

Functional analysis of the U5 snRNA loop 1 in the second catalytic step of yeast pre-mRNA splicing

Raymond T.O'Keefe¹ and Andrew J.Newman²

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

¹Present address: School of Biological Sciences, 2.205 Stopford Building, University of Manchester, Manchester M13 9PT, UK

²Corresponding author
e-mail: newman@mrc-lmb.cam.ac.uk

The U5 snRNA loop 1 interacts with the 5' exon before the first step of pre-mRNA splicing and with the 5' and 3' exons following the first step. These U5–exon interactions are proposed to hold the exons in the correct orientation for the second step of splicing. Reconstitution of U5 snRNPs *in vitro* indicated that U5 loop 1–5' exon interactions are not necessary for the first catalytic step of splicing but are critical for the second step in yeast spliceosomes. We systematically made deletion and insertion mutations in loop 1 then monitored splicing activity and loop–exon interactions by cross-linking. Single nucleotide deletions or insertions in loop 1 permitted both steps of splicing. Larger insertions or deletions allowed the first step but progressively inhibited the second step. Analysis of selected loop 1 insertions and deletions by cross-linking revealed that inhibition of the second catalytic step resulted from misalignment of the 5' and 3' exons. These data indicate that the size of loop 1 is critical for proper alignment of the exons for the second catalytic step of splicing and that the 3' exon is positioned on loop 1 independently of the 5' exon.

Keywords: *in vitro* reconstitution/pre-mRNA splicing/spliceosome/U5 snRNA/yeast

Introduction

Pre-mRNA splicing is carried out within the cell nucleus by means of two transesterification reactions catalysed by a multicomponent complex termed the spliceosome (reviewed in Rymond and Rosbash, 1992; Moore *et al.*, 1993). The spliceosome is composed of small nuclear RNAs (snRNAs) and numerous proteins that associate with the pre-mRNA to facilitate the removal of introns. During splicing, conserved sequences within the introns which define the splice sites and the branchpoint are recognized by the splicing machinery and dictate the site of intron removal. There are five snRNAs (U1, U2, U4, U5 and U6) that are incorporated into the spliceosome as small nuclear ribonucleoprotein particles (snRNPs) and of these, U1, U2 and U6 are directly involved in recognizing the conserved sequences within the introns of pre-mRNA (reviewed in Madhani and Guthrie, 1994).

The recognition of the conserved intronic sequences begins through the base pairing of the 5' end of the U1 snRNA with the 5' splice site (Zhuang and Weiner, 1986; Séraphin *et al.*, 1988; Siliciano and Guthrie, 1988). This in turn leads to association of the U2 snRNA with the branchpoint sequence of the pre-mRNA (Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989), resulting in an RNA helix containing a bulged adenosine required for attack of the 5' splice site (Query *et al.*, 1994). The next step in spliceosome assembly is the incorporation of the U4, U5 and U6 snRNPs as a pre-assembled complex. As a result of this addition, a number of critical RNA–RNA interactions take place within the spliceosome which culminate in the removal of the intron and formation of the mRNA. Base pairing between the U4 and U6 snRNAs is dissolved and the U6 snRNA associates with the intron region adjacent to the 5' splice site (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992; Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993) while also directly contacting the U2 snRNA which is base-paired to the branchpoint region (Madhani and Guthrie, 1992; Sun and Manley, 1995; Field and Friesen, 1996). The U5 snRNA interacts with exon sequences at the 5' and 3' splice sites during splicing (Newman and Norman, 1992; Sontheimer and Steitz, 1993). Following the first step of splicing, the U2 snRNA has also been shown to interact with the first base of the exon at the 3' splice site (Newman *et al.*, 1995).

The U5 snRNA is essential for cell viability in yeast (Patterson and Guthrie, 1987; Séraphin *et al.*, 1991) and is required for mammalian *in vitro* splicing (Winkelmann *et al.*, 1989; Lamm *et al.*, 1991; Ségault *et al.*, 1995). In addition, genetic and cross-linking studies in both yeast and mammalian systems have implicated the invariant loop 1 sequence of the U5 snRNA as the region of U5 that interacts with exon sequences immediately adjacent to the 5' and 3' splice sites (Newman and Norman, 1991, 1992; Wyatt *et al.*, 1992; Cortes *et al.*, 1993; Sontheimer and Steitz, 1993; Newman *et al.*, 1995). These studies revealed that before the first step of splicing, the loop 1 sequence associates with the exon sequences at the 5' splice site. Following the first step of splicing, this interaction continues while an additional interaction of loop 1 occurs with the exon sequences at the 3' splice site. This information has been incorporated into a model for the role of the U5 snRNA loop 1 sequence in splicing which proposes that the U5 loop 1 acts to tether the exons in the correct orientation for the second catalytic step of splicing (Newman and Norman, 1992; Sontheimer and Steitz, 1993). Recently, through the development of a yeast *in vitro* depletion/reconstitution system for the U5 snRNP, we have been able to investigate the role of the exon interactions with the U5 snRNA during the two steps

of splicing. We have shown that in yeast spliceosomes the U5 snRNA loop 1 is not required for the first catalytic step of splicing but is critical for the second catalytic step (O'Keefe *et al.*, 1996). Although the U5 loop 1–5' exon interaction is itself dispensable for the first catalytic step, this association is established prior to the first step presumably in order to retain the 5' exon intermediate in the active site of the spliceosome for the second catalytic step.

Given that the U5 snRNA loop 1 is not required for the first catalytic step of splicing, we have directed our attention to the role of this sequence in the second catalytic step. We have varied systematically the size of the U5 loop 1 sequence and monitored splicing activity as well as loop 1–exon interactions by cross-linking to define the requirements for loop 1 in the second catalytic step of splicing. It was found that deletion or insertion of one nucleotide within the loop 1 sequence is tolerated by the splicing machinery and permits both steps of splicing to occur *in vitro*. Larger deletions or insertions within loop 1, however, progressively inhibit the second step of splicing. Cross-linking revealed that some deletion and insertion mutants still interact with both exons but the exons are mis-positioned with respect to each other on the mutant loop 1. This misalignment of the exons provides an explanation for the second step defect of these mutants. Overall, these data indicate that the size of the U5 snRNA loop 1 is critical for the proper alignment of the two exons for the second catalytic step of splicing.

Results

Splicing activity of U5 loop 1 deletions and insertions assayed by *in vitro* reconstitution

Previously, we have assayed U5 snRNA loop 1 deletions and mutations by *in vitro* reconstitution of U5 snRNPs, concluding that the loop 1 sequence is not required for the first catalytic step of splicing and that both steps of splicing are tolerant of drastic loop 1 primary sequence changes (O'Keefe *et al.*, 1996). Deletion of five or more nucleotides from loop 1, as well as deletion of all of loop 1 and replacement with different tetra loops, allowed the first step of splicing but severely inhibited the second step by failure to interact with the 5' exon. In contrast, any sequence changes made in loop 1 that still retained the wild-type loop size were able to carry out both steps of splicing. This led us to the idea that the size of the U5 loop 1, rather than its sequence, may be the important factor in determining its ability to participate in the second catalytic step of splicing. To pursue this idea, we have made additional deletions and insertions within the loop 1 sequence (Figure 1) to determine their effect on the second catalytic step of pre-mRNA splicing *in vitro*. Splicing is inhibited by depletion of U5 snRNA (Figure 2A and B, lane 1), and addition of *in vitro* transcribed wild-type U5 snRNA (wt) to U5-depleted extract can reconstitute both steps of splicing efficiently (Figure 2A and B, lane 2) (O'Keefe *et al.*, 1996). In addition, deletion of the invariant U5 snRNA loop 1 sequence (Del. 1–9) allows the first step of splicing to occur but severely inhibits the second catalytic step (Figure 2A and B, lane 3) (O'Keefe *et al.*, 1996). However, deletion of only one nucleotide from different locations within the U5 loop 1

sequence (Del. G1, C2, U4, A8 and C9) allows both steps of splicing to occur (Figure 2A, lanes 4–8). Phosphor-imaging quantitation of the splicing activity of these single nucleotide deletions (Table I) reveals that their ability to carry out both steps of splicing is similar to, or slightly less than, that of the wild-type *in vitro* transcribed U5 snRNA.

Deletion of two nucleotides from loop 1 in two different locations (Del. C2,3 and Del. U4,5) also allows both steps of splicing to occur (Figure 2A, lanes 9 and 12). In this case, however, quantitation of splicing activity reveals that there is partial inhibition of the second step of splicing (Table I). Deletion of three (Del. C2,3,U4 and Del. U4,5,6) or four (Del. C2,3,U4,5 and Del. U4,5,6,7) nucleotides from loop 1 in two different locations drastically inhibits the second step of splicing (Figure 2A, lanes 10, 11, 13 and 14). Quantitation of splicing activity and comparison of the step 1/step 2 ratio (Table I) confirm the observed second step inhibition for these deletions. It is evident from this deletion analysis of the U5 loop 1 that single nucleotide deletions can be tolerated by the splicing machinery and allow both steps of splicing to take place. Deletion of two or more nucleotides from loop 1, however, results in the progressive inhibition of the second step, indicating that a smaller U5 loop 1 is defective for the second catalytic step of splicing.

To determine whether increasing the size of loop 1 would have any effect on the second step of splicing, we made insertions within the U5 loop 1 sequence. Addition of one uridine into the loop 1 sequence between positions 3 and 4 (Ins. 1U 3/4) allows both steps of splicing to occur (Figure 2B, lane 4). Quantitation of the splicing activity of this single nucleotide insertion (Table I) reveals that the ability of this mutant to reconstitute both steps of splicing is slightly less than that of wild-type *in vitro* transcribed U5 snRNA though similar to that of the single nucleotide deletions. Addition of two (Ins. 2U 3/4), three (Ins. 3U 3/4) or four (Ins. 4U 3/4) uridines into the loop 1 sequence between positions 3 and 4 allows the first step of splicing but results in the progressive inhibition of the second step of splicing (Figure 2B, lanes 5, 6, and 7). Quantitation of splicing activity and comparison of the step 1/step 2 ratio (Table I) confirm the observed inhibition of the second step for these insertions.

Insertion of three uridines at two different positions in loop 1, 5' and 3' of the nine nucleotide invariant loop sequence, results in very different splicing activities. Insertion of three uridines 3' of the invariant loop sequence (Ins. 3U 3') allows the first step of splicing but severely inhibits the second step (Figure 2B, lane 9). Insertion of three uridines 5' of the invariant loop sequence (Ins. 3U 5'), on the other hand, allows both steps of splicing to occur (Figure 2B, lane 8) at a slightly lower level than wild-type *in vitro* transcribed U5 (Table I). It appears, therefore, that insertions within the 5' region of loop 1 sequence may be tolerated by the splicing machinery whereas insertions within the middle and 3' regions of loop 1 prevent the second step of splicing.

To test further the idea that insertions within the 5' side of loop 1 may be tolerated by the splicing machinery, two additional U5 loop 1 insertion mutants were produced. The insertion mutant (Ins. 3U 5') creates a run of five uridine residues, so it is possible that the additional uridine residues become bulged from stem 1, leaving loop 1

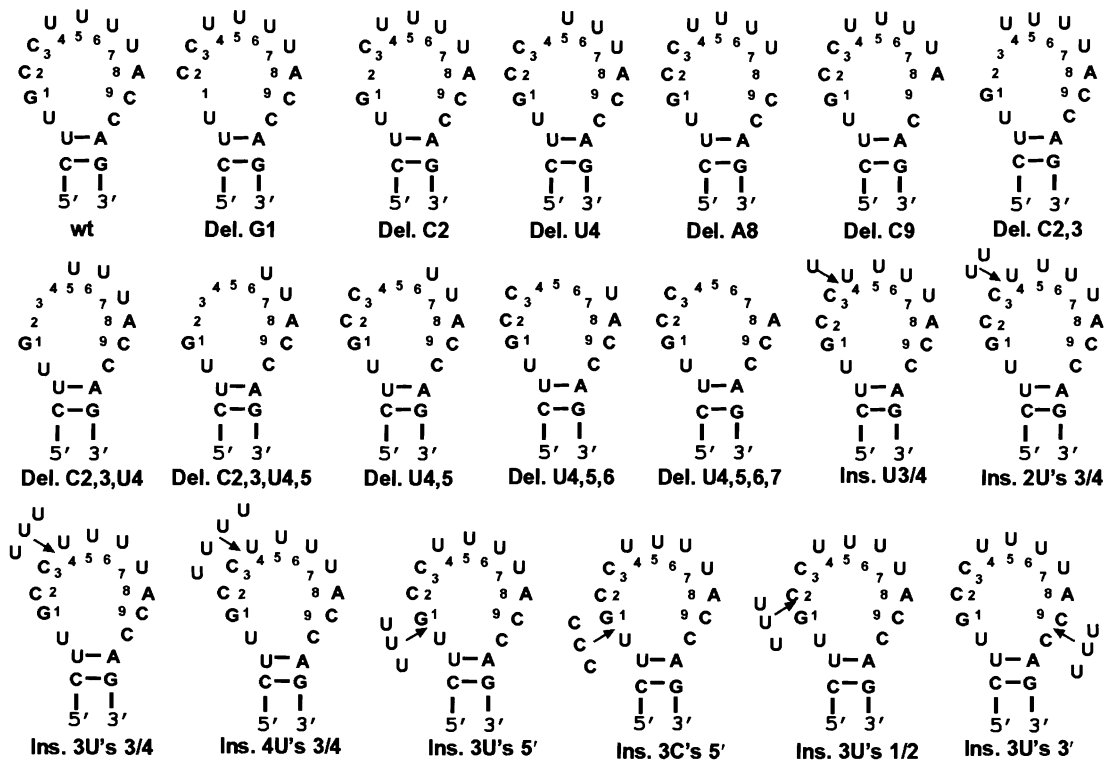


Fig. 1. Diagram of U5 snRNA loop 1 mutants. Comparison of mutations made in U5 snRNA loop 1 with the predicted secondary structure of the wild-type U5 snRNA stem-loop 1. Only nucleotides 90–104 of the *S.cerevisiae* U5 snRNA are shown in this diagram with the nine nucleotide invariant sequence of loop 1 numbered.

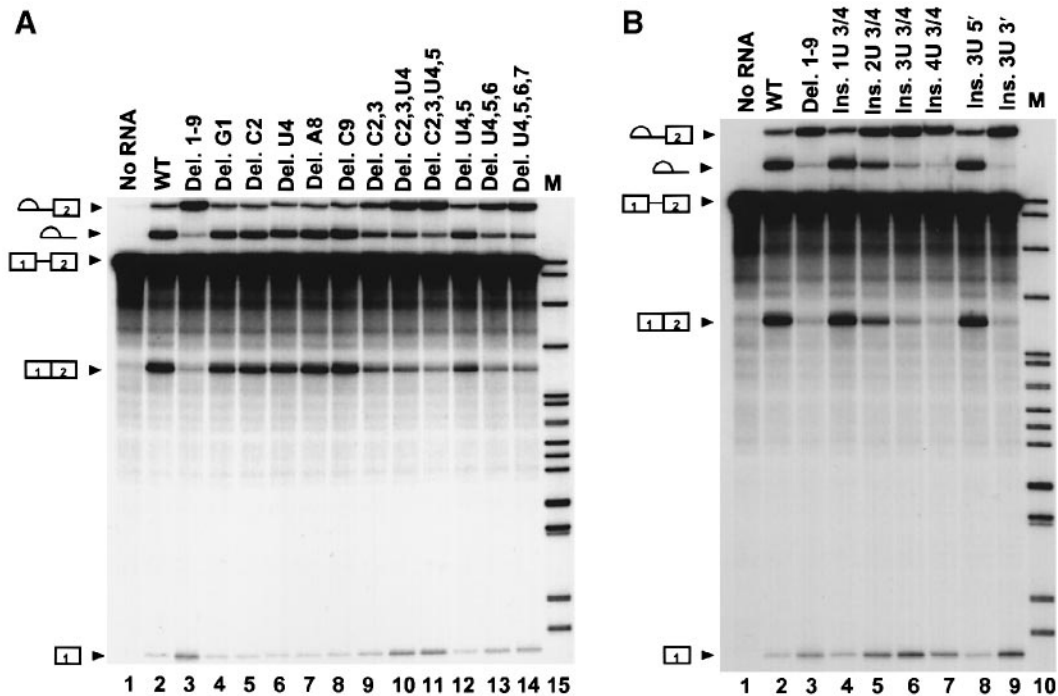


Fig. 2. *In vitro* splicing activity of U5 snRNA loop 1 mutants. Extract depleted of U5 snRNA was reconstituted with different U5 snRNA mutants. Synthetic actin pre-mRNA was added to assay the splicing activity. **(A)** Reconstitution with no RNA (lane 1), *in vitro* transcribed U5 snRNA (WT) (lane 2) or the indicated *in vitro* transcribed deletions (lanes 3–14). pBR322 *Msp*I end-labelled size markers are shown in lane 15. **(B)** Reconstitution with no RNA (lane 1), *in vitro* transcribed U5 snRNA (wt) (lane 2), an *in vitro* transcribed loop 1 deletion (Del. 1–9) (lane 3) or the indicated *in vitro* transcribed insertions (lanes 4–9). pBR322 *Msp*I end-labelled size markers are shown in lane 10. The positions of the *in vitro* transcribed actin pre-mRNA, its splicing intermediates and products are schematically represented on the left side of each panel.

Table I. Quantitation of *in vitro* splicing activity

U5 mutants	Step 1	Step 2	Total splicing	Step 1/step 2
T7-U5 (WT)	0.19	0.81	1.00	0.23
Del. 1-9	0.52	0.13	0.65	4.00
Del. G1	0.15	0.49	0.64	0.31
Del. C2	0.17	0.63	0.80	0.27
Del. U4	0.16	0.60	0.76	0.27
Del. A8	0.16	0.66	0.82	0.24
Del. C9	0.20	0.92	1.12	0.22
Del. C2,3	0.25	0.29	0.54	0.86
Del. C2,3,U4	0.53	0.26	0.79	2.04
Del. C2,3,U4,5	0.66	0.22	0.88	3.00
Del. U4,5	0.17	0.40	0.57	0.43
Del. U4,5,6	0.35	0.21	0.56	1.67
Del. U4,5,6,7	0.40	0.21	0.61	1.90
Ins. 1U 3/4	0.22	0.72	0.94	0.31
Ins. 2U 3/4	0.51	0.37	0.88	1.38
Ins. 3U 3/4	0.66	0.09	0.75	7.33
Ins. 4U 3/4	0.46	0.04	0.50	11.5
Ins. 3U 1/2	0.82	0.07	0.89	11.7
Ins. 3U 5'	0.18	0.62	0.80	0.29
Ins. 3C 5'	0.28	0.55	0.83	0.51
Ins. 3U 3'	0.44	0.02	0.46	22.0

Quantitation is the result of data obtained from a Molecular Dynamics Phosphorimager and is the average of three experiments similar to Figures 2 and 3. The relative values presented in the table have been normalized for the amount of substrate in each lane and the background of the depletion lane has been subtracted. The value of total splicing activity for U5 WT has been set arbitrarily at 1.00.

unchanged in size, and therefore allowing the second step to proceed efficiently. To address this possibility, we made the additional mutant (Ins. 3C 5') which has three cytidines in place of the three uridines. Analysis of the splicing activity of this mutant reveals that insertion of three cytidines 5' of the invariant loop sequence (Ins. 3C 5') still allows both steps of splicing to occur (Figure 3, lane 4), similar to three uridines inserted at this position. The other mutant (Ins. 3U 1/2) inserts three uridines at the next point along the loop between positions 1 and 2 of the invariant loop sequence in order to determine whether the specific position of insertion had any effect on the second step of splicing. Analysis of the splicing activity of this mutant reveals that insertion of three uridines at the next position along the loop (Ins. 3U 1/2) allows the first step of splicing but severely inhibits the second step (Figure 3, lane 5). In summary, insertions 5' of position 1 in the invariant loop sequence (Ins. 3U 5' and Ins. 3C 5') allow the second step of splicing, whereas insertions 3' of this position (Ins. 3U 1/2, Ins. 3U 3/4 and Ins. 3U 3') block the second step of splicing. In other words, position 1 of the invariant loop sequence is the boundary beyond which insertions in the loop inhibit the second catalytic step of splicing.

Interaction of U5 loop 1 deletion and insertion mutants with the 5' exon

To determine the cause of the second catalytic step inhibition displayed by certain U5 deletion and insertion mutants, the interactions between such mutants and the 5' exon were investigated by UV cross-linking. The formation of a cross-link between two molecules indicates that these molecules are in close proximity and may functionally interact. Cross-linking, however, may not detect all interactions, and the absence of a cross-link

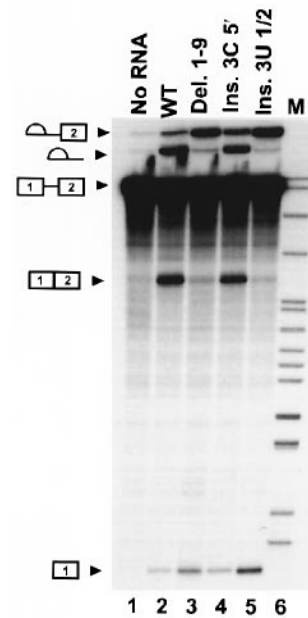


Fig. 3. *In vitro* splicing activity of additional U5 snRNA loop 1 insertion mutants. Extract depleted of U5 snRNA then reconstituted with no RNA (lane 1), *in vitro* transcribed U5 snRNA (WT) (lane 2), an *in vitro* transcribed loop 1 deletion (Del. 1-9) (lane 3) or the indicated *in vitro* transcribed insertions (lanes 3 and 4). pBR322 *MspI* end-labelled size markers are shown in lane 10. The positions of the *in vitro* transcribed actin pre-mRNA, its splicing intermediates and products are schematically represented on the left side of each panel.

does not necessarily indicate that two molecules do not interact. We had found previously that deletion of five or more nucleotides from loop 1 prevented the second catalytic step of splicing (O'Keefe *et al.*, 1996). These mutations also abolished cross-link formation between U5 and the 5' exon, suggesting that inhibition of the second catalytic step resulted from failure to tether the 5' exon intermediate properly. To determine whether a similar situation brought about the inhibition of the second catalytic step with our current mutants, these mutants were added to U5-depleted extract then splicing was initiated with *CYH2* pre-mRNA containing a 4-thiouridine (4-thio-U) at the last nucleotide of the 5' exon (position -1 in exon 1). RNA isolated from splicing reactions following UV irradiation was deproteinized, and the U5-containing species were captured using a biotinylated oligonucleotide complementary to the U5 snRNA and streptavidin-conjugated paramagnetic beads. As an internal reference for the capturing procedure, a biotinylated oligonucleotide complementary to the U1 snRNA was used simultaneously with the U5 biotinylated oligonucleotide to capture the known U1-pre-mRNA cross-links. UV-irradiated reconstitution reactions containing *in vitro* transcribed wild-type U5 incubated with U5 and U1 biotinylated oligonucleotides simultaneously captured three cross-linked species (Figure 4A, lane 1). These correspond to the previously characterized cross-links between U5 snRNA and the pre-mRNA, U5 snRNA and the exon 1 splicing intermediate, and U1 snRNA and the pre-mRNA (Newman *et al.*, 1995; O'Keefe *et al.*, 1996). Deletion of the loop 1 sequence from U5 (Del. 1-9) did not affect the U1-pre-mRNA cross-link but prevented formation of the U5-pre-mRNA and U5-exon 1 cross-links (Figure 4A, lane 2), indicating,

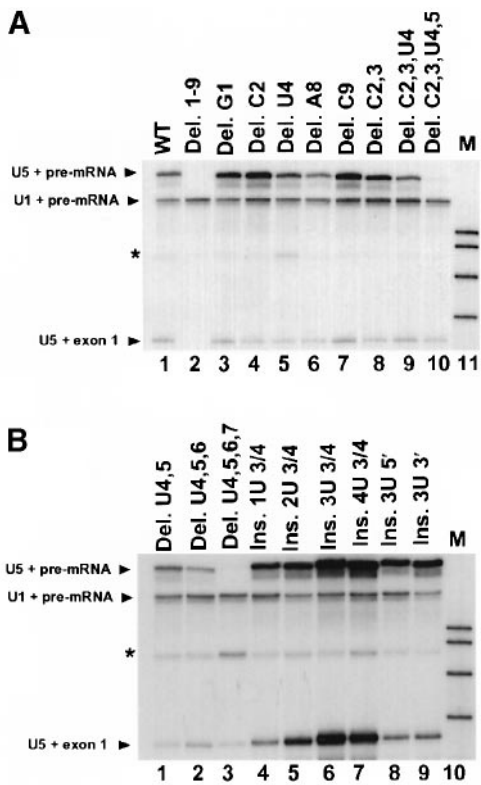


Fig. 4. Cross-linking between U5 snRNA loop 1 mutants and the 5' exon. Reconstitution with different U5 snRNA mutants and pre-mRNA containing a 4-thio-U residue at position -1 in exon 1. RNA species containing U5 and U1 snRNAs were selected from UV-irradiated reactions with biotinylated oligonucleotides and streptavidin paramagnetic particles. (A) Reconstitution with *in vitro* transcribed U5 snRNA (WT) (lane 1), loop 1 deletion (Del. 1–9) (lane 2), one nucleotide loop 1 deletions (lanes 3–7), two nucleotide loop 1 deletion (lane 8), three nucleotide loop 1 deletion (lane 9) and four nucleotide loop 1 deletion (lane 10). pBR322 *MspI* end-labelled size markers are shown in lane 11. (B) Reconstitution with *in vitro* transcribed two nucleotide loop 1 deletion (lane 1), three nucleotide loop 1 deletion (lane 2), four nucleotide loop 1 deletion (lane 3), one nucleotide loop 1 insertion (lane 4), two nucleotide loop 1 insertion (lane 5), three nucleotide loop 1 insertion (lane 6), four nucleotide loop 1 insertion (lane 7), three nucleotide loop 1 insertion 5' (lane 8) and three nucleotide loop 1 insertion 3' (lane 9). pBR322 *MspI* end-labelled size markers are shown in lane 10. The various cross-linked species are indicated at the left of each panel. Asterisks indicate background pre-mRNA captured with streptavidin paramagnetic particles.

as observed previously, that this U5 mutation prevents the U5–exon 1 interaction and, therefore, inhibits the second catalytic step of splicing (O'Keefe *et al.*, 1996). Deletions of one nucleotide from different locations within the U5 loop 1 sequence (Del. G1, C2, U4, A8 and C9), which allow both steps of splicing to occur, produced the same cross-linked species found with *in vitro* transcribed wild-type U5 (Figure 4A, lanes 3–7). Deletions of two nucleotides from loop 1 in two different locations (Del. C2,3 and Del. U4,5), which allow both steps of splicing to occur but have a slight inhibition of the second catalytic step, again produce the same cross-linked species as found with wild-type U5 (Figure 4A, lane 8, and 4B, lane 1). Surprisingly, even when the exon 1 interactions are investigated for the three nucleotide deletions (Del. C2,3,U4 and Del. U4,5,6) that show a substantial inhibition of the second catalytic step, there are still interactions between these U5 mutants and exon 1 before and after

the first step of splicing (Figure 4A, lane 9 and 4B, lane 2). When four nucleotides are deleted from loop 1 (Del. C2,3,U4,5 and Del. U4,5,6,7), the U5–pre-mRNA cross-linked species is of very low abundance (Figure 4A, lane 10 and 4B, lane 3), which may explain, in part, the inhibition of the second catalytic step with these mutants. The finding that the deletion mutations still interact with the 5' exon indicates that the second step defect displayed by some of these mutants is not a result of their inability to interact with exon 1.

Analysis by cross-linking of all the insertion mutations for interaction with position -1 of exon 1 in the pre-mRNA and the exon 1 intermediate revealed that these cross-linked species occur even with the insertions that severely block the second step of splicing (Figure 4B, lanes 4–9). Therefore, the second step defect found with some of these insertion mutations is not due to the inability to interact with the 5' exon, but to some other defect. In fact there is a dramatic increase in cross-link efficiency/yield with a number of these insertion mutants. Currently it is not clear why, or how, this increase occurs.

Mapping the interactions of U5 loop 1 deletions and insertions with the 5' and 3' exons

To determine the molecular basis of the second catalytic step defect of selected U5 deletions and insertions that still appear to interact with the 5' exon (position -1 in exon 1), we have mapped a number of these cross-links by primer extension. In addition, we have mapped the cross-links between the same U5 mutants and the 3' exon (position $+1$ in exon 2) following the first step of splicing to determine the position of both exons in relation to each other before the second step of splicing. Extract depleted of U5 snRNA was reconstituted with a U5 deletion or insertion mutant and incubated with *CYH2* pre-mRNA with a 4-thio-U in the 5' exon (position -1 in exon 1) or the 3' exon (position $+1$ in exon 2). RNA isolated following UV irradiation was captured using a biotinylated oligonucleotide complementary to the U5 snRNA. The captured RNA was then subjected to electrophoresis, the cross-linked species were electroeluted from the gel, and this RNA was used for primer extension analysis with an end-labelled primer specific for the 3' end of the U5 snRNA. The cDNAs resulting from blocked reverse transcription one position from a cross-linked nucleotide were then displayed next to a sequence ladder for the appropriate U5 mutant.

We first analysed the 5' exon cross-links (position -1) in the pre-mRNA before the first step of splicing and in the exon 1 intermediate following the first step of splicing for two insertion mutations that blocked the second step of splicing. With these U5 insertion mutants, one with three uridines inserted in the middle of loop 1 between positions 3 and 4 (Ins. 3U 3/4) and one with three uridines inserted on the 3' side of loop 1 (Ins. 3U 3'), the major cross-links to exon 1 (position -1) in the pre-mRNA were to positions U1 and U2 of loop 1 (Figure 5A and B, top panel). These cross-links are in positions relative to the 5' end of the invariant loop sequence similar to those found with wild-type U5 snRNA (Newman *et al.*, 1995). Following the first step of splicing, the positioning of the two U5 insertion mutants relative to exon 1 (position -1) is maintained (Figure 5A and B, middle panel), again

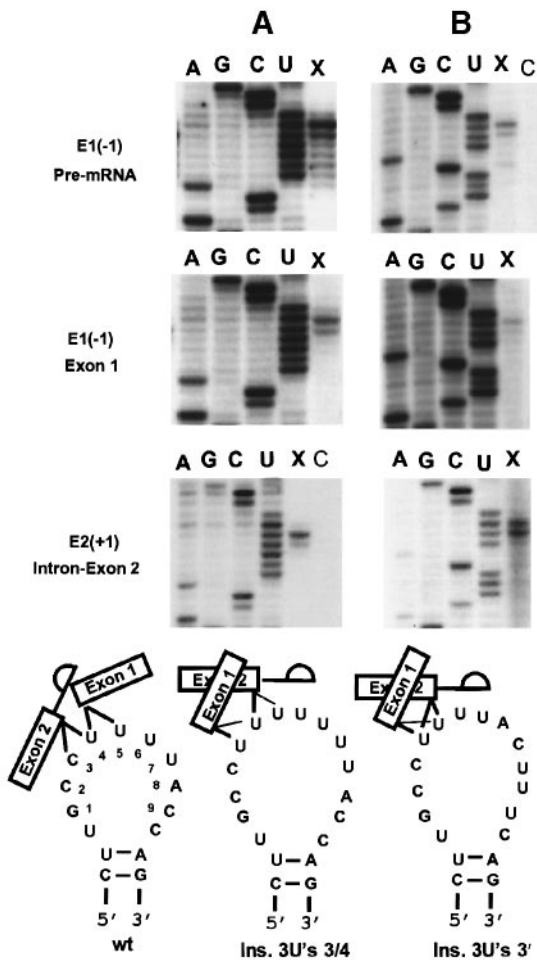


Fig. 5. Primer extension mapping of cross-links between the 5' and 3' exons and U5 snRNA insertion mutants. The U5-containing cross-linked species from UV-irradiated reconstitution reactions using pre-mRNA with 4-thio-U at position -1 in exon 1 or position +1 in exon 2 were isolated with a U5-specific biotinylated oligonucleotide and streptavidin paramagnetic particles. Gel-purified cross-linked species were used as templates for primer extension with an end-labelled U5-specific oligonucleotide. (A) Reconstitution with the U5 insertion mutant (Ins. 3U 3/4). The cross-links (X) between this mutant and position -1 in the pre-mRNA (top panel), position -1 in the exon 1 intermediate (middle panel) and position +1 in the intron-exon 2 intermediate (bottom panel). A schematic representation of these cross-links is displayed below the panels. Dideoxynucleotide sequencing tracks produced using *in vitro* transcribed (Ins. 3U 3/4) RNA are displayed as reference for primer extension mapping. (B) Reconstitution with the U5 insertion mutant (Ins. 3U 3'). The site of cross-links (X) between this mutant and position -1 in the pre-mRNA (top panel), position -1 in the exon 1 intermediate (middle panel) and position +1 in the intron-exon 2 intermediate (bottom panel). A schematic representation of these cross-links is displayed below the panels. Dideoxynucleotide sequencing tracks produced using *in vitro* transcribed (Ins. 3U 3') RNA are displayed for primer extension mapping. Control lanes (A, bottom panel, and B, top panel) from UV-irradiated reconstitution reactions containing no added U5 snRNA indicate there is no background cross-linking with 4-thio-U containing pre-mRNAs in a U5-depleted extract.

similar to the situation found with the wild-type U5 snRNA. On the other hand, the positioning of the 3' exon following the first step of splicing, as revealed by cross-linking of these insertion mutants to exon 2 (position +1) of the lariat intermediate, is quite different from that found with wild-type U5. With these mutants, the cross-links to exon 2 (position +1) have shifted approximately two

nucleotides (Ins. 3U 3/4) and one nucleotide (Ins. 3U 3') on the loop relative to those given by wild-type U5 (Figure 5A and B, bottom panel). The consequence of this shift in the position of exon 2 with these U5 insertion mutants is that the 3' hydroxyl of the exon 1 intermediate is now misaligned with the 3' splice site, thereby providing an explanation for the inhibition of the second step with these mutants.

We also determined the sites of cross-linking to the 5' and 3' exons for two U5 loop 1 deletion mutants (Del. U4,5,6 and Del. C2,3,U4) that inhibited the second catalytic step but still interacted with the 5' exon at position -1 in exon 1. We mapped the U5 snRNA cross-links to the pre-mRNA (position -1 in exon 1) before the first step of splicing and to the exon 1 intermediate following the first step of splicing. The cross-links to pre-mRNA (position -1) for each mutant were found to be distributed widely over a region of the remaining loop 1 sequence. In one case, the cross-links between the pre-mRNA and the deleted loop (Del. U4,5,6) were clustered in a region near the position of the remaining U in the loop but also in a region of two Us which were 5' of this position (Figure 6A, top panel). In the other case, the cross-links in the pre-mRNA to the deleted loop (Del. C2,3,U4) were clustered in the region of the three remaining Us in the loop but again also in a region of two Us which were 5' of this position (Figure 6B, top panel). Following the first step of splicing, for both U5 deletion mutants (Del. U4,5,6 and Del. C2,3,U4) the exon 1 intermediate appears to be tethered to a more restricted region of the deleted loops (Figure 6A and B, middle panel). To compare the positioning of the 5' exon with the 3' exon before the second step of splicing in these U5 deletion mutants, cross-links to exon 2 (position +1) of the lariat intermediate were mapped. These cross-links were very difficult to map because of their low abundance and they required enhancement to visualize the primer extension stops (Figure 6A and B, bottom panel). In both cases, however, the positions of these weak cross-links between exon 2 (position +1) in the lariat intermediate and the U5 mutants (Del. U4,5,6 and Del. C2,3,U4) were separated from the position of the 5' exon cross-links by approximately one nucleotide (Figure 6A and B, bottom panel). This, along with the low abundance of these cross-links, could explain the step two defect of these two U5 deletion mutants. In this situation, when the U5 loop 1 is three nucleotides smaller than the wild-type loop, the exons are held one nucleotide apart on the loop, possibly preventing the attack of the 3' splice site by the 3' hydroxyl of exon 1 and consequently blocking the second step of splicing.

Discussion

We have investigated the role of the U5 snRNA loop 1 in the second catalytic step of pre-mRNA splicing. By mutating the loop 1 region and monitoring splicing activity, it was found that small insertions or deletions of one nucleotide within loop 1 were tolerated by the splicing machinery, whereas increasingly larger changes resulted in the progressive inhibition of the second step of splicing. Analysis of the exon interactions of selected loop 1 mutants by cross-linking to the 5' or 3' exon revealed an explanation for the second step defects. Deletion or

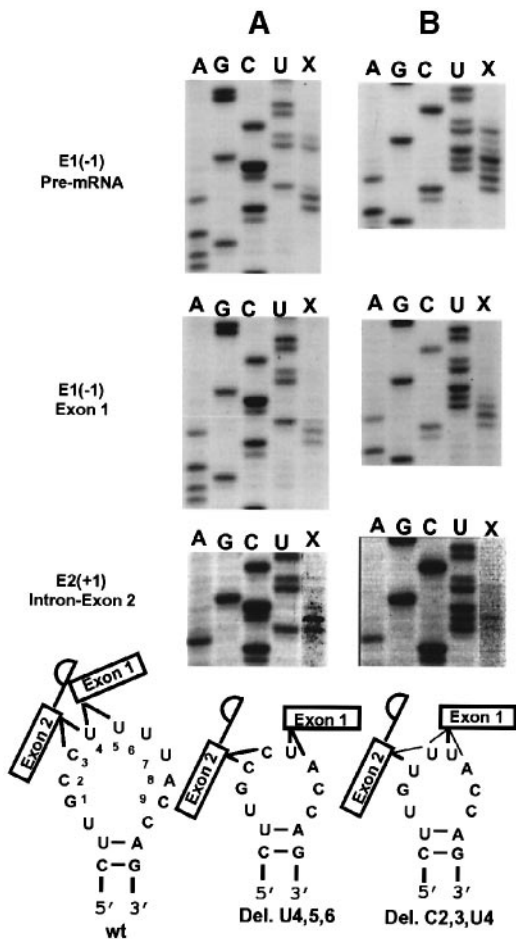


Fig. 6. Primer extension mapping of cross-links between the 5' and 3' exons and U5 snRNA deletion mutants. The U5-containing cross-linked species from UV-irradiated reconstitution reactions using pre-mRNA with 4-thio-U at position -1 in exon 1 or position +1 in exon 2 were isolated with a U5-specific biotinylated oligonucleotide and streptavidin paramagnetic particles. Gel-purified cross-linked species were used as templates for primer extension with an end-labelled U5-specific oligonucleotide. (A) Reconstitution with the U5 deletion mutant (Del. U4,5,6). The cross-links (X) between this mutant and position -1 in the pre-mRNA (top panel), position -1 in the exon 1 intermediate (middle panel) and position +1 in the intron-exon 2 intermediate (bottom panel). A schematic representation of these cross-links is displayed below the panels. Dideoxynucleotide sequencing tracks produced using *in vitro* transcribed (Del. U4,5,6) RNA are displayed as reference for primer extension mapping. (B) Reconstitution with the U5 deletion mutant (Del. C2,3,U4). The site of cross-links (X) between this mutant and position -1 in the pre-mRNA (top panel), position -1 in the exon 1 intermediate (middle panel) and position +1 in the intron-exon 2 intermediate (bottom panel). A schematic representation of these cross-links is displayed below the panels. Dideoxynucleotide sequencing tracks produced using *in vitro* transcribed (Del. C2,3,U4) RNA are displayed as reference for primer extension mapping.

insertion of three nucleotides at certain locations within loop 1 resulted in the misalignment of the exons before the second step of splicing. These results indicate that the size of the U5 snRNA loop 1 is important for the proper alignment of the exons for the second catalytic step of splicing.

The misalignment of the exons found with all the deletion and insertion mutants investigated appears to occur in a predictable manner. Deletion mutants resulted in the exons cross-linking to positions in the remaining

loop that are non-contiguous. This holds the exons apart, preventing the attack of the 3' splice site in the lariat-exon 2 intermediate by the 3' hydroxyl of exon 1. Insertion mutants resulted in the exons cross-linking to positions in the larger loop that were shifted in the opposite direction to that found with the deletion mutants. This held the exons in a crossed over, or overlapping, configuration, again preventing the attack of the 3' splice site in the lariat-exon 2 intermediate by the 3' hydroxyl of exon 1. Therefore, it appears that, in general, deletion mutants that block the second step of splicing hold the exons apart and insertion mutants that block the second step of splicing cross over, or overlap, the exons.

Our results indicate that the positioning of the exons on the U5 loop 1 is related to the size of loop 1. This suggests a possible mechanism in which the exons are positioned with respect to the U5 loop at a particular distance from a fixed point or points within the U5 snRNA. For example, following the first step of splicing, a cross-link to exon 1 is positioned consistently on the U5 loop three nucleotides from the invariant guanosine in all cases investigated so far. The only exception to this idea is when loop 1 is changed to its reverse complement (see O'Keefe *et al.*, 1996) which does not alter the positioning of exon 1 relative to the loop. This indicates that the primary sequence of loop 1 may not be the sole determinant of the exon 1-binding site. At this time, we have not defined a positioning mechanism used to determine the lariat-exon 2 intermediate-binding site on the U5 loop 1. Further analysis will be required to understand the molecular basis of the positioning of this intermediate for the second catalytic step.

We have made two mutants where, strictly speaking, our tenet that the size of loop 1 is important for the second catalytic step of splicing does not hold. Insertions 5' of position 1 in the invariant loop sequence (Ins. 3U 5' and Ins. 3C 5') allow the second step of splicing, even though, in theory, they should make the loop 1 larger. It is not known, however, what the actual size of loop 1 is when it is within the spliceosome. The secondary structure of the U5 snRNA has been predicted by phylogenetic and chemical modification probing analysis (Krol *et al.*, 1981; Branlant *et al.*, 1983; Black and Pinto, 1989; Bach and Lührmann, 1991; Frank *et al.*, 1994). What is evident from these studies is that the U5 snRNA is composed of a highly conserved stem-loop structure that contains the predicted 11 nucleotide loop 1 sequence. However, there are numerous proteins associated with the U5 snRNA which might influence the structure of loop 1 within the spliceosome. Therefore, one cannot predict with absolute accuracy the structure within the spliceosome of the wild-type loop 1 or the insertion and deletion mutants of loop 1. One must assume that the changes made in loop 1 would make the loop larger or smaller to some degree, based on the predicted secondary structure. However, it appears that insertions 5' of position 1 in the invariant loop sequence (Ins. 3U 5' and Ins. 3C 5') do not have a dramatic effect on splicing, indicating that insertions in this region are not detrimental to proper exon alignment.

We have not exhausted the possible mutants that could be made within the U5 loop 1 sequence. So far, the combination of deletion mutants described in this study

and our previous study (O'Keefe *et al.*, 1996) reveals that as the loop 1 sequence is gradually made smaller two different effects are evident. At first, when small deletions are made in loop 1, the exons are able to interact with the smaller loop but they become misaligned, resulting in the inhibition of the second step of splicing. However, as loop 1 is made even smaller, the ability to interact with exon 1 decreases until five or more nucleotides are deleted, at which point these mutants are unable to interact with exon 1 (O'Keefe *et al.*, 1996). Insertion mutants, however, seem to have an opposite effect on the ability to interact with exon 1. Our observations presented here indicate that when loop 1 is made bigger the interactions with exon 1 appear to be more efficient, at least up to four nucleotides. It is not known at this time what effect more than four nucleotides inserted within loop 1 will have on exon 1 interactions.

We can now only speculate as to the mechanisms by which the splicing machinery aligns the exons with respect to the U5 snRNA loop 1. The data presented here do reveal that the 5' and 3' exons are positioned on the U5 loop independently of each other. In other words, the positioning of the 5' exon does not influence the subsequent positioning of the 3' exon. Clearly there must be a complex interplay between both RNA and protein factors that dictates the alignment of the exons with the U5 loop 1. Discovering how the U5 snRNP interacts with the other components of the spliceosome to position the U5 snRNA loop 1 with the exon sequences at the 5' and 3' splice sites may provide information as to the mechanism of exon alignment.

Little information is available about how the U5 snRNA interacts with the other snRNAs during splicing. An interaction between loop 1 of the mammalian U5 snRNA and the U1 snRNA has been detected by psoralen cross-linking before the first catalytic step in mammalian spliceosomes (Ast and Weiner, 1997). This association, however, may not be required in yeast since the first step of splicing can occur without the U5 loop 1 sequence. Following the first step of splicing, it appears that the U5 snRNA and the U2 snRNA are in very close proximity within the yeast spliceosome (Newman *et al.*, 1995). Both the U5 loop 1 and position U23 of the U2 snRNA can cross-link to the first position in exon 2 following the first step of splicing, indicating that these two snRNAs may interact with each other. Our reconstitution system will allow us to determine whether the U5 snRNA loop 1 interacts with other snRNAs by the introduction of site-specific cross-linking groups within the U5 snRNA.

Proteins may also be critically involved in the alignment of the exons with the U5 snRNA loop 1 for the second catalytic step of splicing. Again there is not much information as to how the U5 snRNA loop 1 interacts with the protein components of the spliceosome. The mammalian U5 snRNP has a complex protein composition in both the 20S U5 snRNP and 25S U4/U5/U6 tri-snRNP (Bach *et al.*, 1989; Behrens and Lührmann, 1991). The functions of a number of the U5 snRNP-specific proteins are just coming to light. For example, the highly conserved yeast U5 protein PRP8 and its mammalian counterpart p220 are known to interact with both the 5' and 3' exons during splicing (Wyatt *et al.*, 1992; Teigelkamp *et al.*, 1995; Reyes *et al.*, 1996). In UV cross-linking experiments with

yeast U5 snRNPs reconstituted *in vitro*, the PRP8 protein and another unidentified protein were detected cross-linked to the U5 loop 1 sequence within spliceosomes (I.Dix, R.T.O'Keefe, A.J.Newman and J.D.Beggs, unpublished data). Recently, p220 and two other proteins of 110 and 116 kDa that are thought to be U5 proteins have been found to interact with the 3' splice site region and may be important for the second catalytic step of splicing (Chiara *et al.*, 1997). In addition, two recently identified human U5 snRNP-specific proteins, one which contains domains characteristic of the DEXH-box protein family of putative RNA helicases and RNA-stimulated ATPases (Lauer *et al.*, 1996) and another which is a GTP-binding factor (Fabrizio *et al.*, 1997), have yeast homologues that are likely to function during splicing (Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu *et al.*, 1996). Finally, two other U5-associated proteins, Slu7 and Prp18, are important for the second catalytic step of splicing (reviewed in Umen and Guthrie, 1995). These U5-specific proteins and possibly other spliceosomal proteins may be involved in the intricate RNA-protein interactions required for the alignment of the exons with the U5 loop 1. The discovery of how both the RNA and protein components of the spliceosome interact with the U5 loop 1 will most likely reveal why the size of loop 1 is critical for the proper alignment of the exons for the second catalytic step of splicing.

Materials and methods

Construction of yeast strains

The *Saccharomyces cerevisiae* strain SC261.8 was made from SC261 (*matr ura3-52 leu2 trp1 pep4-3 prb1-1132 prc1-407*) by disruption of the *SNR7* gene for U5 snRNA using a linear fragment of DNA consisting of the *LEU2* gene embedded in *SNR7* flanking sequences as follows: SC261 was co-transformed with the *snr7::LEU2* fragment and pRS316 (Sikorski and Hieter, 1989) carrying *SNR7*. *Leu+* transformants were then transformed with the plasmid pROK8 (O'Keefe *et al.*, 1996) carrying a modified gene for the U5 snRNA with a 30 nucleotide insertion required for U5 depletion. Finally, the pRS316 plasmid carrying *SNR7* was evicted by growth on plates containing 5-fluoro-uracil. The presence of the *snr7::LEU2* disruption was confirmed by PCR analysis of genomic DNA from the SC261.8 strain.

Splicing extract preparation and *in vitro* depletion-reconstitution

Splicing extracts were prepared from the yeast strain SC261.8 as previously described (Newman *et al.*, 1985). Depletion and reconstitution of U5 snRNPs in extracts made from the SC261.8 strain were carried out essentially according to O'Keefe *et al.* (1996) except for the following modification to the depletion procedure. Extracts were depleted at 35°C for 40 min, with addition of half the depletion oligonucleotides at time 0 min and the remaining oligonucleotides at 15 min. The size of the depletion-reconstitution reactions used for different experiments is as follows: 5 µl to assay the splicing activity of U5 mutants (Figures 2 and 3); 15 µl to display U5 and U1 cross-links to position -1 in exon 1 (Figure 4); and 50–400 µl to obtain cross-linked species for primer extension mapping of U5 cross-links to position -1 in exon 1 and position +1 in exon 2 (Figures 5 and 6).

In vitro mutagenesis and transcription of RNA

U5 snRNA deletions and insertions were produced by *in vitro* mutagenesis (Kunkel, 1985) of the U5 snRNA gene in the plasmid m571 (O'Keefe *et al.*, 1996) with the following oligonucleotides: 95-033, 5'CGGATGG-TTCTGAAGAACCATGTT (Del. 1–9); 96-4188, 5'TCTGGTAAAAG-GAAGAACCATGTT (Del. G1); 96-4189, 5'GTTCTGGTAAAAG-CAAGAACCATG (Del. C2); 96-4191, 5'GGTCTGGTAAAAGGCAA-GAACCAT (Del. U4); 96-4195, 5'GGATGGTTCTGGAAAAGGCAA-GAA (Del. A8); 96-4196, 5'CGGATGGTTCTGTAAAAGGCAAGA (Del. C9); 96-4190, 5'GTTCTGGTAAAACAAGAACCATGT (Del.

C2,3); 97-830, 5'GGTTCTGGTAAACAAGAACCATGT (Del. C2,3 U4); 97-831, 5'TGGTTCTGGTAAACAAGAACCATGT (Del. C2,3 U4,5); 96-4192, 5'TGGTTCTGGTAAAGGCAAGAACCAT (Del. U4,5); 96-4193, 5'ATGGTTCTGGTAAAGAAGAACCAT (Del. U4,5,6); 96-4194, 5'GATGGTTCTGGTGGCAAGAACCAT (Del. U4,5,6,7); 96-4977, 5'TGGTTCTGGTAAAAAGGCAAGAACC (Ins. 1U 3/4); 96-4978, 5'TGGTTCTGGTAAAAAGGCAAGAACC (Ins. 2U 3/4); 96-4979, 5'TGGTTCTGGTAAAAAGGCAAGAACC (Ins. 3U 3/4); 96-4980, 5'TGGTTCTGGTAAAAAGGCAAGAACC (Ins. 4U 3/4); 96-5479, 5'CTGGTAAAAGGCAAAAAAGAACCATGTT (Ins. 3U 5'); 96-5480, 5'CGGATGGTTCTGAAAGTAAAAGGCAAG (Ins. 3U 3'); 97-3391, 5'CTGGTAAAAGGCGGGAAGAACCATGTT (Ins. 3C 5'); 97-3392, 5'TCTGGTAAAAGGCAAGAACCATGT (Ins. 3U 1/2). *In vitro* transcription and purification of U5 snRNAs and actin pre-mRNA were as previously described (O'Keefe *et al.*, 1996).

RNA ligation and UV cross-linking

Synthetic *CYH2* pre-mRNAs containing 4-thio-U at position -1 in exon 1 or position +1 in exon 2 were made by RNA ligation as described (Newman *et al.*, 1995) essentially according to the method of Moore and Sharp (1992). The only modification was that the 5' RNA transcript for ligation at position -1 in exon 1 was made by chemical synthesis (95R-108, 5'UAGAAAGCACAGAGGUCACGUCUCA). UV cross-linking and the recovery of RNA from UV-irradiated splicing reactions were as previously described (Newman *et al.*, 1995).

RNA capture using biotinylated oligonucleotides and cross-link mapping by primer extension

Specific RNA species containing U5 and/or U1 snRNA were captured from RNA recovered from UV-irradiated splicing reactions using 5'-biotinylated oligonucleotides (94-6170, 5' biotinATGGCAAGCCACAGTAACGGACAGC for U5 and 97-1062, 5' biotinCGTACTTTGATCAGTAGGACTTCTTGATC for U1) and streptavidin paramagnetic beads (Dyna, Dynabeads M-280 Streptavidin). RNA was annealed to 12.5 pmol of biotinylated oligonucleotide in a 40 µl volume containing 1× SSC by heating to 90°C and cooling to 30°C. Fifty µl of streptavidin paramagnetic beads (0.5 mg) washed with 1× SSC were added and the mixture was incubated at 20°C for 15 min with occasional agitation. The beads were then captured magnetically and washed three times with 0.1× SSC. Following the final wash, the beads were again captured magnetically and all liquid removed. The beads were then resuspended in a small volume (3–5 µl) of formamide gel loading buffer, heated at 100°C for 2 min, spun for 2 min at 13 000 r.p.m. and the supernatant loaded on a 6% polyacrylamide-8 M urea gel to fractionate captured RNAs. For display of captured RNA species, gels were fixed, dried and exposed to film at -70°C with an intensifying screen. For isolation of captured RNA species for cross-link mapping, wet gels were exposed to film overnight and the cross-linked species cut out then electroeluted from the gel slice (Amicon Centrilon, Centricon C30 columns). Cross-link mapping by primer extension was as previously described (Newman *et al.*, 1995) except that the reference ladders were produced from 2.5–7.5 pmol of the appropriate *in vitro* transcribed U5 snRNA mutant.

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