RNA (1995), *1*:968–980. Cambridge University Press. Printed in the USA.

snRNA interactions at 5′ and 3′ splice sites monitored by photoactivated crosslinking in yeast spliceosomes

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ABSTRACT

Splice site recognition and catalysis of the transesterification reactions in the spliceosome are accompanied by a dynamic series of interactions involving conserved or invariant sequences in the spliceosomal snRNAs. We have used site-specific photoactivated crosslinking in yeast spliceosomes to monitor interactions between snRNAs and exon sequences near the 5′ and 3′ splice sites. The last nucleotide of the 5′ exon can be crosslinked to an invariant loop sequence in U5 snRNA before and after 5′ splice site cleavage. The first nucleotide of the 3′ exon can also be crosslinked to the same U5 loop sequence, but this contact is only detectable after the first transesterification. These results are in close agreement with earlier data from mammalian splicing extracts, and they are consistent with a model in which U5 snRNA aligns the 5′ and 3′ exons for the second transesterification. After the first catalytic step of splicing, the first nucleotide of the 3′ exon can also crosslink to nt U23 in U2 snRNA. This is one of a cluster of residues in U2–U6 helix I implicated by mutational analysis in the second catalytic step of splicing. The crosslinking data suggest that these residues in U2–U6 helix I are in close proximity to the scissile phosphodiester bond at the 3′ splice site prior to the second transesterification. These results constitute the first biochemical evidence for a direct interaction between the 3′ splice site and U2 snRNA.

Keywords: site-specific crosslinking; spliceosome; 4-thio-uridine; U2 snRNA; U5 snRNA

INTRODUCTION

Introns are removed from messenger RNA precursors (pre-mRNAs) by splicing in a complex ribonucleoprotein particle called the spliceosome. Two sequential transesterification reactions are involved in pre-mRNA splicing: (1) the 2′OH at the intron branch site attacks the phosphodiester bond at the 5′ splice site, producing exon 1 and lariat intron–exon 2 intermediates; (2) the 3′OH of exon 1 attacks the phosphodiester bond at the 3′ splice site to produce spliced mRNA and lariat intron products (reviewed in Moore et al., 1993). In addition to a large number of protein factors, five small nuclear RNAs (U1, U2, U4, U5, and U6 snRNAs) are essential components of the splicing machinery. These RNA molecules contain short, conserved sequence motifs through which a dynamic network of specific snRNA:substrate and snRNA:snRNA interactions is established in the spliceosome.

Recognition of the 5′ splice site and the intron branchpoint involves base pairing with U1 (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988) and U2 (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989) snRNAs, respectively. An invariant sequence motif in U6 snRNA interacts with the 5′ splice site (Sawa & Abelson, 1992; Sawa & Shimura, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993) and a specific interaction between the first and last G residues of the intron appears to be required for the second transesterification reaction (Parker & Siliciano, 1993; Chanfreau et al., 1994; Scadden & Smith, 1995). Suppression of 5′ and 3′ splice site mutations by specific alterations in yeast U5 snRNA has shown that an invariant loop sequence in U5 interacts with exon sequences at 5′ and 3′ splice sites (Newman & Norman, 1992). Similar interactions have also been detected in mammalian splicing extracts (Sontheimer & Steitz, 1993), allowing the formulation of an explicit model for

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U5 function: the invariant U5 loop sequence is proposed to act as a "tethering" site for exon 1 after the first catalytic step and then to align the two exons for the second catalytic step.

Mutational analysis has shown that conserved sequences in yeast U2 and U6 snRNAs, close to the regions that base pair with the branch site and 5′ splice site, respectively, interact to form a helix (helix I) with a 2-nt bulge (Madhani & Guthrie, 1992). Several of the residues that participate in U2–U6 helix I are essential for viability and for activity in splicing assays in vitro (reviewed in Madhani & Guthrie, 1994a). It is particularly interesting that some mutations in helix I support the first catalytic step, but interfere with the second (McPheeters et al., 1989; Fabrizio & Abelson, 1990; Madhani & Guthrie, 1992; McPheeters & Abelson, 1992). Mutations in one of these residues (G52 in U6 snRNA) can suppress 3′ splice site mutations, albeit in a non-allele-specific fashion (Lesser & Guthrie, 1993). However, it is currently unclear whether U2–U6 helix I interacts directly with the substrate, as might be expected if it plays an intimate role in one or both transesterification reactions.

In summary, it is clear that a complex network of RNA-mediated interactions plays a crucial role in splice site recognition in the spliceosome (reviewed in Madhani & Guthrie, 1994a). The splicing of group II autocatalytic introns occurs via a two-step transesterification pathway, involving lariat intermediates and products, which has the same stereochemical characteristics as nuclear pre-mRNA splicing (Maschhoff & Padgett, 1993; Moore & Sharp, 1993; Padgett et al., 1994). Furthermore, several of the snRNA interactions in the spliceosome appear to have counterparts in group II self-splicing introns. These similarities have led to much speculation that pre-mRNA splicing may be dependent on catalysis by RNA, with conserved sequences in the snRNAs playing a central role in the transesterification reactions (see Steitz & Steitz, 1993; Weiner, 1993; Wise, 1993; Nilsen, 1994; Sharp, 1994).

In the experiments described here, we have used the nucleotide analogue 4-thiouridine (4-thioU) as an intrinsic photoaffinity probe to monitor interactions between snRNAs and exon sequences adjacent to the splice sites in yeast spliceosomes. As in mammalian extracts (Sontheimer & Steitz, 1993), we can detect crosslinks between the U5 snRNA loop sequence and the last nucleotide of exon 1 both before and after 5′ splice site cleavage. Crosslinks between the same invariant U5 sequence and the first nucleotide of exon 2 appear only in the lariat intermediate after the first transesterification. The first nucleotide of exon 2 also forms crosslinks in the lariat intermediate to an invariant residue in U2 snRNA, which has previously been implicated in the second catalytic step by mutational studies. These results place invariant residues in both U2 and U5 snRNAs in close proximity to the scissile phosphate at the 3′ splice site, and they strongly support the view that these snRNAs may be intimately involved in the second transesterification reaction.

RESULTS

Genetic data from studies in yeast (Newman & Norman, 1992), site-specific crosslinking results from experiments using mammalian extracts (Wyatt et al., 1992; Sontheimer & Steitz, 1993), and functional assays in HeLa cells (Cortes et al., 1993) have shown that the loop I sequence in U5 snRNA interacts with exon sequences at the 5′ and 3′ splice sites. The aim of the work described here is to exploit the site-specific crosslinking approach to identify and monitor contacts between snRNA and substrate sequences in yeast spliceosomes. In view of the low abundance of spliceosomal snRNAs in yeast nuclei and splicing extracts, we anticipated that the detection and analysis of substrate:snRNA crosslinks in yeast spliceosomes could prove technically difficult. Therefore, for initial detection of crosslinks, we used a highly active snRNP-enriched fraction from yeast splicing extract, made by ammonium sulfate precipitation (see the Materials and methods).

Contacts between U5 snRNA and 5′ exon sequences near the 5′ splice site

Splicing reactions were performed with *CYH2* pre-mRNA carrying a single 4-thioU residue at position (−1) in exon 1 (immediately upstream of the 5′ splice site). Samples were withdrawn at intervals and irradiated with 365-nm UV light and, after deproteinization, RNA was fractionated on a 6% polyacrylamide–8-M urea gel (Fig. 1A, lanes 1–7). To demonstrate the site-specificity of any crosslinks, a parallel reaction was performed with a similar pre-mRNA containing an unmodified U residue at position (−1) in exon 1 (Fig. 1A, lanes 9–15). Three 4-thioU-specific products are visible in lanes 2–7, migrating more slowly than the pre-mRNA. The lariat intron and lariat intermediate species are not seen because the only radioactive label is in exon 1, immediately upstream of the 4-thioU residue. The kinetics of the appearance of these products are shown more clearly in Figure 1B. The most rapidly migrating of the three species is visible after 1 min at 23 °C, whereas the slower migrating two products appear simultaneously after 2–2.5 min of incubation. All three of the 4-thioU-specific species arise before the exon 1 intermediate and spliced mRNA product (Fig. 1B; data not shown), which appear only after about 4 min at 23 °C.

As a first step in the characterization of the 4-thioU-specific products, the upper two species were gel purified together and subjected to RNaseH cleavage in the presence of a panel of oligonucleotides specific for U1, U2, U5, and U6 snRNAs, and for the exon 1, intron, and exon 2 regions of the substrate (Fig. 2A). The re-

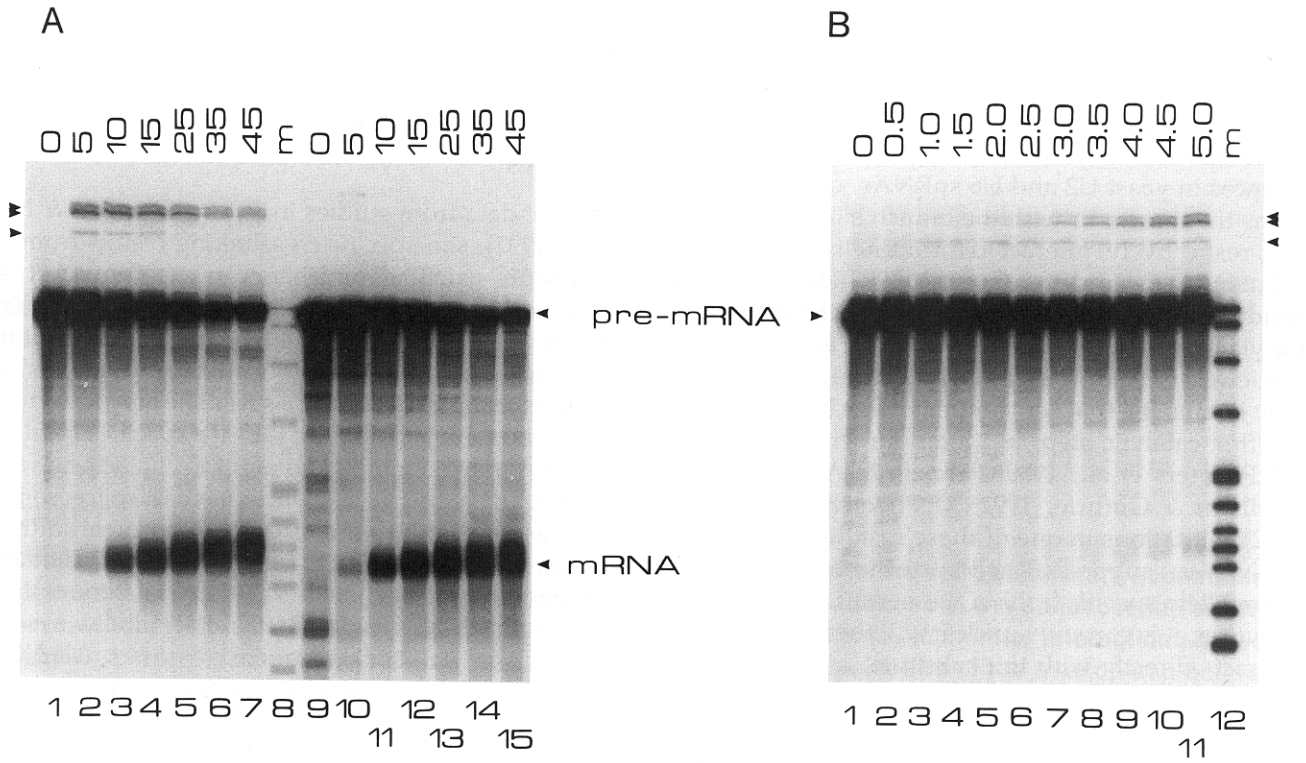

FIGURE 1. In vitro splicing and photoactivated crosslinking of pre-mRNAs carrying a single 4-thioU residue at position (−1) in exon 1 (immediately upstream of the 5′ splice site). **A:** Lanes 1–7, samples were withdrawn from an in vitro splicing reaction after 0, 5, 10, 15, 25, 35, and 45 min of incubation at 23 °C, UV-irradiated, deproteinized, and fractionated on a 6% polyacrylamide–8-M urea gel; lane 8, pBR322.MspI end-labeled size markers; lanes 9–15, samples prepared as in lanes 1–7, but the pre-mRNA contains an unmodified U residue at position (−1) in exon 1, to demonstrate the site-specificity of crosslink formation. Three 4-thioU-dependent species migrating more slowly than the pre-mRNA are indicated by arrowheads. The exon 1 intermediate is not shown, and the lariat intron product and lariat intron–exon 2 intermediate are not visible because the only radioactive phosphate in the pre-mRNA is in exon 1. **B:** Lanes 1–11, samples were withdrawn at 30-s intervals from a splicing reaction containing pre-mRNA with a single 4-thioU residue at position (−1) in exon 1 and analyzed as in A. Three products migrating more slowly than the pre-mRNA (indicated by arrowheads) appear rapidly at 23 °C; lane 12, pBR322.MspI end-labeled size markers.

sults show that these two species result from crosslinks between the pre-mRNA and U5 snRNA. The presence of two species of this product is not unexpected, given that U5 snRNA exists in the cell in two forms with different 3′ ends (U5S and U5L, short and long forms; Patterson & Guthrie, 1987; Frank et al., 1994). A similar RNaseH analysis of the fastest of the three 4-thioU-specific products showed that it was the result of a crosslink between U1 snRNA and the pre-mRNA (data not shown). This crosslink has not been analyzed further, but the early appearance of this species (before the U5:pre-mRNA crosslinks are visible) is consistent with the view that it reflects the well-established interaction of the 5′ end of U1 snRNA with the 5′ splice site (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988; Wassarman & Steitz, 1992).

The two species shown by RNaseH analysis to contain U5 snRNA are specifically enriched by hybridization to a biotinylated oligonucleotide complementary to U5 and capture with streptavidin-conjugated paramagnetic particles (Fig. 2B, lane 1). This U5-specific capture experiment also reveals a faster-migrating pair of products (Fig. 2B, lane 1). The latter two species can alternatively be visualized by fractionation of total RNA from a UV-irradiated reaction by 2D PAGE (Fig. 2C, products marked S and L). The appearance of these products is 4-thioU and UV-dependent (data not shown) and their anomalous mobility in the second dimension is characteristic of nonlinear molecules. RNaseH analysis (Fig. 2D) of the faster-migrating of these two showed that it contains exon 1 and U5 sequences, suggesting that it results from formation of a crosslink between U5 snRNA (S form) and the exon 1 intermediate produced in the first catalytic step of splicing. The slower migrating of the two gave similar results (L form of U5 snRNA; data not shown). In summary, these results show that U5 snRNA contacts the last nucleotide of exon 1 before the first catalytic step, and that this contact persists after 5′ splice site cleavage.

The S forms of the U5:pre-mRNA and U5:exon 1 products were gel-purified to allow mapping of the crosslinks by primer extension blockage. These species

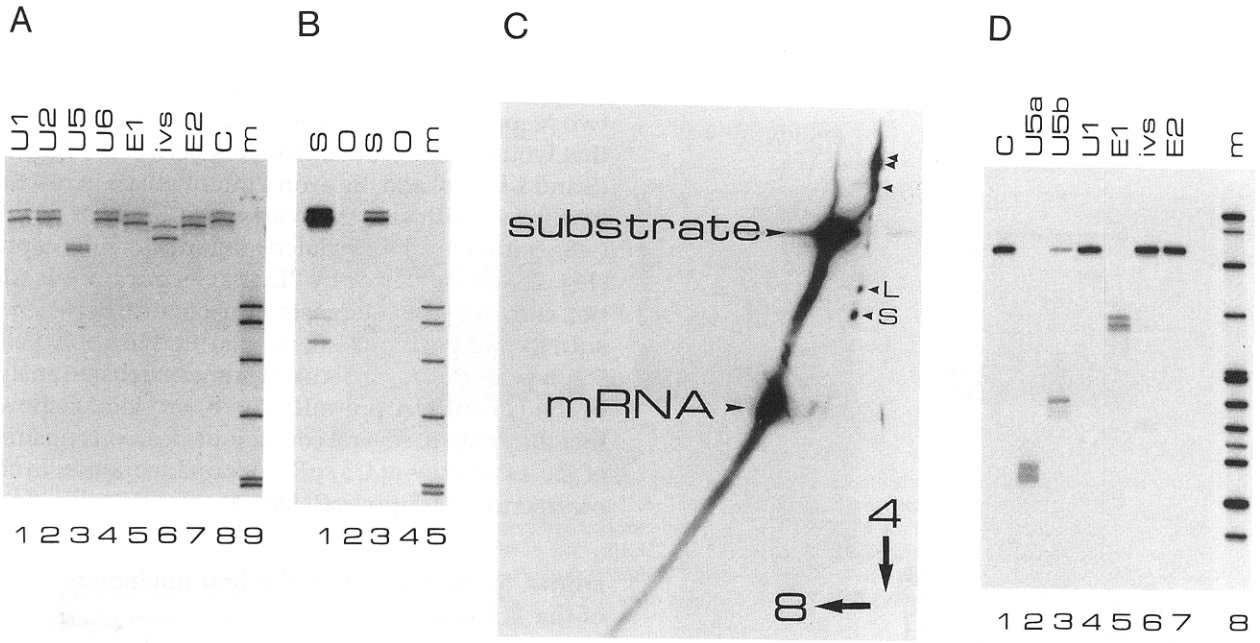

FIGURE 2. Identification of U5:pre-mRNA and U5:exon 1 crosslinked species produced after photoactivation during splicing of pre-mRNA containing a single 4-thioU residue at position (−1) in exon 1 (immediately upstream of the 5′ splice site). **A:** Oligonucleotide-targeted RNaseH cleavage of gel-purified crosslinked species, using oligonucleotides with the following specificities: lane 1, U1 snRNA; lane 2, U2 snRNA; lane 3, U5 snRNA; lane 4, U6 snRNA; lane 5, exon 1; lane 6, intron; lane 7, exon 2; lane 8, no oligo control; lane 9, pBR322.MspI end-labeled size markers. **B:** Streptavidin-paramagnetic particle capture of U5 snRNA-containing species from total RNA derived from UV-irradiated splicing reactions. Lane 1, 4-thioU at position (−1) in exon 1; lane 2, U at position (−1) in exon 1; lane 3, 4-thioU at position (−8) in exon 1; lane 4, U at position (−8) in exon 1. **C:** Visualization of crosslinked species after 2D gel electrophoresis. Total RNA from a UV-irradiated splicing reaction (4-thioU at position (−1) in exon 1) was fractionated in 4% polyacrylamide-8-M urea in the first dimension, and 8% polyacrylamide-8-M urea in the second dimension. Crosslinked species containing the pre-mRNA (migrating more slowly than the substrate) are not well-separated by this gel system, but two additional species (4-thioU-dependent and UV-dependent; data not shown) migrating behind the diagonal of uncrosslinked substrate degradation products are indicated by arrowheads marked S and L. Exon 1 has run off in the first dimension. **D:** Oligonucleotide-targeted RNaseH cleavage analysis of species S (Fig. 1C) using oligonucleotides with the following specificities. Lane 1, control (no oligo); lane 2, U5 snRNA (93-1309); lane 3, U5 snRNA (93-1310); lane 4, U1 snRNA; lane 5, exon 1; lane 6, intron; lane 7, exon 2; lane 8, end-labeled pBR322.MspI size markers.

were used as the templates for reverse transcription reactions with a 5′ end-labeled primer specific for a sequence near the 3′ end of yeast U5 snRNA. For reference, a set of four sequence lanes was produced in parallel, using total RNA from yeast splicing extract as the template (Fig. 3A, lanes 1–4). The results show that the crosslinks map to several contiguous positions in the invariant U5 snRNA loop I motif. In both the U5:pre-mRNA and U5:exon 1 products, the majority of the crosslinks map to the same two positions (Fig. 3A, lanes 5, 7). Assuming that primer extension stops 1 nt short of the actual crosslink, the results show that the major crosslinking sites are positions U4 and U5 in the invariant loop sequence GCCUUUUAC. This is in close agreement with data from mutational analysis in yeast (Newman & Norman, 1992) and with site-specific crosslinking results in mammalian splicing extracts (Sontheimer & Steitz, 1993).

Are the interactions between U5 snRNA and the pre-mRNA restricted to the immediate vicinity of the 5′ splice site? To address this question, we synthesized *CYH2* pre-mRNAs with a single 4-thioU residue positioned either 8 nt upstream of the 5′ splice site (position (−8) in exon 1), or in the intron 4 nt downstream of the 5′ splice site (GUAUGU). Both substrates were processed normally in splicing extracts, producing labeled exon 1 intermediate and mRNA product (for position (−8) in exon 1; data not shown) or labeled lariat intron and lariat intron–exon 2 intermediate (for position 4 in the intron; data not shown). No crosslinks to U5 snRNA were detectable using the substrate carrying 4-thioU at position 4 in the intron, either before or after 5′ splice site cleavage (data not shown). UV-irradiation of splicing reactions performed with substrate carrying 4-thioU at position (−8) revealed several 4-thioU-dependent products migrating more slowly than the pre-mRNA, even at early time points in the reaction (Fig. 4A,B,C, lane 1; data not shown). RNaseH analysis showed that two of these slowly migrating products were targeted in the presence of an oligonucleotide specific for U5 snRNA (Fig. 4B, lane 5). These two species can be explicitly captured from the reaction

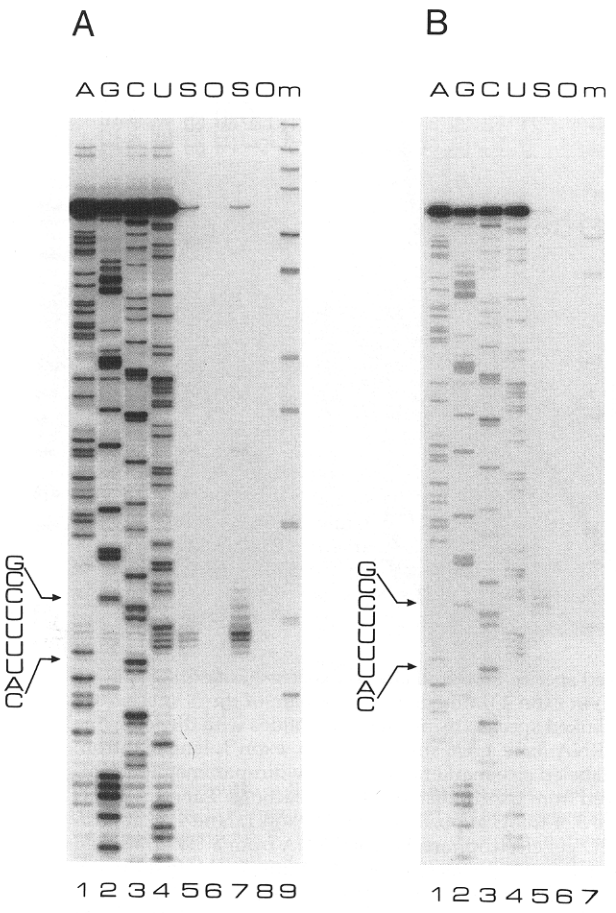

FIGURE 3. Primer extension mapping of crosslinks between U5 snRNA and 4-thioU residues at positions (−1) and (−8) in exon 1. **A:** Lanes 1–4, dideoxynucleotide sequencing tracks produced using total RNA from yeast splicing extract as template; lane 5, gel-purified U5:exon 1 species from splicing of pre-mRNA with 4-thioU at position (−1) in exon 1 as template; lane 6, template RNA gel-purified in parallel from a control reaction using pre-mRNA with unmodified U at position (−1) as template; lane 7, as for lane 5, but the gel-purified template is the U5:pre-mRNA species from splicing of pre-mRNA with 4-thioU at position (−1) in exon 1; lane 8, template RNA gel-purified in parallel from a control reaction using pre-mRNA with unmodified U at position (−1) in exon 1. The 5′ end (^{32}P)-labeled oligonucleotide primer is specific for nt 145–171 of yeast U5 snRNA. **B:** Lanes 1–4, dideoxynucleotide sequencing tracks as in A; lane 5, gel-purified U5:pre-mRNA species from splicing of pre-mRNA with 4-thioU at position (−8) in exon 1 as template; lane 6, template RNA gel-purified in parallel from a control reaction using pre-mRNA with unmodified U at position (−8) in exon 1.

using a biotinylated U5 snRNA-specific oligonucleotide and streptavidin-conjugated paramagnetic particles (Fig. 2B, lane 3). Moreover, they co-migrate with the crosslinked U5:pre-mRNA species produced when the 4-thioU residue is at position (−1) next to the 5′ splice site (Fig. 2B, lanes 1, 3). The appearance of these U5-containing products is dependent on both ATP and on incubation at 23 °C (conditions necessary for spliceosome assembly; Fig. 4).

These results show that U5 snRNA can contact sequences in the pre-mRNA at least 8 nt upstream of the 5′ splice site. This interaction is distinct from the well-established contact between U5 snRNA and the exon nucleotides immediately adjacent to the 5′ splice site in two respects. First, there is no trace of the two species that would result from a crosslink between U5 snRNA (S and L forms) and the exon 1 intermediate in reactions performed with substrate carrying 4-thioU at position (−8), either by biotinylated oligonucleotide capture (Fig. 2B, lanes 1, 3) or by 2D gel electrophoresis (data not shown). This suggests that contact between U5 snRNA and position (−8) in exon 1 occurs only before 5′ splice site cleavage. Second, primer extension analysis of the U5 snRNA:pre-mRNA(−8) crosslinks showed that they map to several contiguous positions upstream of the invariant 9-nt U5 snRNA loop I sequence, in clear contrast to the U5:pre-mRNA(−1) crosslinks (Fig. 3A,B).

snRNA interactions with the first nucleotide of the 3′ exon

In order to monitor snRNA interactions in the vicinity of the 3′ splice site in yeast spliceosomes, a single 4-thioU residue was incorporated into *CYH2* pre-mRNAs as the first nucleotide (+1) of exon 2, immediately downstream of the 3′ splice site. For initial detection and analysis of crosslinks, a single nucleotide mutation (CAG to CAC) was introduced at the 3′ splice site. This leads to the accumulation of lariat intermediates by inhibiting the second transesterification reaction (Fig. 5A,B). Following UV irradiation, four slowly migrating 4-thioU-dependent RNA species appear in these reactions (Fig. 5A,B, species a–d). Species d is present in the zero time sample, and is not targeted by oligonucleotides specific for U1, U2, U5, or U6 snRNAs (data not shown); it may contain an intramolecular crosslink, but it has not been analyzed in detail. Species a–c, however, appear and increase in abundance in parallel with the lariat intermediate (Fig. 5B, lanes 6–10). In order to investigate the composition of species a–c, targeted RNaseH digestion was performed with a panel of oligonucleotides specific for snRNA and substrate sequences (Fig. 5C). An exon 2-specific oligonucleotide was included in the digestions shown in lanes 3–10 to facilitate gel fractionation of the RNaseH cleavage products. The data show that species b and c result from crosslinks between the lariat intermediate and U5 snRNA (L and S forms of U5, respectively; Fig. 5C, lane 7). Species a results from a crosslink between the lariat intermediate and U2 snRNA (Fig. 5C, lanes 5, 6). Furthermore, it is possible to deduce from the mobility of the RNaseH digestion products, which appear after targeting with U2 oligonucleotides U2a and U2b, that the crosslink(s) in species a is near the 5′ end of U2 snRNA.

Splicing and photoactivation of substrates with a wild-type 3′ splice site (CAG), carrying 4-thioU at position (+1) in exon 2, also produces species a–c (Fig. 5D,

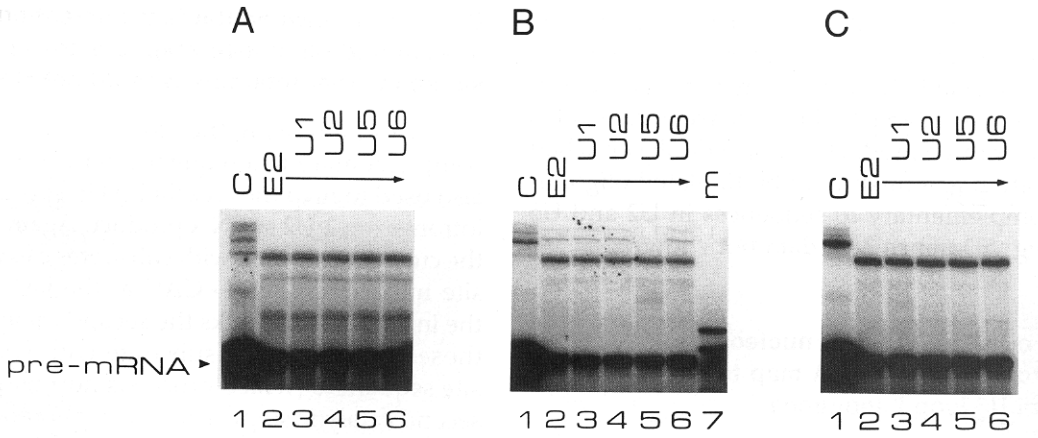

FIGURE 4. Oligonucleotide-targeted RNaseH cleavage of the products of UV-irradiation of splicing reactions containing pre-mRNA with a 4-thioU residue at position (−8) in exon 1. **A:** Lanes 1–6, RNA from a reaction depleted of ATP by incubation with 1 mM glucose before addition of substrate and incubation (−ATP) at 23 °C for 6 min. **B:** Lanes 1–6, RNA from a reaction incubated at 23 °C for 6 min (+ATP); lane 7, pBR322.MspI end-labeled size markers. **C:** Lanes 1–6, zero time sample RNA from a (+ATP) reaction. In each set of six samples, RNaseH cleavage was targeted with oligonucleotides of the following specificities. Lane 1, control (no oligo); lane 2, exon 2; lane 3, exon 2 + U1 snRNA; lane 4, exon 2 + U2 snRNA; lane 5, exon 2 + U5 snRNA; lane 6, exon 2 + U6 snRNA.

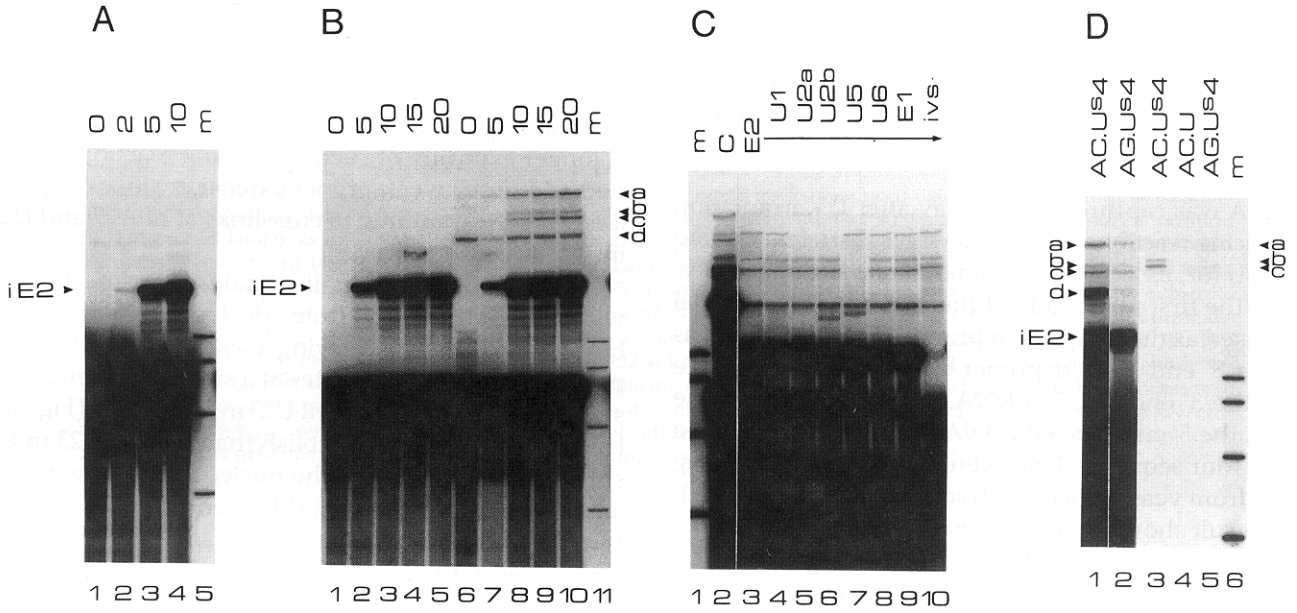

FIGURE 5. Formation and analysis of crosslinks using substrates carrying a single 4-thioU residue at position (+1) in exon 2 (immediately downstream of the 3′ splice site). **A:** Lanes 1–4, time course of splicing (samples taken at 0, 2, 5, and 10 min incubation at 23 °C) of 4-thioU substrate (3′ splice site CAC; no UV irradiation); lane 5, pBR322.Msp size markers; iE2, lariat intron–exon 2 intermediate. **B:** Lanes 1–5, time course of splicing of control substrate (unmodified U at position (+1) in exon 2, CAC 3′ splice site; samples taken at 0, 5, 10, 15, and 20 min at 23 °C, and UV-irradiated for 5 min at 0–4 °C); lanes 6–10, as for lanes 1–5, but using substrate with 4-thioU at position (+1) in exon 2; lane 11, pBR322.MspI end-labeled size markers; iE2, lariat intron–exon 2 intermediate. **C:** Oligonucleotide-targeted RNaseH analysis of crosslinked species produced during splicing of substrate carrying 4-thioU at position (+1) in exon 2. Lane 1, end-labeled pBR322.MspI size markers; lane 2, no oligo. Lanes 3–10, exon 2-specific oligo plus second oligo of the following specificity: lane 3, nil; lane 4, U1 snRNA; lane 5, U2 snRNA oligo a (94-2126); lane 6, U2 snRNA oligo b (94-2127); lane 7, U5 snRNA; lane 8, U6 snRNA; lane 9, exon 1; lane 10, intron. **D:** Capture of crosslinked species a, b, and c using streptavidin-paramagnetic particles and biotinylated oligonucleotides specific for U2 and U5 snRNA. Lane 1, 3′ splice site sequence CAC.4-thioU, total RNA from splicing reaction prior to capture; lane 2, 3′ splice site sequence CAG.4-thioU, total RNA from splicing reaction prior to capture; lane 3, 3′ splice site sequence CAC.4-thioU, after combined U2 and U5 capture; lane 4, 3′ splice site sequence CAC.U, after combined U2 and U5 capture; lane 5, 3′ splice site sequence CAG.4-thioU, after combined U2 and U5 capture; lane 6, end-labeled pBR322.MspI size markers; iE2, lariat intron–exon 2 intermediate. All samples were analyzed by electrophoresis on 5% polyacrylamide–8-M urea sequencing gels.

lane 2) as well as spliced mRNA (data not shown). The lariat intron product does not appear because it is not radioactively labeled. In agreement with the identities of species a–c established by RNAseH digestions (Fig. 5C), these three products are specifically enriched by hybridization to a mixture of biotinylated oligonucleotides complementary to sequences in U2 and U5 snRNAs (Fig. 5D, lanes 3–5; data not shown).

Crosslinks between the first nucleotide of the 3′ exon and U5 snRNA map to the invariant U5 loop I sequence

Because of their exceptionally slow electrophoretic mobility, species a–c (Fig. 5) proved to be difficult to gel purify in quantities necessary for primer extension mapping of the crosslinks. Instead, we exploited the observation (Fig. 5D) that the intron–exon 2:U5 (S and L forms) and intron–exon 2:U2 species were the only detectable U2 and U5 snRNA-containing adducts produced in reactions with substrates carrying a 4-thioU residue at position (+1) in exon 2. Therefore, total RNA isolated from UV-irradiated reactions was hybridized to a biotinylated oligonucleotide complementary to a sequence in the *CYH2* intron, and the oligonucleotide was captured with streptavidin-paramagnetic particles. As expected, the captured RNA species included pre-mRNA, intron–exon 2 lariat intermediate, and species a–c (data not shown).

RNA was captured in this way after UV-irradiation of splicing reactions assembled with substrates carrying 4-thioU (or, as a control to demonstrate site-specificity, U) as the first nucleotide of the 3′ exon. This material was used as the template in primer extension reactions with a 5′ end-labeled primer specific for a sequence near the 3′ end of U5 snRNA. After digestion of the RNA, the 5′ end-labeled cDNAs were run alongside a set of four sequence lanes generated in parallel using RNA from yeast splicing extract as template (Fig. 6A). The result shows that the primer extension stops map to several contiguous positions in the invariant U5 snRNA loop sequence; material derived from wild-type (CAG 3′ splice site) and mutant (CAC 3′ splice site) substrates gave similar results (Fig. 6A; data not shown). Assuming that reverse transcriptase stops 1 nt from the site of the actual crosslink, the majority of the crosslinks map to positions C3 and U4 in the 9-nt invariant sequence. This is in close agreement with genetic data from yeast (Newman & Norman, 1992) and also with crosslinking results from mammalian splicing extracts (Sontheimer & Steitz, 1993). Together, the genetic and biochemical data argue that, after the first catalytic step of splicing, a functionally significant interaction occurs between the first nucleotide of exon 2—immediately adjacent to the 3′ splice site—and the invariant 9-nt loop sequence in U5 snRNA.

Crosslinks between the first nucleotide of the 3′ exon and U2 snRNA map to a conserved sequence that interacts with U6 snRNA

RNA captured using the biotinylated oligonucleotide complementary to a sequence in the *CYH2* intron was also used to map the crosslink(s) in species a, the lariat intron–exon 2:U2 snRNA product. Again we compared the crosslinks formed with substrates carrying a 3′ splice site mutation (CAG → CAC at the last nucleotide of the intron, which blocks the second catalytic step) with those formed using a substrate with wild-type splice site sequences (which proceeds normally through the second catalytic step). The electrophoretic mobilities of species a RNaseH cleavage products (Fig. 5C, lanes 5, 6; data not shown) suggested that the crosslink(s) must involve a region near the 5′ end of U2 (within 45 nt of the 5′ end). Reverse transcription reactions were conducted using a 5′ end-labeled primer specific for a sequence in U2 snRNA downstream of the putative crosslink site(s). After digestion of the RNA, the 5′ end-labeled cDNAs were analyzed alongside a set of four sequence lanes generated in parallel using RNA from yeast splicing extract as template. Figure 6B shows the result obtained using material captured from splicing reactions in which the second catalytic step is blocked by the single nucleotide mutation at the 3′ splice site. Primer extension terminates mainly at a single position corresponding to a crosslink at nt A30 in U2 snRNA (Fig. 6B, lane 5). A longer exposure of such a mapping reaction reveals two additional weak primer extension stops (Fig. 6C, lane 5), corresponding to crosslinks at nt A27 and U23 in U2 snRNA.

Strikingly, when a similar analysis was performed with material captured from splicing reactions assembled with a substrate carrying a wild-type 3′ splice site, primer extension terminates at a single position corresponding to a crosslink at nt U23 in U2 snRNA (Fig. 6D, lane 5). These results establish that residue U23 in U2 snRNA, which is one of the nucleotides in the invariant U2–U6 helix Ib element (Madhani & Guthrie, 1992), is in close proximity to the 3′ splice site prior to the second transesterification.

DISCUSSION

We have used pre-mRNAs containing single photoactivatable 4-thioU residues in the vicinity of the 5′ or 3′ splice sites to monitor and analyze contacts between the substrate and snRNAs in yeast spliceosomes. Irradiation with UV light induces the formation of crosslinks between the last nucleotide of exon 1 and specific positions in an invariant 9-nt loop sequence in U5 snRNA. Such crosslinks can form either before or after the first catalytic step of splicing, consistent with the idea that the exon 1 intermediate is "tethered" to the U5 snRNA loop after 5′ splice site cleavage. Crosslinks

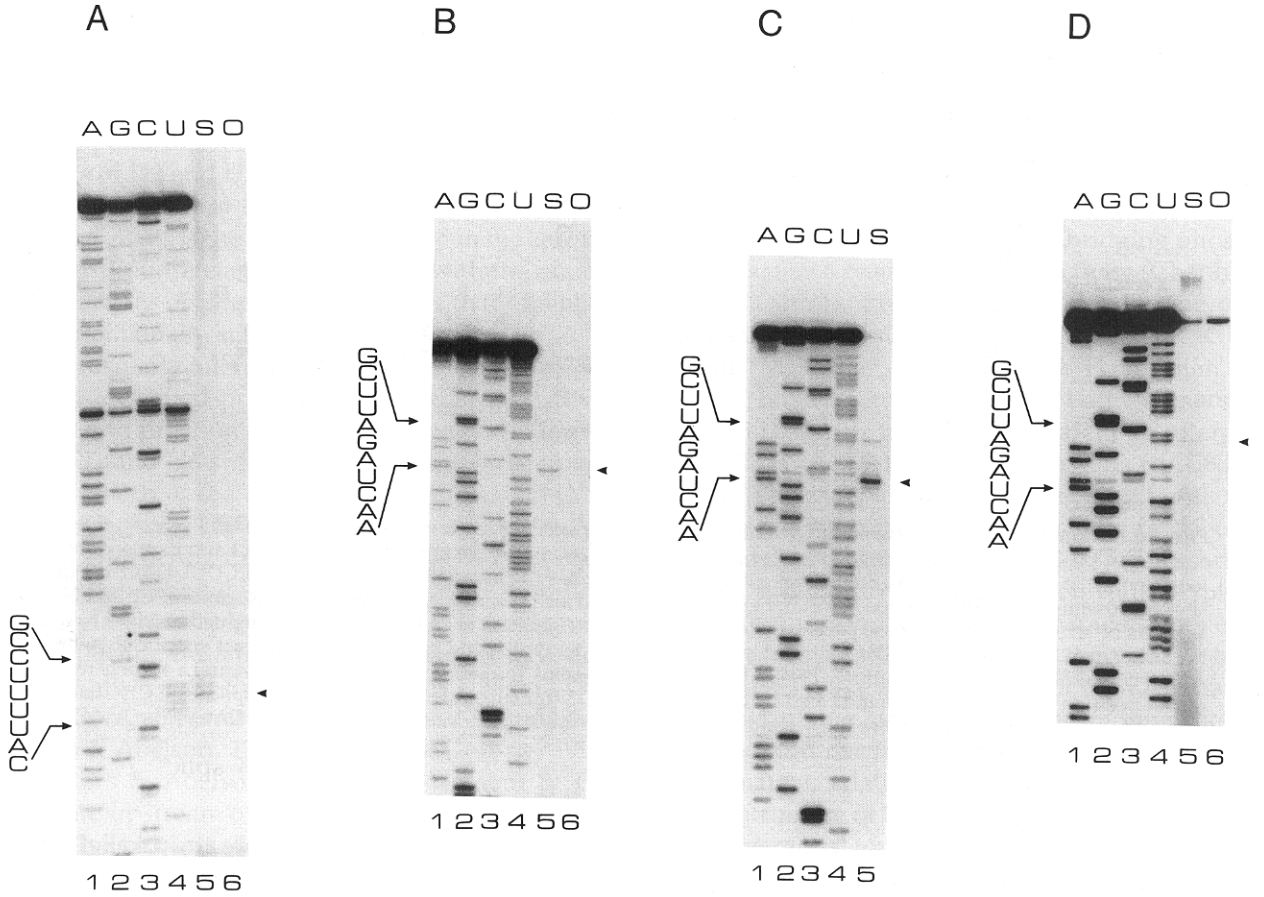

FIGURE 6. Primer extension analysis of crosslinks between a 4-thioU residue at position (+1) in exon 2 and U5 snRNA (A) or U2 snRNA (B–D). **A:** Lanes 1–4, dideoxynucleotide sequencing tracks produced using total RNA from yeast splicing extract as template; lane 5, template RNA from splicing and photoactivation of substrate carrying 4-thioU at position (+1) of exon 2; lane 6, as for lane 5, but substrate with unmodified U at position (+1) in exon 2. The 5′ end (^{32}P)-labeled primer is specific for nt 145–171 of yeast U5 snRNA. **B:** Lanes 1–4, dideoxynucleotide sequencing tracks produced using total RNA from yeast splicing extract as template; lane 5, template RNA from splicing and photoactivation of substrate carrying 4-thioU at position (+1) in exon 2 (3′ splice site CAC.4-thioU); lane 6, as for lane 5, but substrate with unmodified U at position (+1) in exon 2. The 5′ end (^{32}P)-labeled primer is specific for nt 132–153 of yeast U2 snRNA. **C,D:** The 5′ end (^{32}P)-labeled primer is specific for nt 107–124 of U2 snRNA and in each case lanes 1–4 are dideoxynucleotide sequencing tracks produced using total RNA from yeast splicing extract as template. **C:** Lane 5, template RNA for reverse transcription from splicing and photoactivation of substrate with CAC.4thioU 3′ splice site. **D**: Lane 5, template RNA for reverse transcription from splicing and photoactivation of substrate with 3′ splice site CAG.4-thioU; lane 6, template RNA for reverse transcription from splicing and photoactivation of substrate with 3′ splice site CAG.U.

between the first nucleotide of exon 2 and U5 snRNA are detectable only after the first catalytic step. Furthermore, the sites of these crosslinks on the U5 loop I sequence are consistent with the idea that the exons are aligned for the second transesterification reaction by their contacts with U5 snRNA (Fig. 7A). These results are in close agreement with data from similar studies in mammalian splicing extracts (Sontheimer & Steitz, 1993), and they are fully consistent with data on suppression of 5′ and 3′ splice site mutations by U5 snRNA loop mutations in yeast (Newman & Norman, 1992).

Taken together, the available genetic and biochemical data suggest that U5 snRNA and the U5 snRNP protein PRP8 (or its mammalian equivalent p220) make contacts with several positions in exon 1 near the 5′ splice site. Crosslinking experiments in the mammalian system revealed interaction between p220 and position (−2) and between U5 snRNA and positions (−1) and (−2) (Wyatt et al., 1992; Sontheimer & Steitz, 1993). We have now shown that yeast U5 snRNA and the PRP8 protein contact positions (−1), (−2), and (−8) in exon 1 (Fig. 7A; data not shown; Teigelkamp et al., 1995) and genetic data imply that position (−3) also interacts with U5 snRNA (Newman & Norman, 1992). In principle, all these contacts could occur simultaneously if the substrate and U5 sequences were appropriately folded, and, in this case, U5 snRNP may have a more extensive "footprint" on exon 1 than previously suspected. *Alternatively, and especially because the crosslink of* U5 snRNA to position (−8) is only detectable before the

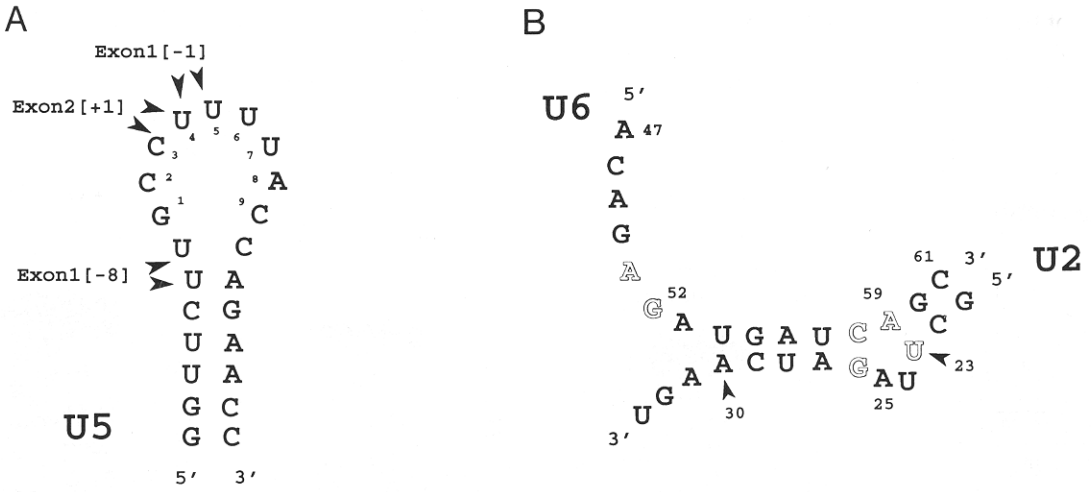

FIGURE 7. Summary of photoactivated crosslinks found in yeast splicing reactions using substrates with single 4-thioU residues at positions (−1) and (−8) in exon 1 or position (+1) in exon 2. **A:** Sequence shown is stem I and its associated loop (loop I) in yeast U5 snRNA (Frank et al., 1994), which includes the invariant 9-nt sequence GCCUUUUAC. Major sites of crosslink formation mapped by primer extension blockage are indicated by arrowheads. **B:** Regions of U2 and U6 snRNAs believed to interact by base pairing in yeast spliceosomes (Madhani & Guthrie, 1992). Highlighted residues have been shown to be important for the second step of splicing (see text). G52 in U6 is thought to interact specifically with A25 in U2 snRNA (Madhani & Guthrie, 1994b). Major sites of crosslink formation between U2 snRNA and a 4-thioU residue at position (+1) in exon 2 are indicated by arrowheads at U23 (for substrate with wild-type 3′ splice site CAG) and at A30 (for substrate blocked before the second catalytic step by the 3′ splice site mutation CAG → CAC).

first catalytic step, this could reflect an early interaction with the pre-mRNA prior to the final positioning of the U5 snRNA loop at the 5′ splice site. Further experiments will be necessary to clarify this issue.

A great deal of evidence shows that multiple interactions between conserved snRNA sequences and the 5′ splice site precede or accompany the first catalytic step of splicing: contacts with U1, U5, and U6 snRNAs can all contribute to 5′ splice site recognition (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988; Newman & Norman, 1992; Wassarman & Steitz, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993; Konforti & Konarska, 1994). In contrast, the only direct snRNA contact at the 3′ splice site that has been shown to play a role in the second transesterification is the interaction between the first nucleotide of exon 2 and the invariant 9-nt loop sequence in U5 snRNA (Newman & Norman, 1992; Sontheimer & Steitz, 1993). The crosslinking data presented in this paper show that one of the invariant residues in U2 snRNA—U23—is also in close proximity to the 3′ splice site prior to the second catalytic step. Indeed, it has been shown previously that mutation of residue U23 in yeast U2 snRNA leads to the accumulation of lariat splicing intermediates, indicating inhibition of the second step of splicing (Madhani & Guthrie, 1992). Sequences in the vicinity of U23 in U2 snRNA are therefore excellent candidates for the roles of recognition or catalytic activation of the 3′ splice site.

We have shown that contact between U2 snRNA and the first nucleotide of the downstream exon is established in the lariat intermediate, in parallel with the well-documented interaction of the U5 snRNA loop sequence at this position. It is intriguing that, when the second catalytic step is blocked by a single base change (G → C) at the last nucleotide of the intron, the pattern of U2 crosslinks alters substantially: contact between exon 2 position (+1) and U2 residue U23 is still detectable, but additional crosslinks also appear, with position A30 in U2 snRNA becoming the major crosslinking site (Fig. 7B). One interpretation of this change in crosslinking pattern is that the G → C 3′ splice site mutation interferes with an interaction between the first and last G residues of the intron in the lariat intermediate (Parker & Siliciano, 1993), when the first G of the intron is joined to the branchpoint A by a 2′–5′ linkage. If this G:G interaction is important for constraining the 3′ splice site, its disruption by the G → C 3′ splice site mutation could loosen the adjacent 4-thioU residue and allow it to crosslink to other sites in the vicinity in addition to position U23 in U2 snRNA. In any case, these results reinforce the view that the underlying U2 snRNA interaction may play a functionally significant role in the second catalytic step of splicing. We have so far been unable to "chase" the U2 snRNA:intron-exon 2 intermediates through the second step of splicing (data not shown), so it remains uncertain whether the crosslink from position (+1) in exon 2 to U23 in U2 snRNA reflects a catalytically active conformation of the spliceosome. However, even if this were so, it would not be surprising if the crosslink interfered with the second catalytic step, perhaps by restricting the mobility

or flexibility of the structure. Finally, we have shown, using a pair of substrates carrying tandemly duplicated 3′ splice sites (with a single 4-thioU residue positioned immediately downstream of the branch point-proximal or branch point-distal 3′ splice sites, respectively), that crosslinks of U2 and U5 snRNAs with the lariat intermediate occur only at the proximal 3′ splice site (data not shown). This is the site actually used in the second transesterification reaction, consistent with the notion that interactions of U2 and U5 snRNAs with the 3′ splice site are functionally significant.

It is particularly interesting that nt U23 in U2 snRNA has been identified previously in studies of snRNA structure and function as one of several invariant residues in U2 and U6 snRNAs whose identity is crucial for one or both of the catalytic steps of splicing (Madhani & Guthrie, 1992). Two clusters of residues in the U2–U6 structure depicted in Figure 7B have been shown by mutational studies and chemical modification experiments to play important roles in the second step of splicing: mutations at positions A51–G52 and C58–A59 in U6 snRNA and at positions U23 and G26 in U2 snRNA all result in specific second-step defects (Fabrizio & Abelson, 1990; Madhani & Guthrie, 1992; McPheeters & Abelson, 1992). Replacement of the phosphate upstream of A59 in U6 with a phosphorothioate also blocks splicing at the second step (Fabrizio & Abelson, 1992; Yu et al., 1995). Recent evidence from an analysis of covariation in viable combinations of U2 and U6 snRNA mutations suggests that there is a direct contact between G52 in U6 snRNA and A25 in U2 snRNA. This could reflect a specific tertiary structure in which the two clusters of residues required for the second step of splicing are juxtaposed (Madhani & Guthrie, 1994b). The crosslinking results presented in this paper represent the first direct biochemical evidence for close contact between the 3′ splice site and one of the invariant nucleotides in the step 2-specific cluster. Furthermore, our results suggest that invariant residues in U2, U5, and U6 snRNAs—that is, all three of the RNA components currently believed to play active roles in the transesterification reactions—are positioned close to the 3′ splice site in the lariat intron–exon 2 intermediate.

Several authors have recently drawn attention to some striking parallels between the invariant helix Ib element in the U2–U6 snRNA structure (nt AGC(59–61) of U6 snRNA and nt GCU(21–23) of U2 snRNA; Fig. 7B) and a similar conserved feature of the domain 5 (D5) helix of group II autocatalytic introns (Chanfreau & Jacquier, 1994; Boulanger et al., 1995; Peebles et al., 1995). D5 is a largely helical structure, essential for splicing activity, with three almost invariant base pairs between the sequences AGC(2–4) and GUU(31–33). Base changes at these six positions do not prevent substrate binding, but many such mutations interfere strongly with splicing activity (Boulanger et al., 1995; Peebles et al., 1995). As with mutations in the U2–U6 helix Ib element, there is a marked asymmetry in the effects of single-base mutations in the D5 AGC:GUU element: mutations on the AGC side of the helix have stronger effects on activity. Similarly, studies using chemical modification–interference and phosphorothioate substitutions have shown that the AGC sequence and one or more of its nonbridging phosphate oxygens play a crucial role in D5 activity (Chanfreau & Jacquier, 1994). Photo-activated crosslinking experiments using 4-thioU residues in the D5 AGC:GUU element (M. Podar, C. Peebles, & P. Perlman, pers. comm.) have recently shown that both partners of the A2:U33 base pair in D5 (the apparent counterpart of A59:U23 in U6–U2 helix Ib) can crosslink to G588 of the intron. This is a particularly telling result with regard to the U2 snRNA:exon 2(+1) crosslinks described here because the adjacent position (A587) is one partner of the γ:γ' interaction that helps to define the 3′ splice site in group II introns. Overall, the parallels between helix Ib in U2–U6 snRNAs and the AGC:GUU helix in group II D5 make a strong case for the idea that these elements play a similar role in promoting the transesterification reactions in both systems.

Ultimately, a detailed description of the three-dimensional structure of the active site(s) of the spliceosome will be essential for understanding the molecular basis of catalysis. It is clear that multiple snRNA sequences come into close contact with the 5′ and 3′ splice sites in active spliceosomes. Added to this already crowded environment are several of the spliceosomal proteins, including PRP8 and its mammalian homologue, which have been shown by crosslinking to interact with substrate sequences close to the splice sites or branch site (Wyatt et al., 1992; MacMillan et al., 1994; Teigelkamp et al., 1995; Umen & Guthrie, 1995). Unravelling the contributions made by protein and RNA components to substrate recognition and catalysis in the spliceosome promises to be a formidable technical challenge.

MATERIALS AND METHODS

Site-specific labeling and RNA ligation

Synthetic pre-mRNAs containing single 4-thioU residues adjacent to the 5′ or 3′ splice site were made by ligation using T4 DNA ligase essentially according to Moore and Sharp (1992). Substrate sequences were derived from the yeast *CYH2* gene, after removal of 203 nt from the central region of the intron via site-directed mutagenesis (oligonucleotide 93-3866). The 3′ section of each synthetic pre-mRNA was made from a template produced by PCR, using T7 RNA polymerase transcription primed with UpG or 4-thioUpG (Sigma) at a UpG:GTP ratio of 5:1. The 5′ end of this RNA was phosphorylated using T4 polynucleotide kinase and γ-(^{32}P)-ATP (Amersham; 3,000 Ci/mmol). Ligation to an appropriate 5′ section of RNA was mediated by a synthetic "bridge" oligonucleotide (see Teigelkamp et al., 1995 for details). Ligated

pre-mRNA was purified by denaturing PAGE and electroelution (Amicon Centrilutor; Centricon C30 columns). Substrates made in this way have a single radioactive phosphate at the site of ligation, immediately 5′ of the 4-thioU residue.

Splicing extract preparation and in vitro splicing

Splicing reactions were carried out at 23 °C essentially according to Newman et al. (1985) using splicing extract prepared from *Saccharomyces cerevisiae* strain SC261 (*mat***a** *ura3-52 leu2 trp1 pep4-3 prb1-1132 prc1-407*). The final *CYH2* substrate concentration was typically 2 nM. Analytical scale reactions in some cases used an snRNP-enriched fraction of the whole-cell extract prepared by ammonium sulfate precipitation. Briefly, ground ammonium sulfate was slowly added to the clear supernatant from the 100,000 × *g* spin (0.246 g/mL of supernatant) and the mixture was stirred gently on ice for 30 min. The precipitate was collected by centrifugation (22,000 × *g* for 20 min at 4 °C) and redissolved in 0.1× original volume buffer D (20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA, 0.5 mM DTT, 50 mM KCl, 20% [v/v] glycerol). After dialysis against buffer D for 3 h at 4 °C, the solution was clarified by centrifugation (2 min at 15,800 × *g*, 4 °C) and frozen in liquid nitrogen in small aliquots.

UV crosslinking and RNaseH analysis

Photo-activated crosslinks were induced by irradiating (typically for 5 min) small droplets (2–25 μL) of splicing reactions on a parafilm-covered aluminium block chilled to 0–4 °C. The light source was a Blak-Ray B100AP ultraviolet lamp with 365-nm filter (UV Products). After irradiation, proteins were removed by addition of 0.25 volumes 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 was recovered by ethanol precipitation and crosslinked RNA species were visualized by autoradiography after fractionation on 5% or 6% polyacrylamide–8-M urea gels. snRNA components of putative crosslinked RNA species were identified by oligonucleotide-targeted RNAseH cleavage using oligonucleotides specific for individual spliceosomal snRNAs, as follows: RNA and oligonucleotide (2.5 μM) were annealed by slow cooling from 90 to 30 °C in 9 μL of a solution containing 40 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 20 ng/μL *Escherichia coli* MRE600 tRNA (Boehringer Mannheim); 1 U *E. coli* SK780 RNaseH (Pharmacia Biotech) was added and the reaction was incubated at 30 °C for 30 min. RNA was recovered by phenol:chloroform:isoamyl alcohol (50:50:1) extraction and ethanol precipitation, and analyzed by fractionation on 5% or 6% polyacrylamide–8-M urea gels.

RNA capture using biotinylated oligonucleotides

Specific RNA species were captured from deproteinized total RNA derived from UV-irradiated splicing reactions using 5′-biotinylated oligonucleotides and streptavidin-derivatized paramagnetic particles (Magnesphere Particles; Promega), as follows. RNA from a 100-μL splicing reaction was annealed to 12.5 pmol biotinylated oligonucleotide in 40 μL of solution containing 0.5× SSC, by slow cooling from 90 to 30 °C; 150 pmol streptavidin paramagnetic particles were added in 50 μL 0.5× SSC and the reaction was incubated at 20 °C for 30 min. The beads were captured magnetically and washed four times in 0.1× SSC. RNA bound to the beads was then recovered by elution in double distilled water at 37 °C followed by ethanol precipitation.

Crosslink mapping by primer extension

Crosslinks in U2 snRNA and U5 snRNA were mapped by primer extension blockage. Crosslinked species were gel purified or captured using specific 5′-biotinylated oligonucleotides and streptavidin-derivatized paramagnetic particles. RNA was then annealed to a specific 5′ end (^{32}P)-labeled primer by slow cooling from 95 to 37 °C in 7.5 μL of 1× rt buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.5, 0.5 mM EDTA, 8 mM $MgCl_2$); 1.5 μL of extension cocktail (1× rt buffer containing 100 mM DTT, 3 U RNasin/μL, and 0.25 U/μL AMV reverse transcriptase [Promega]) were added and 7 μL of the mixture were combined with 7 μL of a solution of all four dNTPs (800 μM each dNTP). Primer extension was at 41 °C for 15 min. The reaction was heated to 100 °C for 30 s and snap-chilled on ice. RNA was then digested by addition of 2 μL of RNase A (2.5 mg/mL) and incubation at 41 °C for 30 min. The reaction was stopped by addition of 4 μL of stop mix (1 mg/mL Proteinase K, 50 mM EDTA, 1% SDS) and incubation at 37 °C for 30 min. The reaction was diluted by addition of 65 μL of extraction buffer (50 mM sodium acetate, pH 5.3, 1 mM EDTA, 0.1% SDS, 25 μg/mL tRNA from *E. coli* MRE 600) and extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (50:50:1). Nucleic acids were ethanol-precipitated and analyzed by fractionation on 8% polyacrylamide–8-M urea gels. Reference sequence ladders were generated in parallel using total RNA prepared from 1 μL of splicing extract as template. Conditions were as described above, except that four separate primer extension reactions were performed with 400 μM ddTTP, ddCTP, ddGTP, or ddATP, respectively, in addition to 800 μM of each dNTP.

Synthetic DNA

Oligonucleotide 93-3866 was used to delete 203 nt from the center of the *CYH2* gene intron: 5′CAG CGA TAA TTA GTG CGT TCG CAA TCA AAA CAC.

The following oligonucleotides were used to synthesize pre-mRNAs labeled at position (−1) in exon 1: 93-1529, 5′TAA TAC GAC TCA CTA TA (T7 promoter top strand); 93-2596, 5′TGA GAC GTG ACC TCT GTG CTT TCT AGT CTT AGT GAA TCT GGA AGG CAT TCC TAT AGT GAG TCG TAT TA (template strand for 5′ transcript); 93-2597, 5′GCG CTA ATA CGA CTC ACT ATA GTA TGT AGT TCC ATT TGG AAG (forward PCR primer for 3′ transcript); 93-2598, 5′CTC TTC CAA ATG GAA CTA CAT ACA TGA GAC GTG ACC TCT GTG CTT (bridge oligonucleotide); 93-451, 5′AAG TAT CTC ATA CCA ACC TTA CCG (reverse PCR primer for 3′ transcript).

The following oligonucleotides were used to synthesize pre-mRNAs labeled at position (+1) in exon 2: 93-163, 5′GCG CTA ATA CGA CTC ACT ATA GAC TAG AAA GCA CAG AGG TC (forward PCR primer for 5′ transcript); 93-142, 5′CTG TAC AAA AAA AAT ATT GTA ATG AAA TAC (re-

verse PCR primer for 5′ transcript; CAG 3′ splice site); 94-4126, 5′GTG TAC AAA AAA AAT ATT GTA ATG AAA TAC (reverse PCR primer for 3′ transcript, CAC 3′ splice site); 93-174, 5′GCG CTA ATA CGA CTC ACT ATA GGC TAA GGG TAG AAT CGG TA (forward PCR primer for 3′ transcript); 93-1526, 5′TTA CCG ATT CTA CCC TTA GCC ACT GTA CAA AAA AAA TAT TGT AA (bridge oligonucleotide for CAG 3′ splice site); 94-4114, 5′TTA CCG ATT CTA CCC TTA GCC AGT GTA CAA AAA AAA TAT TGT AA (bridge oligonucleotide for CAG 3′ splice site).

The following oligonucleotides were used to synthesize pre-mRNAs labeled at position (−8) in exon 1: 94-4155, 5′TGG TAG TAG CGC TTT CTA GTC TTA GTG AAT CTG GAA GGC ATT CCT ATA GTG AGT CGT ATT A (template strand for 5′ transcript); 94-4156, 5′GCT AAT ACG ACT CAC TAT AGG ACC AGG TAT GTA GTT CCA (forward PCR primer for 3′ transcript); 94-4157, 5′TGG AAC TAC ATA CCT GGT CCA TGG TAG TAG CGC TTT CTA G (bridge oligonucleotide).

The following oligonucleotides were used for directing cleavage of specific RNA sequences by RNaseH: 93-4584, 5′TCT TAG TGA ATC TGG AAG G (*CYH2* exon 1); 94-4241, 5′CTG AGA CGT GAC CTC (*CYH2* exon 1); 93-3866, 5′CAG CGA TAA TTA GTG CGT TCG CAA TCA AAA CAC (*CYH2* intron); 90-484, 5′CTT ACC GAT ACG ACC TTT ACC GGC (*CYH2* exon 2); 94-2133, 5′AAA CTT CTC CAG GCA G (nt 263–278 of yeast U1 snRNA); 94-2126, 5′AAC AGA TAC TAC ACT T (nt 30–45 of yeast U2 snRNA); 94-2127, 5′AAG GTA ATG AGC CTC A (nt 76–91 of yeast U2 snRNA); 93-1309, 5′CGT TAT AAG TTC TAT AGG CA (nt 60–79 of yeast U5 snRNA); 93-1310, 5′CCT GTT TCT ATG GAG ACA ACA (nt 115–135 of yeast U5 snRNA; 94-1011, 5′TGC AGG GGA ACT GC (nt 60–73 of yeast U6 snRNA).

The following oligonucleotides have a single biotin residue at the 5′ end and were used for capturing specific RNA sequences via binding to streptavidin-conjugated paramagnetic particles: 95-038, 5′bioCA GCG ATA ATT AGT GCG TTC GCA ATC (*CYH2* intron); 94-6194, 5′bioAA GGT AAT GAG CCT CAT TGA GGT CAT (nt 66–91 of yeast U2 snRNA); 94-6170, 5′bioAT GGC AAG CCC ACA GTA ACG GAC AGC (nt 140–165 of yeast U5 snRNA).

The following oligonucleotides were used as primers for mapping crosslinks with reverse transcriptase: 92-1162, 5′AAG TCT CTT CCC TC CAT TTT A (nt 132–153 of yeast U2 snRNA); 95-2729, 5′GGG TGC CAA AAA ATG TGT (nt 107–124 of yeast U2 snRNA); 93-4696, 5′AAA AAT ATG GCA GGC CTA CAG TAA CGG (nt 145–171 of yeast U5 snRNA).

ACKNOWLEDGMENTS

We thank Kiyoshi Nagai for a gift of T7 RNA polymerase, the Oswel DNA service and the LMB oligonucleotide synthesis group for synthetic DNA, and Ray O'Keefe, Chris Norman, and Ingrid Kelly for suggesting improvements to the manuscript. We also thank Mircea Podar, Craig Peebles, and Phil Perlman for communicating their results to us prior to publication. This work was supported by the Medical Research Council. J.D.B. is supported by a Royal Society Cephalosporin Fund Senior Research Fellowship.

Received September 26, 1995; returned for revision October 17, 1995; revised manuscript received October 30, 1995

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