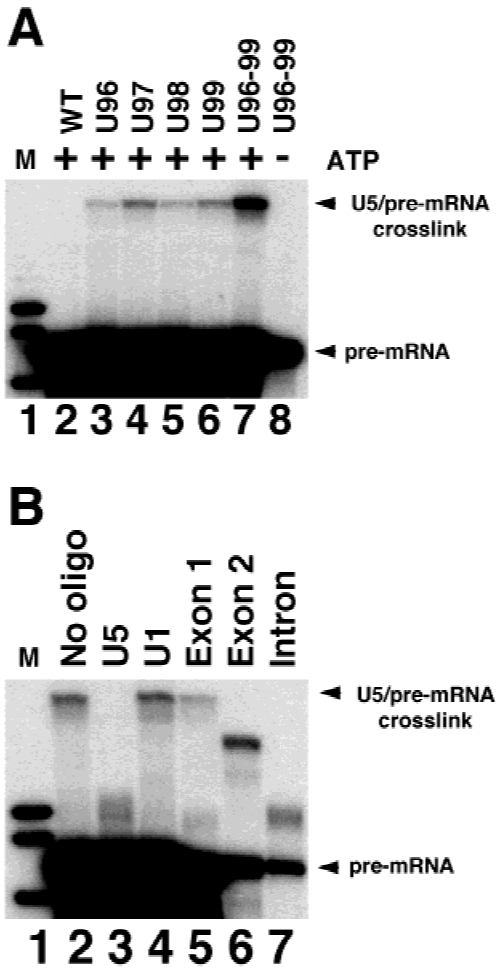

FIGURE 3. Photoactivated crosslinking of U5 snRNA containing 4-thioU in loop 1 and identification of U5/pre-mRNA crosslinks. **A:** Extract depleted of endogenous U5 snRNA was reconstituted with U5 containing 4-thioU in loop 1. Splicing was initiated by addition of *CYH2* pre-mRNA containing a single radioactive phosphate between positions (−1) and (−2) in exon 1 and reactions subjected to UV irradiation. Reconstitution with in vitro-transcribed wild-type U5 snRNA (lane 2) and each U5 containing 4-thioU (lanes 3–8). Splicing was carried out either in the presence (lanes 2–7) or absence (lane 8) of ATP. U5/pre-mRNA crosslinks can be seen as bands migrating slower than the pre-mRNA and are indicated on the right of the panel. The position of *CYH2* pre-mRNA is also indicated on the right of the panel. pBR322 *Msp*I end-labeled size markers (lane 1). **B:** RNase H analysis of crosslinks produced in a reaction containing 4-thioU at position U99. Oligonucleotides were targeted to the following RNAs: no oligo control (lane 2), U5 (lane 3), U1 (lane 4), exon 1 (lane 5), exon 2 (lane 6), and intron (lane 7). pBR322 *Msp*I end-labeled size markers (lane 1). The positions of the *CYH2* pre-mRNA and U5/pre-mRNA crosslink are indicated on the right of the panel. In both **A** and **B** the intron-exon 2 splicing intermediate and the lariat intron splicing product that usually run above the pre-mRNA are not visible as the only label is in exon 1. The exon 1 intermediate and mRNA products are not shown.

results were found when crosslinks produced with 4-thioU in other loop 1 positions (U96, U97, U98, and U96–U99) were examined by RNase H analysis (data not shown). It is interesting to note that the exon 1 oligonucleotide does not fully target the U5/pre-mRNA crosslink (Fig. 3B, lane 5). This is most likely due to overlap between the oligonucleotide binding site and the U5 crosslinking site within exon 1 that protects the exon from total degradation. The exon 1-specific oligonucleotide binds from the (−3) position to the (−17) position of exon 1 relative to the 5′ splice site. These results establish that U5 loop 1 nt U96 to U99 make specific contact with the pre-mRNA before the first step of splicing.

A time course of crosslinking with 4-thioU at positions 96 to 99 in loop 1 revealed that the U5/pre-mRNA crosslink first appeared at 3–4 min following addition of pre-mRNA (Fig. 4A, lanes 5 and 6). The U5/pre-mRNA crosslink increased in intensity up to 20 min (Fig. 4A, lane 9), then decreased by 40 min (Fig. 4A, lane 10). A splicing time course of extract reconstituted with the same 4-thioU-containing U5, carried out in parallel with uniformly labeled *CYH2* pre-mRNA, revealed that the crosslink appears before accumulation of intermediates and products of splicing at 10 min (Fig. 4B, lane 8). All five U5 snRNAs containing 4-thioU in loop 1 crosslinked to the pre-mRNA with similar kinetics (data not shown).

To visualize U5/pre-mRNA crosslinks more clearly, extract depleted of U5 was reconstituted with each 4-thioU-containing U5. Splicing was initiated by addition of single labeled *CYH2* pre-mRNA. RNA isolated following UV irradiation of splicing reactions was hybridized with a biotinylated oligonucleotide complementary to U5 to specifically capture U5 containing molecules. Because ligated U5 snRNAs employed for reconstitution did not contain a radioactive label, the only way captured U5 could be visualized was if it physically crosslinked to the labeled pre-mRNA. Following capture, the U5-containing RNAs were subjected to electrophoresis to visualize U5/pre-mRNA crosslinks. This revealed that the U5/pre-mRNA crosslinks we identified previously were specifically captured (Fig. 5A, lanes 1–4 and 6). This technique also revealed a new crosslink that migrated faster than the pre-mRNA (Fig. 5A, lanes 1–4 and 6). This crosslink, along with the U5/pre-mRNA crosslink, was selected in a reaction with 4-thioU-containing U5 snRNAs but not with in vitro-transcribed wild-type U5 without 4-thioU (Fig. 5A, lane 5). The faster migrating crosslink runs in a similar location to U5 crosslinked to the exon 1 intermediate produced following the first step of splicing (O'Keefe & Newman, 1998). The identity of each captured crosslink for the U5 with 4-thioU at position U98 (Fig. 6) and the other positions (U96, U97, U99, and U96–99; data not shown) was confirmed by RNase H analysis following electroelution from the gel. Analysis of the crosslink presumed to be U5/pre-mRNA showed that this crosslink was targeted by an oligonucleotide specific for U5 (Fig. 6, lane 2) and for exon 1, exon 2, and intron (Fig. 6, lanes 3–5). This confirmed that this crosslink was U5/pre-mRNA. RNase H analysis of the crosslink thought

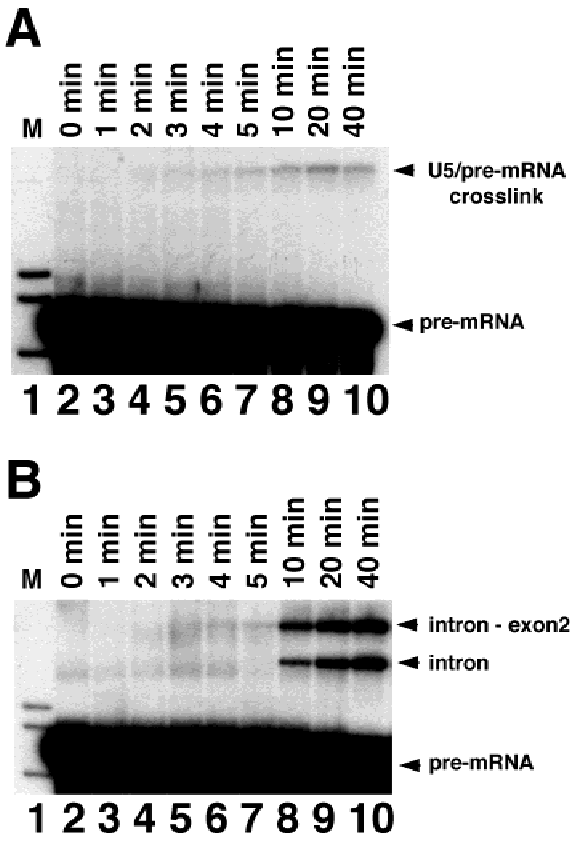

FIGURE 4. Time course of U5/pre-mRNA crosslinks. **A:** Extract depleted of U5 was reconstituted with U5 containing 4-thioU at positions U96–U99. Splicing was initiated by addition of *CYH2* pre-mRNA containing a single radioactive phosphate between positions (−1) and (−2) in exon 1. Samples were withdrawn from an in vitro splicing reaction after 0, 1, 2, 3, 4, 5, 10, 20, and 40 min of incubation at 23 °C and subjected to UV irradiation (lanes 2–10). U5/pre-mRNA crosslinks can be seen as bands migrating slower than the pre-mRNA and are indicated on the right of the panel. The position of *CYH2* pre-mRNA is indicated on the right of the panel. The intron-exon 2 splicing intermediate and the lariat intron splicing product that usually run above the pre-mRNA are not visible as the only label is in exon 1. The exon 1 intermediate and mRNA products are not shown. pBR322 *Msp*I end-labeled size markers (lane 1). **B:** Extract depleted of U5 snRNA reconstituted with U5 containing 4-thioU at positions U96–U99. Splicing was initiated by addition of *CYH2* pre-mRNA containing radioactive phosphate incorporated throughout the pre-mRNA. Samples were withdrawn from in vitro splicing reaction after 0, 1, 2, 3, 4, 5, 10, 20, and 40 min of incubation at 23 °C (lanes 2–10). The positions of the in vitro-transcribed *CYH2* pre-mRNA, the intron-exon 2 splicing intermediate, and the lariat intron splicing product are indicated on the right of the panel. The exon 1 intermediate and mRNA products are not shown. pBR322 *Msp*I end-labeled size markers (lane 1).

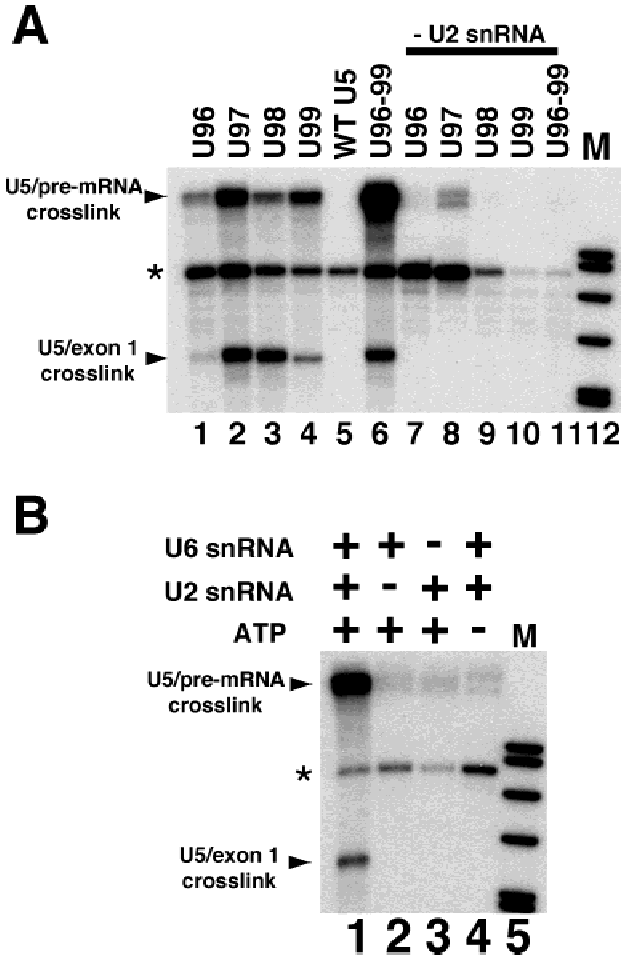

FIGURE 5. U5 loop 1 crosslinking to pre-mRNA and exon 1 is dependent on U2 snRNA, U6 snRNA, and ATP. Reconstitution with U5 containing 4-thioU in loop 1 and *CYH2* pre-mRNA containing a single radioactive phosphate between positions (−1) and (−2) in exon 1. RNA containing U5 was selected from UV-irradiated reactions with a U5 specific biotinylated oligonucleotide and streptavidin paramagnetic particles. **A:** Reconstitution with each U5 containing 4-thioU in loop 1 (lanes 1–4 and 6–11) and in vitro-transcribed wild-type U5 (lane 5). Splicing was carried out either in the presence (lanes 1–6) or absence (lanes 7–11) of U2 snRNA. pBR322 *Msp*I end-labeled size markers (lane 12). **B:** Reconstitution with U5 containing 4-thioU at positions U96 to U99 (lanes 1–4). Splicing was carried out either in the presence of U6 snRNA (lanes 1, 2, 4), U2 snRNA (lanes 1, 3, 4), and ATP (lanes 1–3) or absence of U6 snRNA (lane 3), U2 snRNA (lane 2), and ATP (lane 4). pBR322 *Msp*I end-labeled size markers (lane 5). The U5/pre-mRNA and possible U5/exon 1 crosslinks are indicated at the left of each panel. Asterisks indicate background pre-mRNA captured with streptavidin paramagnetic particles.

to be U5 crosslinked to the exon 1 intermediate showed that this crosslink was targeted by oligonucleotides specific for U5 (Fig. 6, lane 8) and exon 1 (Fig. 6, lane 9) but not the exon 2 or intron (Fig. 6, lanes 10 and 11). This proved that this species was indeed a crosslink between U5 and exon 1 intermediate (U5/exon 1). More importantly, this indicated that each of the uridines in U5 loop 1 could also interact with the exon 1 intermediate following the first step of splicing. It is interesting to note, however, that positions U97 and U98 of loop 1 crosslinked more efficiently to the exon 1 intermediate than the U96 and U99 positions (Fig. 5A, lanes 1–4). This suggests that the interactions of loop 1 with exon 1 change following the first step of splicing. Comparing the RNase H analysis of the U5/pre-mRNA crosslink with the U5/exon 1 crosslink, it was apparent that the U5/exon 1 crosslink can be degraded more efficiently with the

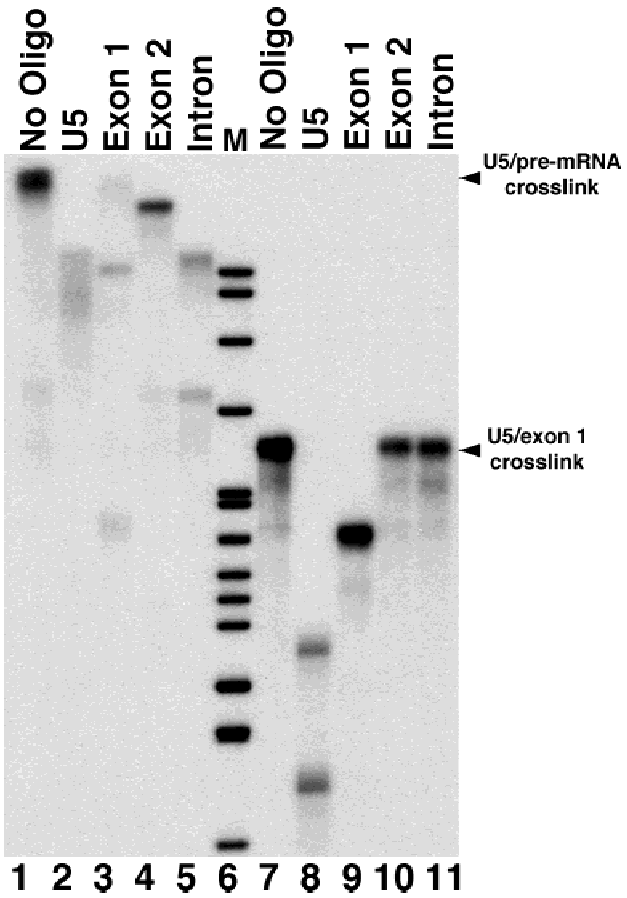

FIGURE 6. U5 loop 1 containing 4-thioU can crosslink to both pre-mRNA and exon 1 intermediate. Reconstitution with U5 containing 4-thioU at position U98 and uniformly labeled actin pre-mRNA. RNA containing U5 was selected from UV-irradiated reactions with biotinylated oligonucleotides and streptavidin paramagnetic particles, then separated by gel electrophoresis. Crosslinked RNAs eluted from the gel were subjected to RNase H analysis with the following targeting oligonucleotides: no oligo control (lanes 1 and 7), U5 (lanes 2 and 8), exon 1 (lanes 3 and 9), exon 2 (lanes 4 and 10), and intron (lanes 5 and 11). pBR322 *Msp*I end-labeled size markers (lane 6). The U5/pre-mRNA and U5/exon 1 crosslinks are indicated at the right of the panel.

exon 1-specific oligonucleotide than the U5/pre-mRNA crosslink (compare Fig. 3B, lane 5, and Fig. 6, lane 3, with Fig. 6, lane 9). This implies that contacts U5 loop 1 forms with the exon 1 intermediate are restricted primarily to the (−1) and (−2) positions of exon 1.

U5 loop 1 interactions with pre-mRNA and exon 1 are dependent on functional U2 and U6 snRNAs

To investigate how U5 loop 1 interacts with the pre-mRNA and the exon 1 intermediate, crosslinking was carried out in extract depleted of specific snRNAs. The U2 and U6 snRNAs can be depleted from extracts by targeted RNase H digestion with oligonucleotides complementary to each snRNA (Fabrizio et al., 1989; McPheeters et al., 1989). To test the effects of U2 depletion on U5/pre-mRNA and U5/exon 1 crosslinks, extract was depleted simultaneously of U5 and U2 snRNAs, then reconstituted with each 4-thioU-containing U5. Depletion of U2 dramatically decreased the amount of U5/pre-mRNA and U5/exon 1 crosslinks with each 4-thioU containing U5 (Fig. 5A, lanes 7–11 and Fig. 5B, lane 2). Extract depleted simultaneously of U5 and U6 snRNAs then reconstituted with U5 with 4-thioU at positions U96–99 also showed a dramatic decrease in U5/pre-mRNA and U5/exon 1 crosslinks (Fig. 5B, lane 3). These results indicate that U5 loop 1 cannot interact with the pre-mRNA or exon 1 intermediate without functional U2 or U6 snRNAs.

U5 loop 1 interacts specifically with sequences at the 5′ splice site including the invariant GU

Having established that uridines U96 to U99 in U5 loop 1 contact the pre-mRNA before the first step of splicing and these interactions were dependent on functional U2 and U6, it was now important to map these contacts. Ideally it would be most appropriate to map the U5 loop 1 crosslinks to a *CYH2* pre-mRNA. Unfortunately, we had difficulty obtaining good primer extension of the *CYH2* pre-mRNA with a number of different primers and reverse transcriptases. Therefore, we changed to actin pre-mRNA for crosslink mapping. The actin pre-mRNA crosslinked to each of the 4-thioU-containing U5 snRNAs in a similar manner as the *CYH2*.

Extract depleted of U5 was reconstituted with each 4-thioU-containing U5 and incubated with uniformly labeled actin pre-mRNA. RNA isolated following UV irradiation of splicing reactions was captured using a biotinylated oligonucleotide complementary to U5. Captured RNA was subjected to electrophoresis, exposed to X-ray film, and crosslinks electroeluted from the gel. RNA isolated by this method was used for primer extension analysis with an end-labeled primer specific for the intron region of actin pre-mRNA near the 5′ splice site. The cDNAs resulting from blocked reverse transcription one base from a crosslinked nucleotide were then displayed beside a sequence ladder of actin pre-mRNA utilizing the same primer.

Mapping of crosslinks between U96 to U99 of U5 loop 1 and the pre-mRNA revealed that loop 1 interacted specifically with a region of predominantly 7–8 nt around the 5′ splice site (Fig. 7). Each uridine in loop 1 that was tested resulted in a slightly different pattern of crosslinking. The U96 position crosslinked predominantly with two guanosine residues on either side of the 5′ splice site and extended to a lesser extent into exon 1 (Fig. 7A, U96). This position gave the weakest crosslink of all the variants and was difficult to map.

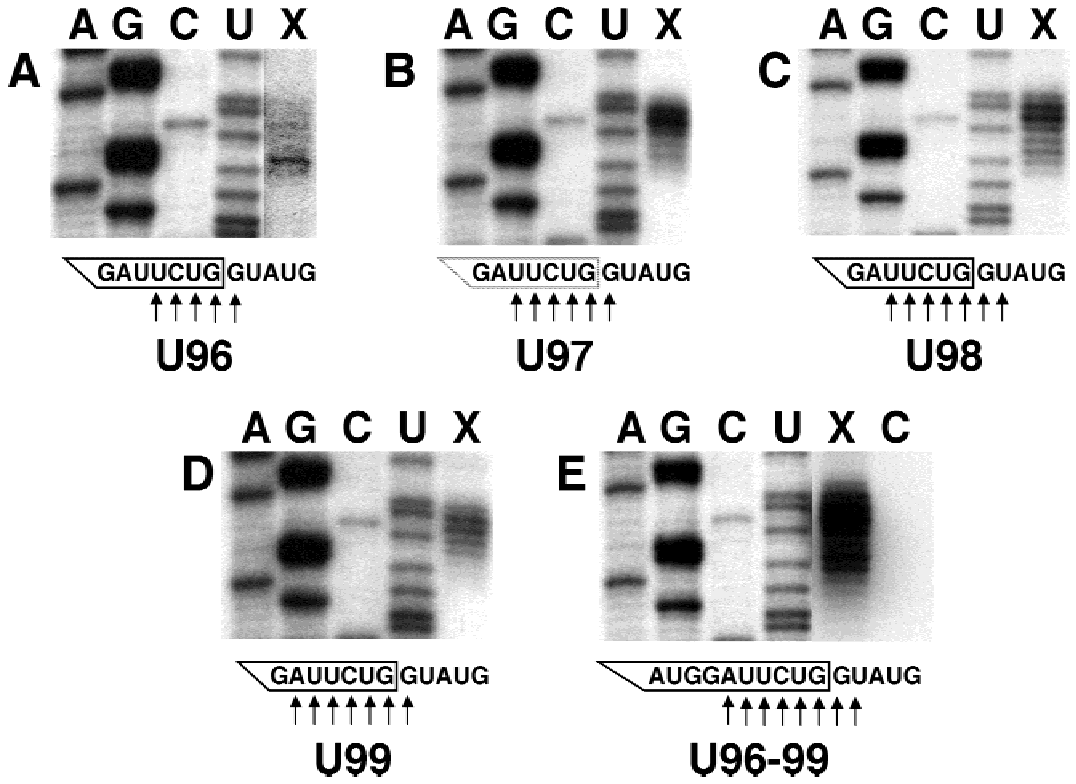

FIGURE 7. Primer extension mapping of crosslinks between U5 loop 1 and pre-mRNA. U5-containing crosslinked species from UV-irradiated reconstitution reactions with uniformly labeled actin pre-mRNA were isolated with a U5-specific biotinylated oligonucleotide and streptavidin paramagnetic particles. Gel-purified crosslinks or corresponding controls were used as templates for primer extension with an end-labeled oligonucleotide specific to the actin intron. **A:** Reconstitution with U5 containing 4-thioU at position U96. The crosslinks (X) between this U5 and actin pre-mRNA are represented below the panel. In this panel the crosslinks were specifically overexposed to enable better visualization of the U5 U96/pre-mRNA crosslinks. **B:** Reconstitution with U5 containing 4-thioU at position U97. The crosslinks (X) between this U5 and actin pre-mRNA are represented below the panel. **C:** Reconstitution with U5 containing 4-thioU at position U98. The crosslinks (X) between this U5 and actin pre-mRNA are represented below the panel. **D:** Reconstitution with U5 containing 4-thioU at position U99. The crosslinks (X) between this U5 and actin pre-mRNA are represented below the panel. **E:** Reconstitution with U5 containing 4-thioU at positions U96–U99. The crosslinks (X) between this U5 and actin pre-mRNA are represented below the panel. Control lane (C) from UV-irradiated reconstitution reaction containing in vitro-transcribed wild-type U5 indicates that the mapped crosslinks are 4-thioU specific. In all panels, dideoxynucleotide sequencing tracks were produced in parallel with actin pre-mRNA as reference for primer extension mapping.

The next position, U97, crosslinked predominantly with two uridines upstream of the 5′ splice site, positions (−4) and (−5) relative to the 5′ splice site, and extended to a lesser extent to the first guanosine of the intron (Fig. 7B, U97). Analysis of the U98 crosslink (Fig. 7C, U98) revealed that this position mainly interacted, like U97, with two uridines upstream of the 5′ splice site. However, the U98 crosslinks also extended to the conserved GU within the intron (Fig. 7C, U98). Visualization of the U99 crosslink revealed a pattern of crosslinking almost identical to the U98 position. The U99 position in loop 1 contacted two uridines upstream of the 5′ splice site and extended to the GU (Fig. 7D, U99). Mapping of the U96–U99 crosslink to the pre-mRNA exhibited extensive interactions within the region of the 5′ splice site (Fig. 7E, U96–99). These interactions were concentrated within 7 nt around the 5′ splice site composed of contacts with five exon nucleotides adjacent to the 5′ splice site and the GU (Fig. 7E, U96–99). Taken together, this mapping of U5/pre-mRNA crosslinks indicated that, before the first step of splicing, the conserved uridines in loop 1 make specific contacts with a small region around the 5′ splice site. Interestingly, not only were known contacts of loop 1 with the exon at the 5′ splice site identified but novel interactions between loop 1 and the invariant GU at the 5′ splice site were revealed.

DISCUSSION

In yeast, interaction of U5 loop 1 with the 5′ and 3′ exons is critical for the second step of pre-mRNA splicing. Even though contacts between loop 1 and the 5′ exon are not required for the first step, they must occur before the first step to align the 5′ exon intermediate for the second step of splicing (O'Keefe et al., 1996; O'Keefe & Newman, 1998). Site-specific crosslinking was applied to characterize how U5 loop

1 establishes contacts with the 5′ exon within the pre-mRNA. We replaced four conserved uridine residues in loop 1 with 4-thioU and used these modified U5 snRNAs to reconstitute functional U5 snRNPs in vitro. By UV irradiating reconstituted reactions we induced 4-thioU-specific crosslinks between U5 loop 1 and the pre-mRNA. We found that loop 1 makes specific contacts with approximately 8 nt around the 5′ splice site including the invariant GU residues. These interactions were ATP dependent and required functional U2 and U6 snRNAs. These results back a model where the U5 snRNA interacts in two steps with the 5′ exon prior to the first catalytic step of pre-mRNA splicing.

The dependence of the U5 loop 1 on functional U2 snRNA for crosslinking to the 5′ splice site indicates that there may be communication between these snRNAs or their protein components at the 5′ splice site. In yeast, U5 loop 1 nt U97, U98, and U99 we replaced with 4-thioU have been shown to interact genetically with a region of U2 that forms helix I with U6 (Xu et al., 1998). Part of helix I, nt U23 of yeast U2 in helix Ib, has been shown to have a role in 5′ splice site selection (Luukkonen & Séraphin, 1998). Both the U5 loop 1 and the U23 residue of U2 have been crosslinked to the same site in the 3′ exon following the first step of splicing (Newman et al., 1995). It has also been suggested that the U5 snRNP is positioned with respect to the 3′ splice site by direct or indirect interactions with U2 snRNP bound at the branchpoint (Chiara et al., 1997). This evidence, and ours, points to interactions between the U5 and U2 snRNPs being important for the first and/or second step of splicing.

Recently, however, interaction of the human homolog of the yeast Prp8 protein with the 5′ splice site was found to be independent of U2 snRNA binding to the branchpoint (Maroney et al., 2000). Prp8 is a component of the U5 snRNP and is known to be intimately associated with U5 loop 1 (Hinz et al., 1996; Dix et al., 1998; Urlaub et al., 2000). Prp8 also appears to play a critical role in a number of aspects of pre-mRNA splicing (Collins & Guthrie, 2000). In yeast, Prp8 and U5 are known to crosslink to the (−1) and the (−8) positions of the 5′ exon (Newman et al., 1995; Teigelkamp et al., 1995). The interaction of U5 with the (−8) position occurs through 1 nt at the bottom of loop 1 (U92) and an adjacent nucleotide within stem 1 (U91). These interactions do not continue following the first step of splicing. As proposed by Maroney et al. (2000), the interaction of U5 snRNA and Prp8 at the (−8) position may occur early in the splicing reaction independently of U2 snRNP binding.

Our results, which at first appear to contradict this proposal, are actually complementary if U5 is thought to interact with the 5′ exon in two steps. In the first step, U5 and Prp8, as part of the U4/U6.U5 tri-snRNP, interact with the 5′ exon independently of U2 binding. In this step, U5 nt U91 and U92 would contact the 5′ exon upstream of the 5′ splice site. In the second step, the loop 1 nt U96 to U99 would be positioned on the 5′ splice site in a U2-dependent manner. This positioning of loop 1 could come about through an exchange of the U91 and U92 interaction upstream for the U96 to U99 at the 5′ splice site. Or the U96 to U99 region could initially be masked from binding the 5′ splice site, with subsequent structural rearrangements unmasking these sequences for interaction with the 5′ splice site.

Interestingly, our results indicate that nt U96 to U99 only efficiently interact with (−1) to (−5) in the 5′ exon consistent with this model. In addition, a model where U5 snRNA loop 1 interacts with the 5′ exon in two steps, with two different regions of loop 1, may explain some puzzling previous results. Insertions in U5 loop 1, at a number of different sites, inhibit the second step of splicing (O'Keefe & Newman, 1998). However, insertions in one region of loop 1 (between U92 and G93) are tolerated by the splicing machinery and can carry out both steps of splicing. Therefore, it appears that there may be two functionally distinct regions of loop 1, one region that may interact initially with the 5′ exon that is tolerant of insertions and another region that interacts specifically with the 5′ splice site that is sensitive to insertions.

The engagement of U5 loop 1 with the 5′ exon would most likely occur when the conserved 5′ splice site GU is identified. Identification of the 5′ splice site is thought to take place in steps with sequential inspection of this splicing signal. To date, a number of factors have been implicated in 5′ splice site selection. Initial recognition of the 5′ splice site is through binding of the U1 snRNP and associated proteins to the 5′ splice site (Reed, 2000). Interactions of the U1 snRNA with the U5 snRNA in mammalian cells (Ast & Weiner, 1997) and the yeast U1 snRNP protein Prp40 with Prp8 (Abovich & Rosbash, 1997) suggest an early interaction between these snRNPs. These interactions may take place before U5 loop 1 nt U96 to U99 are positioned at the 5′ splice site.

Prior to activation of the spliceosome it appears that the U6 snRNA and Prp8 may play important roles in identifying the GU at the 5′ splice site. Mutations in U6 can compensate for mutations in the G residue suggesting a role of U6 in recognition of this nucleotide during splicing (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993). In addition, a direct interaction between U6 and the invariant GU has been shown by crosslinking (Kim & Abelson, 1996). Prp8 can also crosslink to the invariant GU (Reyes et al., 1996) and certain alleles of Prp8 can suppress mutations of the U residue at the 5′ splice site (Collins & Guthrie, 1999; Siatecka et al., 1999). Our results show, for the first time, that the U5 loop 1 nt U96 to U99 are also in intimate contact with the GU at the 5′ splice site. The U6 snRNA is thought to have a role in catalysis at the 5′ splice site,

which was recently given further support when yeast U6 was shown to coordinate a metal ion required for catalytic activity of the spliceosome (Yean et al., 2000). The U5 loop 1 is, therefore, well placed for receiving the 5′ exon intermediate, resulting from the first catalytic step, for alignment with the 3′ exon prior to the second catalytic step.

The interactions of U5 loop 1 nt U96 to U99 with the 5′ splice site we identified may occur immediately before the first step of splicing. As proposed, it is possible that the U5 snRNA makes contacts upstream of the 5′ splice site preceding these interactions. This could be determined, using our system, by incorporating 4-thioU at positions U91 and/or U92 (positions of U5 known to interact upstream in 5′ exon) and identifying the factors involved in this interaction of U5 with the 5′ exon. Ultimately, we would like to ascertain the exact timing and mechanisms by which the U5 snRNA is positioned at the 5′ exon and the U5 loop 1 uridines targeted to the 5′ splice site prior to the first step of pre-mRNA splicing.

MATERIALS AND METHODS

Splicing extract preparation and in vitro splicing

Splicing extract was prepared from yeast strain SC261.8 either by lysis of spheroplasts (Newman et al., 1985) or lysis in liquid nitrogen with a mortar and pestle (Ansari & Schwer, 1995). Depletion and reconstitution of U5 snRNPs in extract made from SC261.8 was carried out according to O'Keefe et al. (1996). Depletion of U2 or U6 snRNAs from splicing extract was carried out simultaneously with U5 depletion using oligonucleotides SRU2, 5′-CAGATACTACACTTG, for U2 (McPheeters et al., 1989) and d1, 5′-ATCTCTGTATTGTTT CAAATT, for U6 (Fabrizio et al., 1989). UV crosslinking and recovery of RNA from UV-irradiated splicing reactions were carried out according to Newman et al. (1995). ATP was depleted by omitting ATP from splicing buffer and incubating extract at 23 °C for 5 min with 1 mM glucose.

RNA ligations

U5 snRNAs containing 4-thioU in loop 1 were produced by RNA ligation based on the method of Moore and Sharp (1992) with one chemically synthesized RNA containing 4-thioU and two in vitro-transcribed RNAs. Chemically synthesized RNAs representing nt 93–103 of U5 were the following sequences with X representing 4-thioU: 12581, 5′-GCCXUUUACCA, (U96); 12582, 5′-GCCUXUUACCA, (U97); 12385, 5′-GC CUUXUACCA, (U98); 12583, 5′-GCCUUUXACCA, (U99); 12386, 5′-GCCXXXXACCA, (U96–99). RNA representing nt 1–92 of U5 was transcribed in vitro from PCR templates with T7 polymerase. The PCR primers (62707–001, 5′-GCGC TAATACGACTCACTATAGGAAGCAGCTTTACAGATCAAT, forward; 17462–001, 5′-TCGGGGCGACGACGCCCTTAA GAACCATGTTCGTTAT, back) contained a T7 promoter in the forward primer and a recognition sequence for the VS ribozyme in the back primer (Guo & Collins, 1995). The PCR template was cotranscribed with a linearized plasmid (pTZ19R) encoding the VS ribozyme under the following conditions: 40 mM Tris-HCl, pH 8, 2 mM spermidine, 10 mM dithiothreitol (DTT), 20 mM $MgCl_2$, 1 mM each NTP, 0.05 μg/μL PCR template, 0.05 μg/μL pTZ19R plasmid, 1 U/μL RNasin (Promega), 1.5 U/μL T7 RNA polymerase (Promega). The VS ribozyme cleaves the U5 RNA during the transcription reaction after nt 92, avoiding 3′ heterogeneity by T7 polymerase. RNA was purified from the VS recognition sequence on a 6% polyacrylamide-7 M urea gel, then cut from the gel and electroeluted. VS ribozyme cleavage leaves a 3′ cyclic phosphate that was removed by treatment for 30 min at 37 °C with 0.5 U/μL T4 polynucleotide kinase (New England Biolabs) in a buffer containing 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 1 U/μL RNasin. The pH of this buffer was adjusted to 8.0 by addition of Tris-HCl, pH 8.5, then 0.2 U/μL calf intestinal alkaline phosphatase (New England Biolabs) was added and incubated for 30 min at 37 °C. Proteins were removed by addition of 0.1 vol Proteinase K stop mix (1 mg/mL Proteinase K, 50 mM EDTA, 1% SDS) followed by incubation at 37 °C for 15 min and extraction with phenol:chloroform:isoamyl alcohol (50:50:1). RNA representing nt 104–214 of U5 was transcribed in vitro from a PCR template with T7 polymerase. The PCR primers (62707–003, 5′-GCGCT AATACGACTCACTATAGAACCATCCGGGTGTTGT, forward; 62707–002, 5′-ACGCCCTCCTTACTCATTGAG, back) contained a T7 promoter in the forward primer. Transcription conditions were the same as above except the reaction contained 5 mM guanosine 5′-monophosphate and ribozyme was omitted from the reaction.

Three-way RNA ligation between the middle chemically synthesized RNA and the two in vitro-transcribed RNAs was carried out by phosphorylating 500 pmol middle RNA (U5 nt 93–103) with 0.5 U/μL T4 polynucleotide kinase in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 ng/μL bovine serum albumin and 1 U/μL RNasin for 30 min at 37 °C. To visualize RNA ligation intermediates and products [γ-^{32}P]-ATP was used in place of ATP. Following phosphorylation, 500 pmol each of in vitro-transcribed RNAs (U5 nt 1–92 and 104–214) and bridging DNA oligonucleotide (59879–007, 5′-GG AGACAACACCCGGATGGTTCTGGTAAAAGGCAAGAAC CATGTT) was added to the phosphorylated middle RNA with approximately 9% PEG 6000. RNAs and bridging oligonucleotide were heated to 90 °C and allowed to cool to 25 °C. Ligase buffer, RNasin, and T4 DNA ligase (New England Biolabs) were added to give a reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 ng/μL bovine serum albumin, 1 U/μL RNasin, and 40 U/μL T4 DNA ligase, then incubated at 37 °C for 1 h. The reaction was phenol extracted and RNAs precipitated with ammonium acetate and ethanol. Ligated RNA was purified by electrophoresis in a 6% polyacrylamide–7 M urea gel, RNA visualized by UV shadowing, and electroeluted from the gel.

Ligation to introduce a single radioactive-labeled phosphate between the (−1) and (−2) position of exon 1 in the *CYH2* pre-mRNA was carried out as previously described (O'Keefe & Newman, 1998) using UpG in place of 4-thioUpG to prime transcription of the 3′ RNA.

RNA capture using biotinylated oligonucleotides and crosslink mapping

For visualization or mapping of crosslinked RNA species containing U5, RNA isolated from UV-irradiated splicing reactions was captured using a 5′ biotinylated oligonucleotide complementary to U5 (67736–001, 5′-biotinATGGCAAGC CCACAGTAACGGACAGC) and streptavidin paramagnetic beads according to O'Keefe and Newman (1998). Primer extension mapping of U5/pre-mRNA crosslinks was carried out as described (Newman et al., 1995) using an oligonucleotide complementary to the actin intron (69327–001, CGTGGTTATTACAGATCAGTCA) and 2.5 pmol of in vitro-transcribed actin pre-mRNA for reference sequence ladder.

RNase H analysis

RNA captured with biotinylated oligonucleotides and streptavidin paramagnetic beads was gel purified then electroeluted. To identify the specific RNA species of which each crosslink was composed, electroeluted crosslinked RNA was mixed with a specific oligonucleotide and annealed by slow cooling from 90 °C to 30 °C in a reaction containing 40 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$ and 20 ng/μL tRNA. Then 0.2 U/μL RNase H (Promega) was added and incubated at 30 °C for 30 min. RNA was recovered by phenol extraction and ethanol precipitation, then fractionated on a 6% polyacrylamide-7 M urea gel. Oligonucleotides used for RNase H were complementary to different regions of the *CYH2* pre-mRNA or the U5 snRNA and were of the following sequences: 37067-001, 5′-GAGACGTGACCTCTG (exon 1); 37067-002, 5′-CAGCGATAATTAGTG (intron); 37067-003, 5′-CTTACCGATACGACC (exon 2); 49506-005, 5′-CGTTATA AGTTCTATAGGCA (U5).

In vitro transcription of RNA

Crosslink mapping was carried out with body-labeled actin pre-mRNA that was produced by in vitro transcription from the plasmid p283 as previously described (O'Keefe et al., 1996). *CYH2* pre-mRNA was transcribed under identical conditions from PCR-generated template with primers (36614-001, 5′-GCGCTAATACGACTCACTATAGACTAGAAAGCAC AGAGGTC, forward; 32389-001, 5′-AAGTATCTCATACCAA CCTTACCG) and a plasmid containing the *CYH2* gene with a 203-nt deletion from the center of the intron (Newman et al., 1995). Wild-type U5 snRNA transcription was as previously described (O'Keefe et al., 1996).

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