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Protein–RNA interactions in the U5 snRNP of *Saccharomyces cerevisiae*

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ABSTRACT

We present here the first insights into the organization of proteins on the RNA in the U5 snRNP of *Saccharomyces cerevisiae*. Photo-crosslinking with uniformly labeled U5 RNA in snRNPs reconstituted in vitro revealed five contacting proteins, Prp8p, Snu114p, p30, p16, and p10, contact by the three smaller proteins requiring an intact Sm site. Site-specific crosslinking showed that Snu114p contacts the 5′ side of internal loop 1, whereas Prp8p interacts with five different regions of the 5′ stem-loop, but not with the Sm site or 3′ stem-loop. Both internal loops in the 5′ domain are essential for Prp8p to associate with the snRNP, but the conserved loop 1 is not, although this is the region to which Prp8p crosslinks most strongly. The extensive contacts between Prp8p and the 5′ stem-loop of U5 RNA support the hypothesis that, in spliceosomes, Prp8p stabilizes loop 1–exon interactions. Moreover, data showing that Prp8p contacts the exons even in the absence of loop 1 indicate that Prp8p may be the principal anchoring factor for exons in the spliceosome. This and the close proximity of the spliceosomal translocase, Snu114p, to U5 loop 1 and Prp8p support and extend the proposal that Snu114p mimics U5 loop 1 during a translocation event in the spliceosome.

Keywords: crosslinking; pre-mRNA splicing; Prp8p; Snu114p; yeast

INTRODUCTION

In nuclear pre-mRNA splicing, intronic regions of pre-mRNAs are removed by two sequential transesterification reactions. These reactions are catalyzed by a ribonucleoprotein complex known as the spliceosome, which consists of four distinct small nuclear ribonucleoprotein particles (snRNPs; U1, U2, U5, U4/U6) and a large number of proteins that assemble on the pre-mRNA in an ordered fashion (reviewed by Moore et al., 1993; Madhani & Guthrie, 1994; Nilsen, 1994; Krämer, 1995). The spliceosome is a highly dynamic structure that undergoes a number of complex conformational changes prior to, between, and subsequent to the two splicing reactions. For a better understanding of how the splicing machinery works, a detailed knowledge of its composition is essential. The U5 snRNP is required for both steps of splicing (Patterson & Guthrie, 1987), interacting with both splice sites of the substrate RNA in the reactive centers, thus making it an important snRNP for analysis (reviewed by Newman, 1997).

Newman and Norman (1991, 1992) first demonstrated a role for the U5 snRNA, in particular the conserved loop 1 sequence, in splice site selection through genetic studies, screening for suppressors of mutant 5′ and 3′ splice sites in yeast. The observation of a similar situation in mammalian cells (Cortes et al., 1993) led to the theory that the invariant loop 1 of U5 contacts both the exons, tethering them for the second transesterification reaction. Subsequent crosslinking experiments in both yeast and mammalian systems added weight to this idea because U5 loop 1 could be crosslinked to exon sequences at both the 5′ and 3′ splice sites (Wyatt et al., 1992; Sontheimer & Steitz, 1993; Newman et al., 1995). Also supporting this theory was the discovery that the loop is essential for the second transesterification reaction in vitro, although substitution experiments indicated that the primary sequence is not important in these interactions (O'Keefe et al., 1996). More important is the loop size, which is critical for correct alignment of the exons for the second catalytic step of splicing (O'Keefe & Newman, 1998).

Consistent with the U5 snRNA being essential for splicing, its secondary structure is highly conserved

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across all the species thus analyzed, although the conservation is less pronounced at the primary sequence level (Frank et al., 1994). The 214-nt U5 snRNA (U5L) of *Saccharomyces cerevisiae* consists of two stem-loop structures flanking an Sm site, with the larger 5′ stem-loop conventionally being divided into seven subdomains as shown in Figure 1. A functional short form (U5S) also exists, which lacks the 3′ stem-loop. Frank et al. (1994) found that only the loop 1, the internal loop 1 (IL1), and the Sm site sequences were evolutionarily conserved among different species, suggesting a role for these sequences in either snRNP biogenesis or function. In vivo deletion studies investigating the role of the various domains of the U5 snRNA in *S. cerevisiae* demonstrated that only loop 1, stem 1, IL1, stem 2, and the Sm site were required for a functional U5 snRNA (Frank et al., 1994). Further analyses in transiently transfected human cells have also demonstrated the importance of the 5′ stem-loop in U5 function (Hinz et al., 1996).

As with other spliceosomal snRNPs, the U5 snRNA is associated with two types of proteins, the core or Sm proteins and the snRNP-specific proteins. There are seven distinct Sm proteins (B/B′, D1, D2, D3, E, F, and G) that are common to the U1, U2, U4/U6, and U5 snRNP particles (Lührmann et al., 1990). These small proteins associate with the snRNA to form the snRNP core particle, presumably binding to a conserved sequence element present in the U1, U2, U4, and U5 snRNAs. In *Homo sapiens*, nine U5-specific proteins have been observed with molecular weights of 15, 40, 52, 100, 102, 110, 116, 200, and 220 kDa (Will et al., 1993). The 110-, 116-, and 220-kDa proteins have all been observed to contact the pre-mRNA between the branchpoint sequence (BPS) and the 3′ splice site AG (Chiara et al., 1996, 1997), whereas only the 220-kDa protein has been shown to crosslink to the 5′ splice site (Wyatt et al., 1992; Chiara et al., 1996; Reyes et al., 1996). The 116-kDa protein is a second step splicing factor that, due to its high homology to the GTP binding ribosomal translocase EF-2 and the dependence of splicing in vitro on GTP hydrolysis, is thought to promote conformational changes in the spliceosome (Fabrizio et al., 1997). Liu et al. (1997) crosslinked this protein to a stable hairpin structure between the BPS and the 3′ splice site AG, which inhibits the second step of splicing, leading them to suggest that the 116-kDa protein is involved in 3′ splice site selection, possibly by a scanning mechanism. The 220-kDa protein is the homologue of the highly conserved yeast Prp8p protein, thought to be central to the splicing reactions (see below) (Anderson et al., 1989).

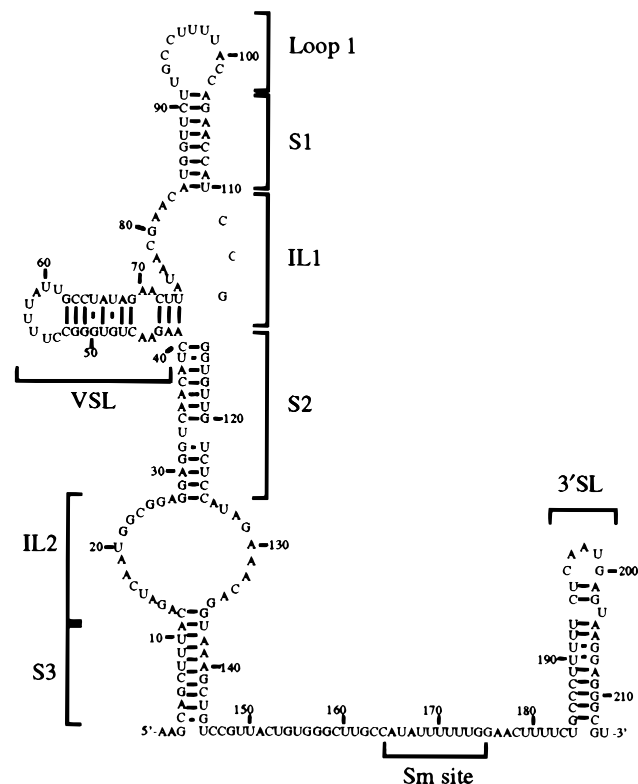


FIGURE 1. U5 snRNA of *S. cerevisiae*. Features of the predicted U5 snRNA secondary structure (after Frank et al., 1992) that are refered to in this work are indicated. IL, internal loop; S, stem; SL, stem-loop; VSL, variable stem-loop.

The protein composition of the yeast U5 snRNP is less well characterized, with only four proteins having been identified: Prp18p, Snu114p, Snu246p, and Prp8p. The 29-kDa nonessential Prp18p associates only weakly with the U5 snRNP and is involved in the second step of splicing (Vijayraghavan & Abelson, 1990; Horowitz & Abelson, 1993a, 1993b). The mammalian equivalent of Prp18p is also a second step splicing factor, but it is not associated with the free U5 snRNP in HeLa extracts (Horowitz & Krainer, 1997). Snu114p, the yeast homologue of the HeLa 116-kDa protein (Fabrizio et al., 1997), and Snu246p, the yeast homologue of the HeLa 200-kDa protein (Lauber et al., 1996), are both essential for splicing. Prp8p (280 kDa) is also essential for splicing in vivo and in vitro (Jackson et al., 1988) and, like its HeLa homologue p220, contacts the 5′ splice site and 3′ splice site, the association with the 3′ splice site occurring only after the first step of splicing (Teigelkamp et al., 1995a, 1995b; Umen & Guthrie, 1995a). This crosslinking pattern of Prp8p on the pre-mRNA is reminiscent of the U5 loop 1–exon interactions, leading to the proposition that Prp8p may stabilize the fragile interactions between the U5 snRNA and the nonconserved exon sequences at the splice sites, anchoring them in the catalytic center of the spliceosome (Beggs et al., 1995). Mutagenesis of Prp8p has shown that it is involved in the recognition of the poly-pyrimidine tract (PPT) and the 3′ splice site, suggesting a role in the second step of splicing (Umen & Guthrie, 1995b, 1996). However, its highly conserved sequence contains no

distinct RNA binding or other recognizable motifs that might give clues to its function (Hodges et al., 1995).

The U5 snRNA is found in three interrelated RNP particles, the U5 snRNP, the U4/U6.U5 tri-snRNP, and the spliceosome. The composition of the yeast tri-snRNP and the proteins in proximity to the U5 snRNA in the spliceosome are even less well defined than the U5 snRNP. In HeLa extracts, five tri-snRNP-specific proteins have been observed: 15.5, 20, 27, 61, and 63 kDa (Will et al., 1993). Of these, only the 27-kDa protein has been identified (Fetzer et al., 1997), although, to date, no potential yeast homologue has been proposed. Nothing is known about which proteins interact directly with the U5 snRNA in the spliceosome, although genetic screens in yeast revealed that Prp8p, Prp16p, Prp17p, Prp18p, and Slu7p interact functionally with the U5 snRNA (Frank et al., 1992).

Here data are presented from studies directed at elucidating the protein–snRNA interactions in the U5 snRNP. A U5 snRNP reconstitution technique was used in combination with UV crosslinking to detect proteins that contact the U5 snRNA directly. Protein binding sites were localized on the snRNA by site-specific crosslinking. It was found that Prp8p and a number of smaller proteins contact the U5 snRNA in the U5 snRNP. We identified one of these proteins as Snu114p, which binds to IL1, in line with the model proposed by Staley and Guthrie (1998), in which it is suggested to mimic the U5 loop 1 structure while performing a translocation function in the spliceosome. Mutant U5 snRNA analysis revealed that, although loop 1 is a strong binding site for Prp8p, it seems not to be the main determinant of Prp8p's U5 association and, even in the absence of loop 1, Prp8p crosslinks to the exons near the 5′ and 3′ splice site in spliceosomes. We therefore propose that Prp8p may be the factor that tethers the exons in the catalytic centers of the spliceosome, whereas the U5 loop plays the more specialized role of aligning the exons for the second step of splicing.

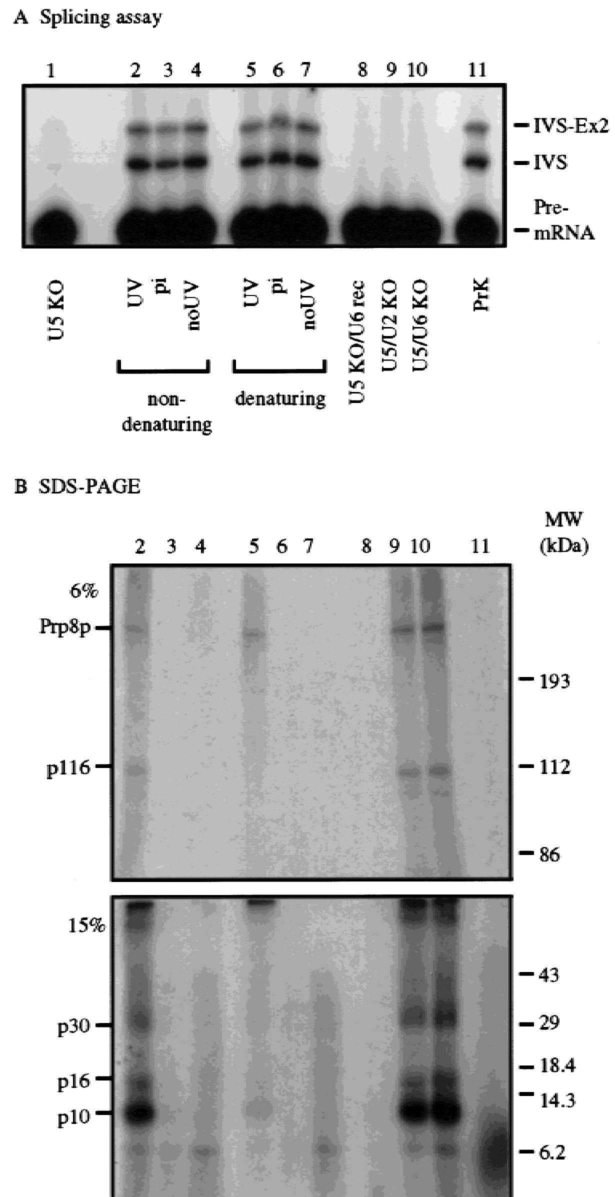


FIGURE 2. Reconstitution of wild-type U5 snRNPs in U5-depleted SC261.8 extracts using in vitro-transcribed uniformly ^{32}P-labeled U5 RNA. **A:** Aliquot (10%) of each reconstituted extract was assayed for function in splicing reactions. Only the pre-mRNA, lariat intron–exon 2 (IVS-Ex2) and lariat intron (IVS) species are shown due to space limitations. **B:** Remaining 90% of each was UV crosslinked and immunoprecipitated using anti-Prp8p antibodies. Following digestion with RNases, the proteins were fractionated by 6% and 15% SDS-PAGE and RNA-crosslinked proteins were visualized by autoradiography (lane 2). Lanes 1, 3, 4, 8, and 11 are controls: No U5 reconstitution (lane 1), pre-immune antibodies (lane 3), no UV crosslinking (lane 4), U5 depletion followed by addition of U6 RNA instead of U5 RNA (lane 8), and proteinase K digestion of the final sample (lane 11). Lanes 5–7 are the same as lanes 2–4, but, prior to immunoprecipitation, the samples were subjected to strong denaturants and heated to disrupt any complexes. Lanes 9 and 10 are the same as that described for lane 2, but, in addition to U5, U2 (lane 9) or U6 (lane 10) was ablated using antisense oligos. Bands at the top of the 15% gel correspond to Prp8p and p116 identified on the 6% gel.

RESULTS

Random crosslinking of the U5 snRNA to proteins in U5 snRNPs

To identify the proteins in contact with the U5 snRNA, we used the U5 snRNP depletion/reconstitution system developed by O'Keefe et al. (1996). U5 snRNPs were reconstituted with uniformly labeled U5 RNA made by (T7) transcription in vitro and tested for function by assaying the extracts for ability to splice actin pre-mRNA (Fig. 2A, lanes 2–7, 11). When no U5 RNA was added back (Fig. 2A, lanes 1 and 8), or when U2 snRNA (Fig. 2A, lane 9) or U6 snRNA (Fig. 2A, lane 10) was depleted in addition to U5, the extracts did not support splicing, confirming the efficacy of the RNase H ablation step and that the splicing activity in reconstituted

extracts was due to the in vitro-transcribed U5 RNA. Following UV irradiation, anti-Prp8p antibodies were used to immunoprecipitate the reconstituted U5 particles, revealing five proteins crosslinked to the U5 RNA: p280, p116, p30, p16, and p10 (Fig. 2B, lane 2). None of these proteins was detected upon immunoprecipitation with pre-immune serum (Fig. 2B, lane 3) or in the absence of UV irradiation (Fig. 2B, lane 4). p280 was identified as Prp8p, because it alone was immunoprecipitated with anti-Prp8p antibodies following the disruption of the snRNP under denaturating conditions. When the U2 or the U6 snRNA was depleted as well as U5 (Fig. 2B, lanes 9 and 10, respectively), the same crosslink pattern was obtained, indicating that the proteins contact the U5 RNA in the absence of intact spliceosomes or tri-snRNPs, presumably in the U5 snRNP itself. Immunoprecipitations using anti-Prp18p antibodies gave the same crosslinking pattern as for anti-Prp8p (data not shown) although weaker in intensity due to the poor association of Prp18p with the U5 RNA, confirming that these crosslinks originate in the U5 snRNP.

Site-specific crosslinking of proteins to the U5 snRNA

To determine the crosslink sites of the proteins on the U5 RNA, reconstitution was performed with U5 RNAs containing the photoactivatable uridine analogue 4-thiouridine (4-thioU) at unique sites with an adjacent ^{32}P radiolabel. Because the method (Moore & Sharp, 1992) used to produce the RNAs involves the incorporation of 4-thioUpG into the RNA, some of the U5 RNAs contain a point mutation to comply with this UG sequence constraint. These mutated RNAs (C79U, U98G, C112U, or A134U) splice actin pre-mRNA in vitro as wild-type (data not shown).

The positions chosen for 4-thioU incorporation were in the conserved, single-stranded loops (positions 20, 79, 97, 112, 134, 173, and 199). The largest protein to crosslink at any position was Prp8p, as confirmed by immunoprecipitation under denaturing conditions, when this was the only crosslinked protein (Fig. 3, lane 29). Prp8p was observed to crosslink to five of the seven positions tested: 20n, 79n, 97n, 112n, and 134n, all of which are in the 5′ stem-loop structure (Fig. 3, lanes 1, 5, 9, 13, and 21). Parallel reactions using RNAs with ^{32}P and an unmodified U at the same position resulted in no crosslinked proteins being observed (Fig. 3, lanes 3, 7, 11, 15, 19, and 23), confirming the site-specificity of the crosslinks. The two positions that did not demonstrate any Prp8p crosslinks (173n and 199n) are both without this 5′ stem-loop structure, indicating that Prp8p directly contacts the 5′ domain only. Densitometric analysis (normalizing the intensity of the cross-

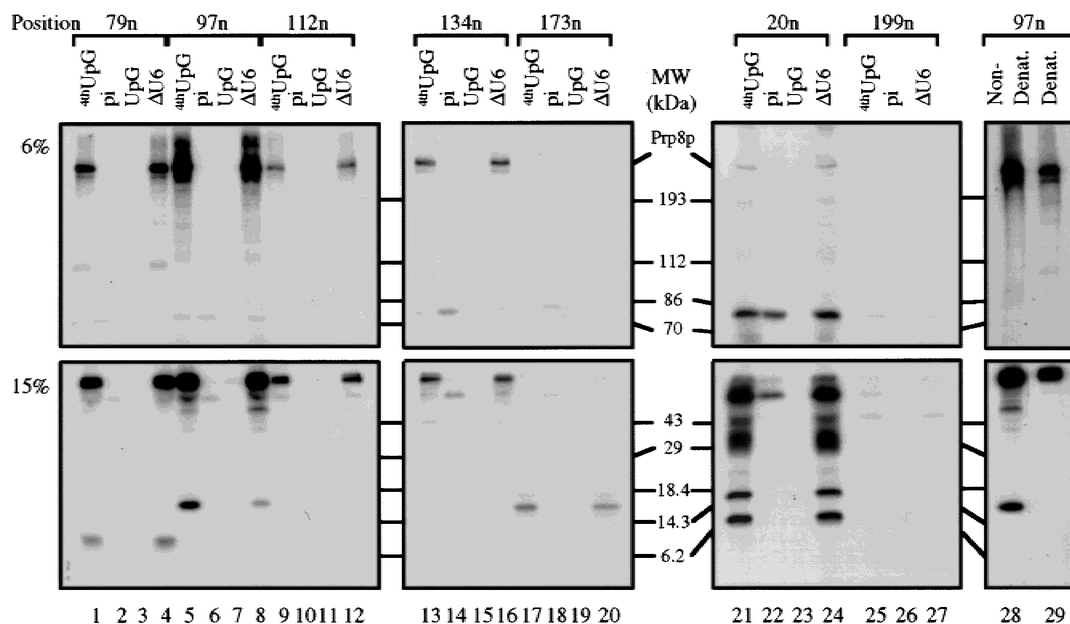


FIGURE 3. Site-specific crosslinking of proteins to U5 RNA. U5-depleted SC261.8 extracts were reconstituted with various U5 snRNAs such that each contained a single ^{32}P-labeled 4-thioU residue at the position indicated. A 10% aliquot of each was assayed for the ability to splice actin pre-mRNA (data not shown), whereas the other 90% was UV irradiated and crosslinked proteins were analyzed as before. Lanes 1, 5, 9, 13, 17, 21, 25, 28, and 29: U5 RNA contained ^{32}P-labeled 4-thioU at the position indicated above the figure. Lanes 2, 6, 10, 14, 18, 22, and 26: Immunoprecipitation was with pre-immune antibodies. Lanes 3, 7, 11, 15, 19, and 23: Controls in which the U5 RNAs contained ^{32}P at the relevant position, but no 4-thioU. Lanes 4, 8, 12, 16, 20, 24, and 27: As for lanes 1, 5, etc., but U5 and U6 snRNAs were depleted and only U5 RNA was replaced. Lane 29: The crosslinked sample was denatured prior to immunoprecipitation with anti-Prp8p antibodies.

linked bands against the input RNA) revealed that the Prp8p crosslink at position 97n was reproducibly strongest, whereas that at position 20n was weakest. Although the chemistry of the different sites in the RNA and protein may affect the intensities of the crosslinks, the pattern suggests that Prp8p crosslinks strongest in the U5 loop 1 structure, the predicted site of action of Prp8p.

Depletion of the U6 snRNA had no effect on any of these crosslinks, indicating that Prp8p contacts the U5 RNA at these positions in the U5 snRNP in the absence of tri-snRNP formation. The persistence of this interaction in tri-snRNPs was not tested, however, in immunoprecipitated spliceosomes, a crosslink (albeit weaker) of Prp8p was detected at position 97 (work in progress), suggesting that this represents a functionally significant interaction.

In addition to Prp8p, a number of other proteins were observed to crosslink to specific positions in the U5 RNA (summarized in Fig. 7; the band observed at ca. 80 kDa was nonspecific). Position 199 revealed only very weak crosslinks, suggesting that this region makes no close protein contacts. Some or all of the smaller proteins (30 kDa and under) may be the same as the crosslinked proteins observed with uniformly labeled RNA. The observation that the crosslink of the 16-kDa protein at position 97 is severely reduced when the U6 snRNA is depleted (Fig. 3, lane 8) or when anti-Prp8p antibodies that disrupt the tri-snRNP are used for the immunoprecipitation (data not shown) indicates that this is a tri-snRNP-specific crosslink, the only one detected. The identity of this protein is unknown (it may derive from the U5 snRNP, the U4/U6 snRNP, or one of the tri-snRNP specific proteins), but it is of considerable interest because, if it remains in contact with U5 loop 1 in spliceosomes, it may play a role at the catalytic centers of splicing.

Identification of p116

The p116 species detected with uniformly labeled RNA may be identical to the 79n:116 kDa species (the 116-kDa crosslink with 4-thioU at position 79), and might correspond to one of four proteins of about 100 kDa present in human U5 snRNP (the 100-, 102-, 110-, and 116-kDa proteins). So far, only the 100- and 116-kDa human proteins have been identified. The 100-kDa protein has been reported to be the likely homologue of the yeast DEAD-box protein Prp28p (Teigelkamp et al., 1997), however, yeast Prp28p is much smaller (67 kDa) and is not U5-associated (Strauss & Guthrie, 1994). On the other hand, the 114-kDa yeast Snu114p is a more promising candidate. Strain IDY1 was therefore constructed, in which the genomic copy of *SNU114* is tagged to increase the molecular weight of Snu114p by a predicted 14 kDa and facilitate its identification.

In crosslink experiments with IDY1 extracts and either uniformly labeled or 4-thioU (position 79n)-containing U5 RNA, the Prp8p crosslinked species migrated in SDS-PAGE as normal. However, with both labeled forms of U5 RNA, the 116-kDa crosslinked species was absent in the IDY1 extract, being replaced by a slower-migrating (125–130 kDa) protein band (Fig. 4). Thus, the 116-kDa species observed in the random crosslinking experiments and the 4-thioU 79n:116 kDa are the same protein, Snu114p. These data indicate that Snu114p contacts the U5 5′ IL1 and so is in very close proximity to Prp8p, which also crosslinks to this loop.

Effects of mutations and deletions in the U5 snRNA

To obtain information about the domains of U5 RNA that are important for snRNP function, a number of U5 mutations were generated (Table 1). The uniformly radiolabeled mutant RNAs were used to reconstitute extracts that were assayed subsequently for (1) function in vitro (splicing assays; Fig. 5A), (2) for the ability of the U5 RNA to form a Prp8p-containing particle (coprecipitation of the RNA with Prp8p; Fig. 5B), and (3) for protein–RNA contacts (crosslinking; Fig. 5C). Deletion of the 3′ SL or the VSL had no significant effect on splicing, on the ability of U5 RNA to form a Prp8p-containing particle, or on the pattern of crosslinked proteins (Fig. 5, lanes 10 and 13, respectively). Deletion of the Sm site led to the in vitro transcript becoming liable to degradation in the extract (Fig. 5, lane 9 and data not shown). However, this RNA was able to support splicing to a variable low level (Fig. 5, lane 9; O'Keefe et al., 1996) and was apparently crosslinked to some of the same proteins as wt U5 RNA, including Prp8p and the p116 protein, but not the smaller proteins (p30, p16, and p10). The observation that these small proteins

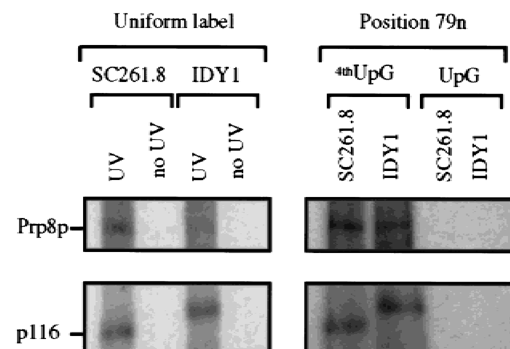


FIGURE 4. Crosslinking of size-shifted Snu114p to U5 RNA. Extracts from SC261.8 and IDY1 cells were U5-depleted and reconstituted with U5 RNA that was either uniformly ^{32}P-labeled (left panel) or contained a single ^{32}P-labeled residue at position 79 (right panel) next to either 4-thioU or unmodified uridine as indicated. The samples were UV irradiated at 254 nm (left panel) or 360 nm (right panel), and crosslinked proteins were analyzed as before.

TABLE 1. Mutants of U5 RNA used in this study.

U5 Sequence	Region modified	Splicing in vitro	Growth at 30 °C	Reference
Substitution of 13–27	IL 2	Very poor	No	This work
Del. 41–74	VSL	Wild-type	Yes	O'Keefe et al. (1996)
Del. 41–83	VSL and 5′ IL1	None	No	This work
Del. 41–74 and Del. 111–113	VSL and 3′ IL1	None	No	This work
Del. 84–110	S1 and Loop 1	None	No	O'Keefe et al. (1996)
Del. 93–101	Loop 1	2nd step block	No	O'Keefe et al. (1996)
Reverse complement 92–102	Loop 1	Wild-type	No	O'Keefe et al. (1996)
Del. 111–113	3′ IL 1	None	No	This work
Del. 166–175	Sm site	Poor[a]	Yes	O'Keefe et al. (1996)
Del. 179–214	3′ SL	Almost wild-type	Yes	This work
Substitution of 13–27 and Del. 179–214	IL2 and 3′SL	Very poor	ND	This work

[a]Limited by U5 RNA stability.

require the Sm site to contact the U5 RNA, and preliminary crosslinking data using tagged proteins (I. Dix, C. Russell, J.D. Beggs, E. Bragado-Nilsson, & B. Séraphin, unpubl. data), suggests that they may be Sm proteins. Crosslinking of Prp8p and p116 to the U5 RNA may not require the presence of these proteins, although it cannot be ascertained using this assay whether the smaller proteins are still present in these mutant snRNPs but not contacting the RNA. These data indicate a role for the Sm site and/or Sm proteins in stabilizing the RNA against degradation rather than for assembly of a functional snRNP in vitro. This seems to contrast with the requirement for Sm proteins for functional reconstitution of HeLa U5 snRNPs (Segáult et al., 1995), but resembles the situation with HeLa U4 snRNP reconstitution, for which the Sm site was found to be dispensable (Wersig & Bindereif, 1992).

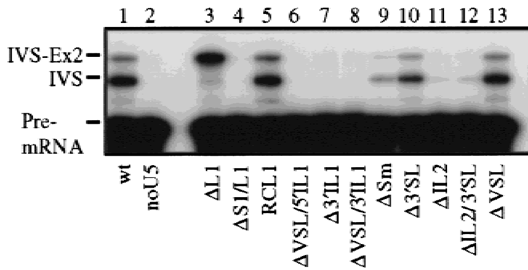
Deletion of loop 1 nt 93–101 (ΔL1) or substitution of the reverse complement of nt 93–101 (RCL1), both of which drastically alter the highly conserved loop 1 sequence, had no effect on the ability of U5 RNA to form a Prp8p-containing particle (Fig. 5B, lanes 3 and 5). Both of these mutations resulted in a reproducibly weaker Prp8p crosslink, with about half the intensity of that with wild-type U5 RNA (Fig. 5C, compare lane 1 with lanes 3 and 5) compatible with the site-specific crosslinking data. The crosslinking of the other proteins appears unaffected. These loop 1 mutations appear to have no defect in the ability of the U5 RNA to be incorporated into tri-snRNPs [as assayed by immunoprecipitations with antibodies against the U6-specific protein, USS1 (Cooper et al., 1995; data not shown)]. When both stem 1 and loop 1 were deleted, splicing was abolished (Fig. 5A, lane 4). This mutant RNA coprecipitated with Prp8p only weakly (Fig. 5B, lane 4), leading to weaker crosslinks being observed for all the proteins. Mutations affecting the structurally conserved internal loops, 5′ IL1, 3′ IL1, and IL2 (Fig. 5, lanes 6–8, 11, and 12) severely inhibited splicing and prevented the U5 RNA from forming Prp8p-containing particles, allowing only a trace or no Prp8p to crosslink to the RNA. These data suggest that both internal loops of the 5′ stem are important for Prp8p–U5 RNA association.

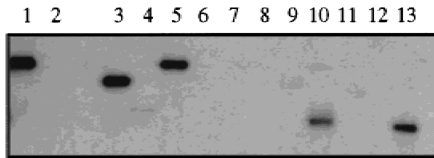
Effect of U5 snRNA mutations on Prp8p crosslinking to the pre-mRNA

The finding that Prp8p and U5 loop 1 both interact with the exons at the splice sites led to the proposal that the U5 snRNP tethers the exons in the spliceosome, with Prp8p possibly stabilizing the loop 1–exon RNA interactions (reviewed Beggs et al., 1995). If the U5 snRNA is the primary determinant of exon binding by the U5 snRNP, then the presence of loop 1 might be necessary for Prp8p interaction with the exons. To test this, extracts were reconstituted with wt or mutant U5 snRNA and incubated with CYH2m pre-mRNA containing 4-thioU at position −1 to the 5′ splice site or +1 to the 3′ splice site. Following UV irradiation, the Prp8p-containing complexes, including spliceosomes, were precipitated with anti-Prp8p antibodies (Fig. 6). When no U5 RNA was added (Fig. 6, lane 4), only a background level of Prp8p interaction with either splice site was observed, due to the low level of residual undigested U5 snRNA in the extract. Reconstitution with U5 RNA containing either the loop 1 deletion or the loop 1 substitution mutation resulted in levels of Prp8p crosslinking comparable to wild-type for both the 5′ and 3′ splice site. Thus, although the interaction of Prp8p with each splice site depends on the presence of U5 RNA, loop 1 itself is not required. This suggests that Prp8p is the main determinant of exon binding by the U5 snRNP.

DISCUSSION

Although it has been known for years that the U5 snRNP is essential for pre-mRNA splicing, relatively little is





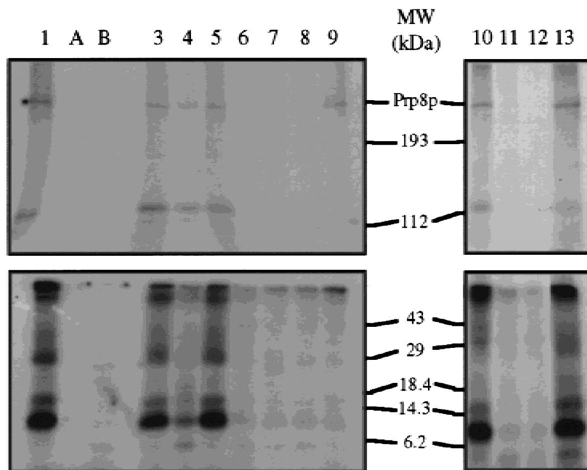


FIGURE 5. Effect of U5 RNA mutations. U5-depleted SC261.8 extracts were reconstituted using in vitro-transcribed uniformly ^{32}P-labeled U5 RNAs containing mutations as indicated. **A:** Aliquots (10%) of reconstituted extracts were assayed for functional reconstitution in splicing reactions. Only the pre-mRNA, lariat intron–exon 2, and lariat intron species are shown. **C:** Remaining 90% of each was UV irradiated and crosslinked proteins were analyzed as in Figure 2. **B:** Corresponding unlabeled mutant U5 snRNAs were used to reconstitute depleted SC261.8 extracts and U5 snRNPs were immunoprecipitated using anti-Prp8p antibodies. The U5 RNA in the immunoprecipitates was detected by northern analysis. Controls: Panels A and B, lane 2, no U5; panel C, lane A, no UV crosslinking; panel C, lane B, pre-immune antibodies were used.

known about the U5 proteins and their arrangement in the snRNP. Here we have used the recently developed *S. cerevisiae* U5 snRNP reconstitution procedure (O'Keefe et al., 1996) and photo-crosslinking to analyze protein–RNA interactions in the U5 snRNP. In particular, we have focused on the highly conserved Prp8 protein thought to be at the catalytic centers of both steps of splicing (Beggs et al., 1995). Crosslinking experiments, using uniformly labeled U5 RNA, revealed that Prp8p contacted the RNA directly in the U5 snRNP, along with at least four other proteins of about 116 kDa, 30 kDa, 16 kDa, and 10 kDa. Further experiments using site-specifically labeled U5 RNA provided information about the positions of the binding sites on the RNA and detected more, weakly crosslinked proteins (Fig. 7). These are the first details about any proteins contacting the U5 snRNA.

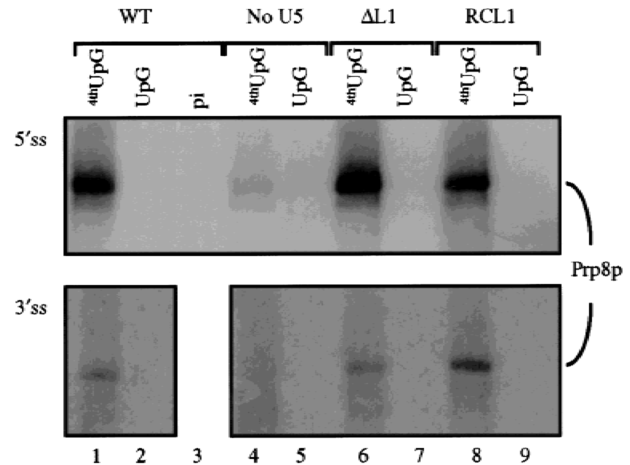


FIGURE 6. Prp8p reconstituted with U5 loop 1 mutant RNAs crosslinks to the exons. Crosslinking of Prp8p to exon position −1 at the 5′ splice site (upper panel) and exon position +1 at the 3′ splice site (lower panel) of the substrate RNA in the presence of either wt, mutant (DL1, loop 1 deleted; RCL1, reverse complement loop 1 sequence), or no U5 RNA. SC261.8 extract depleted of U5 snRNA was reconstituted with either wt, mutant, or no U5 snRNA and incubated with CYH2 pre-mRNA containing ^{32}P-labeled 4-thioU as indicated. Reactions were then crosslinked and digested with RNase T1. Prp8p-containing complexes were immunoprecipitated using anti-Prp8p antibodies and the proteins were fractionated by 6% SDS-PAGE (lanes 1, 4, 6, and 8). Control reactions contained CYH2 RNA ^{32}P-labeled at the 5′ splice site or 3′ splice site but lacking 4-thioU (lanes 2, 5, 7, and 9). The sample in lane 3 was the same as that described for lane 1, but pre-immune serum was used.

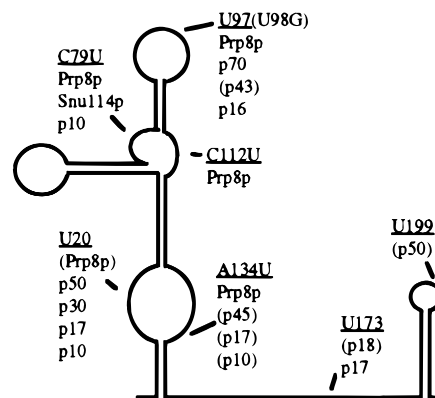


FIGURE 7. Summary diagram of the site-specific crosslinking data. Very weak crosslink signals are indicated in brackets. Where base substitutions were made to create a UG in the sequence, these are indicated.

U5 snRNA–Prp8p interactions

To determine the regions of U5 RNA required for Prp8p association, and where Prp8p directly contacts the U5 RNA, a number of mutant RNAs and RNAs containing single 4-thioU residues (enabling site-specific crosslinking) were generated. Deletion of the VSL, Sm site, or the 3′ SL, none of which is required for in vitro splicing, had no effect on the assembly of the RNA into Prp8p-containing particles. Similarly, no crosslinking of Prp8p to the Sm site or 3′ SL was detected in site-specific crosslinking (the VSL region was not assayed). The failure to detect any strong crosslink signals at position 199 suggests that no proteins bind tightly to the loop of the functionally redundant 3′ SL structure. Both in vivo and in vitro experiments demonstrated that IL1 and IL2 were essential, with Prp8p only associating with those mutant RNAs that retained both these internal loops. Crosslinking with 4-thioU-containing U5 RNAs demonstrated that Prp8p and several other proteins contact both of these internal loops directly. The IL1 data are supported by previous reports that this region is essential in yeast cells (Frank et al., 1994), is important for p220 association with HeLa U5 snRNPs (Hinz et al., 1996), and is protected against chemical modification and nuclease digestion in HeLa U5 snRNPs (Bach & Lührmann, 1991). However, in contrast, Frank et al. (1994) found that IL2 was dispensable for yeast cell viability, possibly reflecting differences in the mutations used. Ast and Weiner (1997) observed that binding of an antisense 2′-*O*-methyl RNA oligonucleotide to HeLa U5 RNA at the base of the 5′ stem on the 3′ side (corresponding to the region in yeast U5 from 3′ IL2 up to the Sm site) caused conformational changes resulting in disruption of tri-snRNPs and exposure of loop 1 to chemical modification. Assuming that HeLa p220 binds to similar regions of U5 RNA as does Prp8p, the antisense oligonucleotide may displace p220 binding to the 5′ stem-loop, thereby disrupting the tri-snRNP, the stability of which is dependent on Prp8p in yeast (Brown & Beggs, 1992).

Mutations in loop 1 in the 5′ stem-loop revealed a more complex situation. Loop 1 is the region in U5 RNA most strongly crosslinked by Prp8p, however, a complete deletion or substitution of the loop 1 sequence had no effect on co-precipitation of the RNA with Prp8p. Thus, the strong binding of Prp8p to loop 1 is not essential for U5 snRNP integrity, nor for the first step of splicing. The simplest interpretation of these data is that other proteins mediate or stabilize the Prp8p association with U5 RNA, possibly through interactions involving IL1 and IL2, thus allowing Prp8p to associate with the snRNP in the absence of its strongest RNA contact site. Nevertheless, Prp8p is the protein that makes the most extensive contacts with the U5 RNA, making direct contact with virtually the entire 5′ stem-loop, not just loop 1; it is the strongest crosslinking protein on 5′ IL1 and 3′ IL2 and is the only protein detected to contact the 3′ IL1. Thus, IL1 and IL2 may be part of a large recognition site for direct Prp8p binding.

It remains to be determined whether any feature of loop 1 other than its position at the end of the 5′ stem is important for Prp8p binding there. Experiments with 4-thioU-containing loop 1 substitution mutants showed that Prp8p also binds to other than wild-type loop 1 sequences (UUGU and GUGG instead of UUUU; data not presented). The recent report of effects of U5 loop 1 size on the alignment of the exons for step 2 of splicing (O'Keefe & Newman, 1998) makes loop size a candidate determinant for its binding by Prp8p.

These results correlate well with genetic data of Frank et al. (1992), who found synthetic lethality between *prp8-1* and U5 loop 1 mutations and suggested the existence of a direct or indirect association. Hinz et al. (1996) also suggested an association between loop 1 and mammalian Prp8p (p220) when, in contrast to the data presented here, they observed that deletion of loop 1 reduced p220 association with the snRNA fivefold. It should be noted that these were in vivo experiments and direct contact between p220 and the U5 RNA was not investigated. Additionally, chemical and nuclease protection experiments using HeLa U5 particles revealed that, although the loop 1 in 20S U5 snRNPs was accessible to chemical modifications, it was less accessible to nucleases than in core snRNPs, leading to the suggestion that U5-specific proteins may be in very close proximity to loop 1, possibly blocking access by other factors (Bach & Lührmann, 1991). Indeed, in experiments presented here, another protein of approximately 16 kDa was observed to crosslink strongly to loop 1 only in tri-snRNPs, indicating that a conformational change may occur upon tri-snRNP formation, in which the Prp8p–loop 1 contact is relaxed or otherwise altered to permit binding of the 16-kDa protein.

Based on the similar crosslinking patterns of Prp8p and U5 loop 1 to the exons prior to and subsequent to the first step of splicing, it has been proposed that Prp8p may stabilize the intrinsically fragile RNA–RNA interactions between the exons and U5 loop 1. Data presented here support such a model and, moreover, the observation that Prp8p binds to the exons even in the absence of loop 1, suggests that Prp8p may be the principal anchoring factor, tethering the exons (and possibly the region between the BP and the 3′ splice site; Teigelkamp et al., 1995a) at the catalytic centers. This resolves the quandary of how the conserved loop 1 could tether all exons regardless of their sequence (Newman, 1997). It is attractive to think of Prp8p as providing a binding pocket into which U5 loop 1 fits to confer a more precise alignment of the exons for the second step of splicing. Thus, U5 mutants with altered loop 1 size that do not fit snugly into the Prp8p pocket would be unable to align the exons correctly (O'Keefe & Newman, 1998).

p116 is Snu114p

The crosslinked protein p116 was identified as Snu114p, the yeast homologue of human p116, that shares strong sequence similarity to the ribosome translocating factor EF-2 (Fabrizio et al., 1997). The crosslinking of this protein to U5 RNA, and its co-precipitation with Prp8p in the absence of tri-snRNPs (Fig. 3, lanes 1 and 4), provides the first evidence that this protein is a U5 snRNP component in yeast. This protein crosslinked very specifically to position 79 in the 5′ IL1 region. The Snu114p crosslink was relatively weak at this position compared to that of Prp8p, suggesting that binding to U5 RNA may not be the primary determinant for Snu114p association with the U5 snRNP. Unlike Prp8p, Snu114p was not found to crosslink to loop 1 and deletion of loop 1 had no effect on Snu114p crosslink levels.

The close proximity of Prp8p and Snu114p is also suggested by two-hybrid data. In an exhaustive two-hybrid screen of a very large yeast genomic library (Fromont-Racine et al., 1997), Prp8p was the predominant interacting factor identified by Snu114p (I. Dix, C. Russell, J.D. Beggs, unpubl. results). The two-hybrid assay can detect indirect as well as direct protein interactions, however, no other known splicing factors were detected in the screen, and Snu114p did not interact with other U5 snRNP proteins tested: Snu246p, Prp18p. Because Prp8p and Snu114p both contact the 5′ IL1 region of U5, it seems highly likely that these two large proteins interact directly. The crosslinking data indicate that Prp8p is the single protein that makes the most extensive contacts with the U5 RNA, and it is tempting to propose that Prp8p may mediate the association of other U5-specific proteins, including Snu114p, with the U5 snRNP.

A model for U5 snRNA, Prp8p, and Snu114p function

The strong sequence similarity between Snu114p and the ribosome translocation factor, EF-2, led to the proposal that Snu114p may be part of a “processivity center” in the spliceosome, functioning ahead of a distinct catalytic center in a simple scanning model for 3′ splice site selection (Fabrizio et al., 1997; Liu et al., 1997). It has been postulated that EF-2 promotes translocation in the ribosome by mimicking the tRNA; in particular, the functionally important domain IV is proposed to mimic the anticodon stem-loop of the tRNA (Nissen et al., 1995). Fabrizio et al. (1997) noted the conservation of domain IV in U5-116 kDa/Snu114p, theorizing that this may also function by RNA mimicry. In yeast, there is evidence against a simple scanning model for 3′ splice site selection, although an adapted model has been proposed (Luukkonen & Séraphin, 1997) in which scanning identifies a region containing the 3′ splice site, with the actual cleavage site being determined by a subsequent recognition step.

At the second step of splicing, the alignment of U5 loop 1 with the exons at the splice sites is reminiscent of the tRNA–mRNA interaction in ribosomes and it has been suggested that Snu114p might mimic U5 loop 1 during a spliceosomal translocation of exon 1 into the catalytic center for the second step of splicing (Staley & Guthrie, 1998). The evidence presented here that Prp8p and loop 1 of U5 snRNA are very closely associated with Snu114p supports this proposal, and our data showing that Prp8p binds to the exons even in the absence of U5 loop 1 leads to a more detailed model. During the translocation of exon 1 toward the 3′ splice site, presumably the intron sequence is moved through a Prp8p pocket, with loop 1 rocking in and out of this pocket as it is displaced by Snu114p. Multiple rounds of translocation could occur until the 3′ splice site reaches the pocket, when the exons are aligned and step 2 occurs. The evidence that the stability of the Prp8p–U5 RNA interaction does not depend on the binding of Prp8p to loop 1 fits well with this model, in that conformational changes in Prp8p–loop 1 interactions during translocation need not destabilize the U5 snRNP. Prp8p can then be viewed as a pivotal protein holding the splicing intermediates, the U5 snRNA, and possibly Snu114p and other step 2 splicing factors in the catalytic center while the 3′ splice site is sought. A similar model could be constructed to represent events prior to the first catalytic step, with Prp8p holding the 5′ splice site region of the pre-mRNA while the 5′ cleavage site is located. Hopefully, further details will come from future crosslinking to investigate the dynamic interactions of the U5 RNA and U5 proteins during the splicing process.

MATERIALS AND METHODS

Strains

SC261.8 is yeast strain SC261 with the genomic copy of the *SNR7* gene disrupted (genotype: *mat***a**, *ura3-52*, *leu2-3*, *trp1-289*, *pep4-3*, *prb1-1132*, *prc1-407*, *snr7::LEU2*) with the *SNR7* gene function being provided by the pROK8 plasmid (O’Keefe & Newman, 1998). The strain IDY1 was created from SC261.8 by targeted integration at the 3′ end of the chromosomal *SNU114* ORF of a linear DNA fragment containing the protein A sequence and the *Kluyveromyces lactis URA3* gene produced from the plasmid pTL54 (Lafontaine & Tollervey, 1996; Lafontaine et al., 1998). The genotype of IDY1 is *mat***a** *ura3-52*, *leu2-3*, *trp1-289*, *pep4-3*, *prb1-1132*, *prc1-407*, *snr7::LEU2*, *snu114::Prot.A::URA3* (*K. lactis*).

Oligonucleotides used in this work

Oligonucleotides 94–6299 and 95–2211 (O’Keefe & Newman, 1998) were used for RNase H cleavage of U5 snRNA in SC261.8 extracts. Oligonucleotides for the mutagenesis of the U5 snRNA were as described by O’Keefe et al. (1996).

Oligonucleotides used for the PCR of the full-length U5 RNA sequence were 93–4548 (5′-GCGCTAATACGACTCACTATA GGAAGCAGCTTTACAGATCAAT) and 89–1959 (5′-AACGC CCTCCTTACTCATTG). To produce U5 species with 4-thioU incorporated at specific sites, oligos 93–4548, 89–1959 were used in combination with three other oligos per position: RNA oligo 96R-146 (5′-AAGCAGCUUUACAGAUCAA), v5994 (5′-GCGCTAATACGACTCACTATAGGCGGAGGGAGGTCAAC ATC), and v5995 (5′-GATGTTGACCTCCCTCCGCCATTGA TCTGTAAAGCTGCTTC). Position 79: v3375 (5′-TTATAAG TTCTATAGGCAATA), v3378 (5′-GCGCTAATACGACTCACT ATAGAACATGGTTCTTGCCTTTTA), and v3379 (5′-AAA AGGCAAGAACCATGTTCATTATAAGTTCTATAGGCAATA). Position 97: v3374 (5′-AGGCAAGAACCATGTTCGTT), v3377 (5′-GCGCTAATACGACTCACTATAGTACCAGAACCATCCG GGTG), and v3376 (5′-CACCCGGATGGTTCTGGTACAAGG CAAGAACCATGTTCATTA). Position 112: v1885 (5′-GATGG TTCTGGTAAAAGGC), v1887 (5′-GCGCTAATACGACTCA CTATAGGGTGTTGTCTCCATAGAA), and v1888 (5-GTTTC TATGGAGACAACACCCAGATGGTTCTGGTAAAAGGCA). Position 134: v4519 (5′-GATTCTATGGAGACAACACC), v4522 (5′-GCGCTAATACGACTCACTATAGGTAAAGCTGT CCGTTACTG), and v4523 (5′-CAGTAACGGACAGCTTTA CCAGTTTCTATGGAGACAACACC). Position 173: v4520 (5′-AAAAATATGGCAAGCCCACAG), v4524 (5′-GCGCTAATA CGACTCACTATAGGAACTTTTTGCCCTTTTTCTC), and v4525 (5′-GAGAAAAAGGGCAAAAAGTTCCAAAAAATATGG CAAGCCCACAG). Position 199: v5997 (5′-TTGAGAAAAA GGGCAGAAA), RNA oligo 96R-150 (5′-^{4th}UGAGUAAGGA GGGCGU), and v5998 (5′-ACGCCCTCCTTACTCATTGA GAAAAAGGGCAGAAA). The oligonucleotides used for the elongation of the genomic copy of the *SNU114* gene were: SnugA (5′-GCGCTGAATTATACGCTCAATTAAGCGAAAA TGGCTTAGTACCGGGCGTGGACAACAAATTC) and SnugB (5′-TGCTGAATAAAAATATTGTGGACATATTGCTTAATTCTT ATGCGCTGGGTAGAAGATCGGTC). The oligonucleotide used to probe for the U5 RNA in northern blot assays was W0679 (5′-CAAGCCCACAGTAAC)

In vitro transcription, site-specific labeling, and ligation of RNA substrates

Uniformly labeled actin pre-mRNA was produced by T7 run-off transcription in vitro from linearized p283 as described by O'Keefe et al. (1996). Uniformly ^{32}P-labeled U5 RNA was transcribed from PCR products containing the T7 promoter in reactions consisting of 40 mM Tris-Cl, pH 7.5, 2 mM spermidine, 10 mM DTT, 10 mM NaCl, 6 mM $MgCl_2$, 0.5 mM ATP, CTP, and GTP, 13.3 μM [$\propto$-^{32}P]UTP (400 Ci/mmol), 0.2 μg/μL DNA, 1.5 U/μL RNasin, 5 U/μL T7 RNA polymerase that were incubated for 30 min at 37 °C. Unlabeled U5 RNAs and 5′ and 3′ fragments of U5 RNA and CYH2 pre-mRNA for site-specific labeling were produced from PCR-generated templates as per Teigelkamp et al. (1995a). All RNAs were purified by denaturing PAGE and electroelution (Amicon Centrilutor; Centricon C30 columns). The 5′ and 3′ RNAs were then ligated as per Teigelkamp et al. (1995a) except the 3′ RNA fragments were kinased using 1.5 μCi/μL [γ-^{32}P] ATP (1.6 pm ATP/μL) in 30-μL reactions. In the case of positions 20 and 199, synthetic oligoribonucleotides were used for the 5′ and 3′ components, respectively.

Splicing extract preparation and in vitro depletion–reconstitution reactions

Yeast whole-cell extracts were prepared as described by Lin et al. (1985). U5 depletion and reconstitution was as described by O'Keefe et al. (1996), except typical reaction volumes were 64 μL and U5 was reconstituted to a final concentration of 5 nM. In all experiments, the long form (214n) of U5 RNA was used.

Protein crosslinking and immunoprecipitations

UV light-induced crosslinking of uniformly ^{32}P-labeled RNA to protein and subsequent immunoprecipitations were performed as described by Teigelkamp et al. (1995a) using polyclonal anti-Prp8p antibodies except that the samples were not RNase digested before the immunoprecipitation step due to the U5 snRNA, proving resistant to RNase treatment. Denatured samples were heated to 90 °C for 2 min in the presence of 2% SDS, 1% Triton X-100, and 100 mM DTT prior to immunoprecipitation, when they were diluted 10-fold in immunoprecipitation buffer (6 mM Hepes, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, and 0.05% NP-40). After the washes, all immunoprecipitates were boiled for 2 min in 30 μL of 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 200 mM NaCl, then incubated in a cocktail of 5 U/μL RNase T1, 5 μg/μL RNase A, and 0.5 U/μL RNase V_1 for 30 min at 37 °C. Site-specifically labeled U5 snRNAs containing 4-thioU were treated similarly, except they were crosslinked for only 5 min at 360 nm and an appropriate RNase was used depending on the base preceding the 4-thiouridine to avoid cleavage of the labeled phosphate from the 4-thiouridine residue.

Northern analysis of the U5 RNA

U5 RNA was immunoprecipitated using polyclonal anti-Prp8p antibodies (anti-8.6) as described by Teigelkamp et al. (1995a). Immunoprecipitates were deproteinized by SDS/proteinase K treatment followed by phenol–chloroform–isoamylalcohol extraction. The resulting RNA was fractionated on a 6% polyacrylamide gel and U5 RNA was detected by northern analysis using [^{32}P]-5′ end-labeled oligo W0679 (Cooper et al., 1995).

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