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Prp31p Promotes the Association of the U4/U6 · U5 Tri-snRNP with Prespliceosomes To Form Spliceosomes in *Saccharomyces cerevisiae*

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The *PRP31* gene encodes a factor essential for the splicing of pre-mRNA in *Saccharomyces cerevisiae*. Cell extracts derived from a *prp31-1* strain fail to form mature spliceosomes upon heat inactivation, although commitment complexes and prespliceosome complexes are detected under these conditions. Coimmunoprecipitation experiments indicate that Prp31p is associated both with the U4/U6 · U5 tri-snRNP and, independently, with the prespliceosome prior to assembly of the tri-snRNP into the splicing complex. Nondenaturing gel electrophoresis and glycerol gradient analyses demonstrate that while Prp31p may play a role in maintaining the assembly or stability of tri-snRNPs, functional protein is not essential for the formation of U4/U6 or U4/U6 · U5 snRNPs. These results suggest that Prp31p is involved in recruiting the U4/U6 · U5 tri-snRNP to prespliceosome complexes or in stabilizing these interactions.

The precise excision of intervening sequences, or introns, from precursor mRNA transcripts (pre-mRNAs) is a critical step in the pathway of gene expression, as evidenced by the resources that the cell invests in the splicing machinery. Five RNA species, the U1, U2, U4, U5, and U6 small nuclear RNAs (snRNAs), are essential to the process; at least four of these interact directly with the splicing substrate and may catalyze the cleavage and ligation events (for reviews, see references 6, 26, 39, and 41 to 43). Unlike the removal of group I or group II introns, splicing of nuclear pre-mRNA transcripts requires a large number of protein factors (reviewed in references 25, 28, 31, 37, 41, 51, and 53). Some of these proteins are integral components of the ribonucleoprotein particles in which the snRNAs are found. Other gene products are less tightly associated with the snRNAs and may serve to recruit splicing factors onto the assembling splicing complex or stabilize interactions between splicing components.

The multicomponent structure in which splicing occurs is termed the spliceosome (13, 21, 24). This complex assembles onto the pre-mRNA substrate and catalyzes the two cleavage-ligation reactions of pre-mRNA splicing. Prior to the initial cleavage reaction, several events occur to ensure identification of introns and accurate splicing of pre-mRNAs. The first defined step of spliceosome assembly involves formation of a base-pairing interaction between the U1 snRNA and the highly conserved 5′ splice site of the pre-mRNA to form the commitment complex, or complex E; this association is sufficient to commit the substrate to the splicing pathway (for reviews, see references 31, 47, and 48). The U2 snRNP associates with the commitment complex in an ATP-dependent step that involves base-pairing of the U2 snRNA with the branchpoint sequence. The complex formed by this interaction is termed the prespliceosome, or complex A (reviewed in references 27, 31, and 47). The U4/U6 and U5 snRNPs form a single tri-snRNP that interacts with the prespliceosome to form the mature spliceosome, or complex B (9, 16, 29, 46, 55). Whether initial interactions between the U4/U6 · U5 tri-snRNP and the prespliceosome involve specific base-pairing between the U4, U5, or U6 snRNAs and RNA components of the prespliceosome is not yet known. Upon assembly of the spliceosome, a series of conformational rearrangements that are presumed to expose catalytic regions of the snRNAs and allow the cleavage-ligation reactions of splicing to occur then take place (reviewed in references 6, 26, 39, and 41 to 43).

Although snRNA/pre-mRNA base-pairing is integral to spliceosome assembly and splicing, the RNA duplexes formed are short, ranging from 3 to 6 bp, and therefore unlikely to be sufficient to recruit the snRNPs onto the assembling spliceosome and to stabilize snRNP-substrate interactions (reviewed in references 6, 26, 39, 41, and 43). In metazoan systems, the SR family of proteins may function to recruit the U1 snRNP to the splicing substrate (reviewed in references 22 and 58). Establishment of the commitment complex also involves recognition of the branchpoint sequence or 3′ splice site by binding of protein factors, some of which are bridged to the U1 snRNP by protein-protein interactions (reviewed in references 3, 31, 47, and 58). As with U1 snRNA/pre-mRNA associations, stable U2 snRNA-branchpoint sequence interactions in yeast or mammals require protein components in addition to the U2 snRNP, including SR proteins in mammals (reviewed in references 27 and 47). Association of the U4/U6 · U5 tri-snRNP with prespliceosomes to form spliceosomes has been less well characterized, although recent studies of mammalian cell extracts have shown that SR proteins are necessary for this step (49).

In the yeast *Saccharomyces cerevisiae*, over 40 gene products that are required for pre-mRNA splicing have been defined (for reviews, see references 28, 31, 51, and 53). The *PRP31* gene was identified in a screen of a temperature-sensitive bank of yeast strains for those defective in pre-mRNA processing after a shift to the nonpermissive temperature (38). Extracts derived from a *prp31-1* strain are temperature sensitive for splicing in vitro, providing evidence that Prp31p is directly involved in pre-mRNA splicing (60). Here, we show that Prp31p is required in yeast extracts for association of the U4/U6 · U5 tri-snRNP with the prespliceosome to form the mature spliceosomal complex. Coimmunoprecipitation experi-

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TABLE 1. Strains used

Strain	Relevant genotype	Source
JWY2857	*MAT***a** *prp31-1 ade1 leu2-3,112 trp1 ura3-52*	This study
JWY2964	*MAT***a** *prp31::TRP1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* (*PRP31*/pRS316)	This study
BJ2168	*MAT***a** *leu2 ura3-52 trp1 prb1-1122 pep4-3 prc1-407 gal2*	E. Jones
JWY2985	*MAT***a** *prp31::TRP1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* (*PRP31*-HA/pRS316)	This study

ments indicate that the U4, U5, and U6 snRNAs are associated with Prp31p. Coimmunoprecipitation experiments further reveal an association of the *PRP31* gene product with the pre-mRNA substrate that is dependent on U2 snRNP addition but is independent of the presence of the U6 snRNA. Taken together, these results demonstrate that Prp31p interacts with both the tri-snRNP and the prespliceosome and may serve as a stabilizing and/or recruiting factor that promotes the association of the U4/U6 · U5 tri-snRNP with the assembling spliceosome.

MATERIALS AND METHODS

In vitro splicing. Cell extracts were prepared as described previously (34). The following strains were used for generation of splicing extracts (Table 1), with relevant genotypes indicated in parentheses: BJ2168 (JWY2878; wild type), JWY2857 (*prp31-1*), JWY2985 [*prp31::TRP1* (*PRP31*-HA/pRS316)], and JWY2964 [*prp31::TRP1* (*PRP31*/pRS316)]. Radiolabeled substrate was produced by in vitro transcription of a synthetic actin substrate from the SP6 promoter (34). Splicing reactions were performed in a total volume of 25 μl, as described in reference 34. Splicing intermediates and products were separated by denaturing gel electrophoresis on 6% polyacrylamide (acrylamide-bisacrylamide, 29:1)–8 M urea gels in 100 mM Tris-borate-EDTA (TBE) at 450 V for 2.5 h.

Native gel electrophoresis. Endogenous ATP was depleted from splicing extracts by addition of 10 mM glucose (in place of 2 mM ATP) to the reaction mix and incubation at room temperature for 30 min. For analysis of commitment complexes, 5-μl aliquots of splicing reaction mixtures were quenched at the indicated time points with an equal volume of cold R buffer (2 mM magnesium acetate, 50 mM HEPES [pH 7.5]). One microliter (1 to 4 μg) of total yeast RNA and 2.5 μl of loading dye (50% glycerol, 250 mM TBE, 0.5% xylene cyanol, 1% bromophenol blue) were then added. Commitment complexes were resolved as described elsewhere (54), with the following modifications: the acrylamide-to-bisacrylamide ratio was 60:0.88, the gels were prerun at 135 V for 3.5 h at 4°C, and the gels were run at 65 V for 24 h at 4°C.

Vertical polyacrylamide gel electrophoresis was used to visualize prespliceosomes and spliceosomes, as described previously (5). Five-microliter aliquots of splicing reaction mixtures were quenched with an equal volume of cold stop mix (60 mM KPO_4 [pH 7.0], 3 mM $MgCl_2$, 3% polyethylene glycol 8000, 8% glycerol, 8 mg of heparin per ml); 1 μl of dye mix (stop mix with 0.5% xylene cyanol and 1% bromophenol blue) and 1 μl of total yeast RNA were added, and splicing complexes were resolved by electrophoresis on a 3% polyacrylamide (acrylamide-bisacrylamide, 19:1) gel in TAE buffer (Tris-acetate-EDTA [pH 8.0]) at 200 V for 5 h at 4°C, with one change of buffer.

Gel electrophoresis of U4/U6 complex. Assays were performed as described previously (20), using ~5 μg of RNA per sample. RNA complexes were separated by electrophoresis on 9% polyacrylamide (30:0.8)–50 mM TBE gels run at 300 V for 3 h at 4°C.

Coimmunoprecipitation and detection of snRNAs. Coimmunoprecipitation reactions were performed essentially as described previously (5). Briefly, protein A-Sepharose (PAS) beads (Sigma, St. Louis, Mo.) were reconstituted overnight at 4°C in 1 ml of NET (50 mM Tris [pH 7.5], 1 mM EDTA, 0.1% Nonidet P-40, 0.02% sodium azide) containing 150 mM KCl (NET-150). The beads were washed three times with 750 μl and suspended to ~800 μl in NET-150. One microgram of purified antihemagglutinin (anti-HA) antibodies (Berkeley Antibody Co., Richmond, Calif.) or 2 μg of anti-trimethyl guanosine antibody (a gift from J. Friesen) were added per 15 μl of beads; binding was permitted to occur for 4 h at 4°C. Antibody-bead complexes were washed three times in 500 μl of NET-150 and suspended to the original volume in SPL-150 without heparin (50 mM K_2HPO_4 [pH 7.0], 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 150 mM KCl). Splicing reactions were carried out as described above, except that the volumes were doubled. Following incubation at 18°C for 30 to 45 min, the reactions were stopped by addition of 10 μl of IP stop buffer (60 mM K_2HPO_4 [pH 7.0], 3 mM $MgCl_2$, 3% polyethylene glycol 8000, 8% glycerol, 150 mM KCl). Thirty microliters of PAS-antibody was added; binding was permitted for 2 h at 4°C. The beads were washed twice (5 min each wash) in 500 μl of NET-150 and once (5 min) in 500 μl of NET; all washes were done at 4°C with gentle shaking. One hundred microliters of NET supplemented with 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10 μg of *Escherichia coli* tRNA per ml, and 10 μg of proteinase K per ml was added to pellet and supernatant fractions, and samples were incubated at 37°C for 10 min. RNA was then extracted and precipitated. RNA species were resolved by electrophoresis on 6% polyacrylamide (acrylamide-bisacrylamide, 29:1)–8 M urea gels in 100 mM TBE at 450 V for 2.5 h. To test the specificity of RNA-protein interactions (see Fig. 3), 25 mg of heparin per ml was included in the IP stop buffer and 5 mg of heparin per ml was included in the SPL-150 buffer.

For oligonucleotide-directed cleavage reactions, 5.6 pmol of U6d1 or U2 delete oligonucleotide (Table 2) per μl of extract was added and samples were incubated at 30°C for 15 min. Reaction mix and radiolabeled substrate were then added, and samples were processed as described above.

Analysis of coimmunoprecipitation of snRNAs was performed as described previously (5), except that 30 μl of beads and an equal volume of splicing extract derived from yeast strain JWY2985 or JWY2964 were used. Following precipitation and extraction, RNA species were resolved by electrophoresis on denaturing polyacrylamide gels. RNA samples were suspended in 10 μl of diethyl pyrocarbonate (DEPC)-treated distilled water, heated at 95°C for 3 min, and quick-chilled on ice. An equal volume of urea dye (8 M urea, 20% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol, 2 M TBE [pH 8.0]) was added prior to loading. U4, U5, and U6 snRNAs were assayed by electrophoresis on 7% polyacrylamide (acrylamide-bisacrylamide, 30:0.8)–4 M urea gels in 100 mM TBE at 200 V for 3 h. RNA species were electroblotted onto a Nytran Plus membrane at 60 V for 45 to 60 min at 4°C in 100 mM TBE. The blots were prehybridized for 1 h at 23°C in 5× Denhardt's solution–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.1% SDS–100 μg of denatured salmon sperm DNA per ml. The hybridization solution was identical to that used for prehybridization but contained labeled oligonucleotides complementary to the snRNAs; oligonucleotide labeling was done as described below, using U4D, U5ew, or U6ew oligonucleotide (Table 2). The blots were hybridized overnight at 23°C; one 15-min wash and one 30-min wash were done, both in 5× SSPE at 23°C.

In order to detect the U1, U2, U4, U5, and U6 snRNAs simultaneously, samples were analyzed as described previously (50). Blots were hybridized with PCR-amplified fragments of the snRNA genes or with gene fragments derived from restriction enzyme digestion of plasmid constructs.

Oligonucleotide labeling. Oligonucleotides were end labeled by incubation with 10 U of T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and 50 μCi of [γ-^{32}-P]ATP in 1× polynucleotide kinase buffer (70 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 5 mM dithiothreitol) at 37°C for 1 h. The reactions were stopped by addition of an equal volume of stop mix (50 mM Tris [pH 7.5], 50 mM NaCl, 5 mM EDTA [pH 8.0], 0.5% SDS). Radiolabeled probes were separated from unincorporated nucleotides by column chromatography through 1 ml of Sephadex G-25 resin (Sigma).

Glycerol gradient fractionation of snRNP complexes. Eleven-milliliter continuous 10 to 30% glycerol gradients in buffer A (50 mM Tris [pH 7.4], 25 mM

TABLE 2. Oligonucleotides used

Oligonucleotide	Sequence	Description[a]
U2delete	5′ GAACAGACACTACACTTG 3′	nt 46–29 of U2 snRNA
U6ew	5′ CGGTTCATCCTTATGCAGGGG 3′	nt 86–66 of U6 snRNA
U5ew	5′ CTATGGAGACAACACCCGG 3′	nt 129–110 of U5 snRNA
U4D	5′ AGGTATTCCAAAAATTCCCTAC 3′	nt 158–137 of U4 snRNA
U6D	5′ AAAACGAAATAAATCTCTTTG 3′	nt 112–92 of U6 snRNA
SNR190	5′ GGCTCAGATCTGCATGTGTTGTATAACACTGG 3′	nt 158–190 of snR190
U6d1	5′ ATCTCTGTATTGTTTCAAATTGACCAA 3′	nt 54–28 of U6 snRNA

[a] nt, nucleotide.

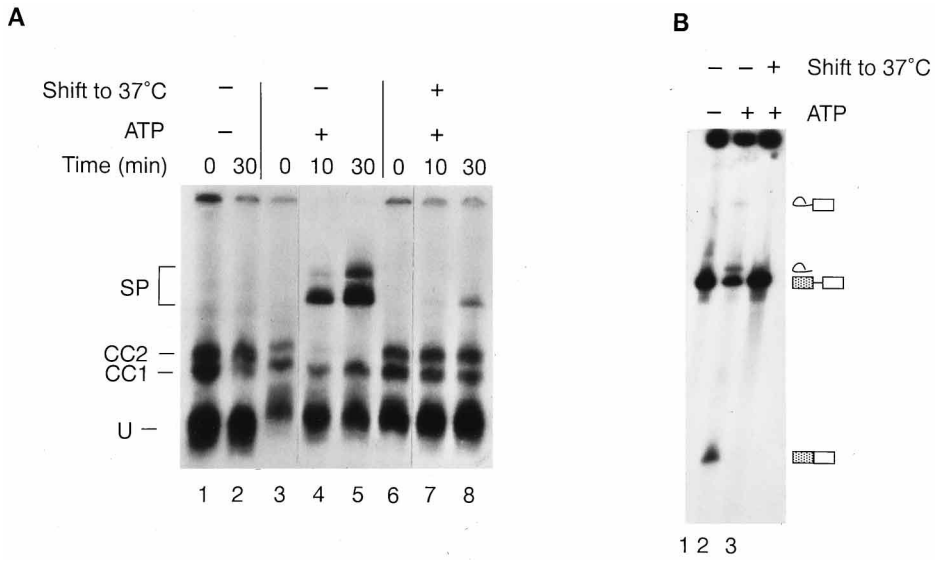

FIG. 1. Commitment complexes form in heat-inactivated *prp31* extracts. (A) Assay of commitment complex formation in heat inactivated *prp31-1* extracts. Extracts derived from a strain bearing the *prp31-1* mutant allele were either maintained on ice or heat inactivated at 37°C for 30 min. Radiolabeled pre-mRNA substrate was then added, and portions were withdrawn at the times indicated above the lanes and quenched on ice; samples were subjected to electrophoresis on nondenaturing composite gels. Splicing complex formation was also monitored in extracts depleted of ATP. Resolution of the higher-molecular-weight spliceosomal complexes (SP) varied in this gel system; levels of these species were not consistently depleted in heat-treated *prp31* extracts. CC1 and CC2, commitment complexes; U, nonspecific complexes, labeled as described in reference 45. (B) Splicing activity of the samples was monitored by subjecting RNA from a portion of the reactions to denaturing gel electrophoresis. ▨—□, pre-mRNA; ⌓—□, lariat intron–3′-exon intermediate; ⌓, lariat intron product; ▨□, spliced mRNA.

NaCl, 5 mM $MgCl_2$) were poured into Beckman 14- by 89-mm tubes by using an SG 15-ml linear gradient maker (Hoefer Scientific Instruments, San Francisco, Calif.). Splicing extract (90 μl) was combined with stock solutions so that final concentrations of 2.5 mM $MgCl_2$, 60 mM KPO_4, and 2 mM ATP were obtained; the volume was then adjusted to 100 μl with DEPC-treated distilled water. Samples were incubated at 23 or 37°C for 30 min prior to being loaded. Two hundred microliters of cold buffer A (12) was then added, and extracts were layered onto the gradients. The samples were spun at 37,000 rpm in an SW41 swinging-bucket rotor for 14 h at 4°C. Fractions of 0.4 ml were collected from each gradient; RNA was extracted from even-numbered fractions by addition of 35 μg of glycogen per ml and 0.4 ml of phenol-chloroform-isoamyl alcohol (50:49:1) and precipitated at −20°C. snRNA species were analyzed by Northern (RNA) analysis, as described previously (12).

RESULTS

Functional Prp31p is not required for commitment complex formation. Previous analyses had indicated that the Prp31 protein is required before or concomitant with the first cleavage reaction of splicing both in vivo and in vitro (38, 60). To distinguish which, if any, spliceosomal complexes form in heat-inactivated *prp31* extracts, nondenaturing (native) gel electrophoresis was utilized. Formation of commitment complexes was analyzed by subjecting splicing reaction mixtures to electrophoresis on a composite acrylamide-agarose gel. Extracts from *prp31-1* strain JWY2857 were maintained on ice or heat inactivated by incubation at 37°C. Synthetic actin transcript was then added; after incubation for the times indicated (Fig. 1), the reaction mixtures were quenched and subjected to gel electrophoresis. The two commitment complexes, CC1 and CC2, as well as more slowly migrating splicing complexes, were observed. Both commitment complexes contain the U1 snRNA (54); CC2 has, in addition, the Mud2 protein (2). Both commitment complexes formed in an ATP-independent fashion in vitro, whereas the larger spliceosomal complexes required ATP for formation (Fig. 1A, cf. lanes 1 and 2 with lanes 3 to 8). Commitment complexes were rapidly formed in *prp31* extracts regardless of heat treatment (Fig. 1A, lanes 3 to 8). These results contrast with those seen for extracts derived from strains containing mutant alleles of the *MUD1* or *PRP39* gene, each of which encodes U1 snRNP proteins, or of the *MUD2* gene, the product of which is also required for commitment complex formation (2, 33, 35), and indicate that Prp31p is not required for commitment complex assembly in yeast. The accompanying splicing assay demonstrates that although commitment complexes formed in *prp31* extracts depleted of ATP or heat inactivated prior to substrate addition, no lariat intron–3′-exon splicing intermediate or spliced mRNAs were detected, indicating that the splicing reaction was blocked prior to cleavage at the 5′ splice site (Fig. 1B, lane 3).

Functional Prp31p is required for assembly of the mature spliceosome from prespliceosomes. The requirement for Prp31p function in later stages of spliceosome assembly was assessed by examining the ability of the heat-inactivated *prp31* extracts to form prespliceosome and spliceosome complexes in vitro, utilizing a gel system that better resolves higher-order spliceosomal complexes (Fig. 2A). The prespliceosome corresponds to complexes containing the pre-mRNA substrate and the U2 snRNA, while the spliceosome contains unspliced message, splicing intermediates and products, and the U2, U5, and U6 snRNAs (45, 46). Formation of both the prespliceosome and the spliceosome requires ATP, indicated by the lack of these complexes in extracts that were depleted of ATP (data not shown). *prp31* extracts maintained on ice were able to form both prespliceosomes and mature spliceosomes (Fig. 2A, lanes 1 to 4), consistent with the splicing activity of these extracts (Fig. 2B, lane 2). Extracts that were heat inactivated prior to substrate addition formed prespliceosomes that appeared to migrate similarly to those detected in unheated extract; however, formation of mature spliceosomes was not detected in these extracts (Fig. 2A, lanes 5 to 8). As previously determined, the heat treatment also abolished the splicing activity of the extract (Fig. 2B, lane 3). These defects are not a general effect of heat treatment, as demonstrated by the abilities of heated

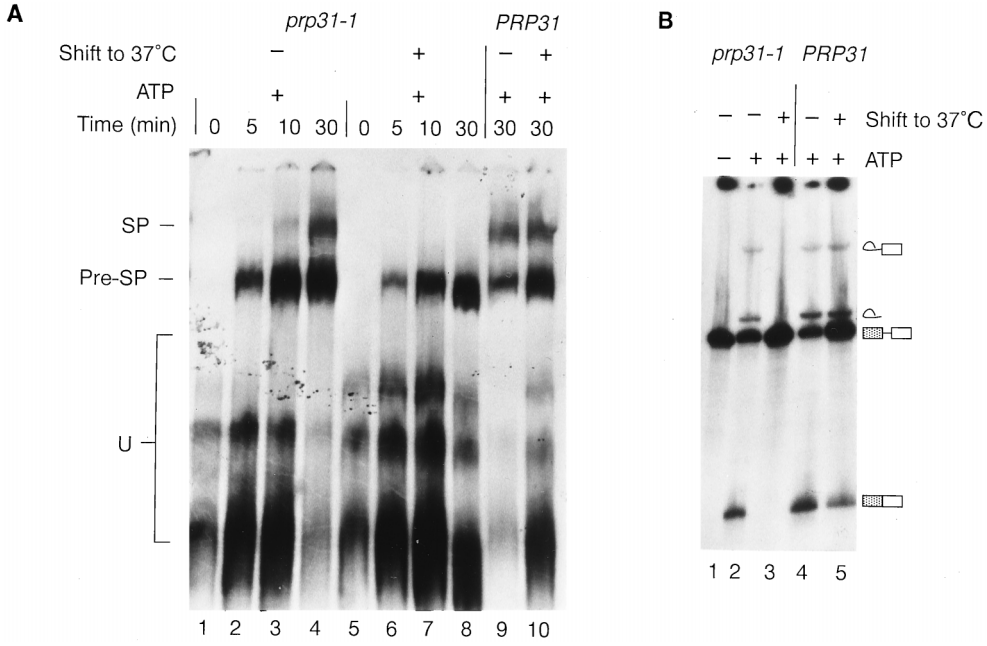

FIG. 2. Prespliceosomes, but not mature spliceosomal complexes, form in heat-inactivated *prp31* extracts. (A) Wild-type and *prp31* extracts were incubated as indicated above the lanes and assayed for splicing complex formation at various times after addition of radiolabeled substrate. Samples were subjected to electrophoresis on a nondenaturing 3% polyacrylamide gel. SP, mature spliceosome; Pre-SP, prespliceosome; U, nonspecific pre-mRNA complexes (54). (B) Denaturing gel electrophoresis of RNA from portions of the same reaction mixtures to assay formation of splicing intermediates and products. ▨—□, pre-mRNA; ⌓—□, lariat intron–3′-exon intermediate; ⌓, lariat intron product; ▨□, spliced mRNA.

wild-type extracts to form mature spliceosomes (Fig. 2A, lane 10) and to splice the radiolabeled substrate (Fig. 2B, lane 5).

Coimmunoprecipitation of U4, U5, and U6 snRNAs with the Prp31 protein. The requirement for Prp31p in assembly of spliceosomes, but not commitment complexes or prespliceosomes, suggests that Prp31p may be required for the association of the U4/U6 · U5 tri-snRNP with the assembling splicing complex. Similar inhibition of mature spliceosome formation has been seen in heat-inactivated extracts derived from *prp3*, *prp4*, *prp6*, and *prp8* mutants (4, 15, 23). Each of these Prp proteins is associated with the U5, U4/U6, or U4/U6 · U5 snRNP (1, 4, 7, 23, 36, 44). To examine whether Prp31p is a U5, U4/U6, or U4/U6 · U5 snRNP protein, immunoprecipitation of snRNA species with Prp31p was assayed. For this purpose, we used a completely functional allele of the *PRP31* gene bearing a triple repeat of the HA epitope (*PRP31*-HA) (60). Immunoprecipitation of the tagged protein was detected upon incubation of cell extracts derived from a *PRP31*-HA strain with antibodies that recognize the HA epitope (59). Extracts derived from a strain bearing the HA-tagged allele of the *PRP31* gene were immunoprecipitated with anti-HA antibodies in buffers containing concentrations of KCl ranging from 100 to 750 mM. Coimmunoprecipitated RNA species were extracted, separated by electrophoresis on polyacrylamide gels, and analyzed by Northern analysis using radiolabeled fragments from the snRNA genes as probes. In buffer containing up to 200 mM KCl, the U4, U5, and U6 snRNAs were coimmunoprecipitated with Prp31p-HA; U1 and U2 snRNAs were not detected in the immunoprecipitated pellet (Fig. 3, lane 1, and data not shown). In pellet fractions from immunoprecipitation samples containing 300 to 500 mM KCl, U5 snRNA was greatly decreased or no longer detectable, and the amount of U4 and U6 snRNAs immunoprecipitated was reduced (Fig. 3, lanes 2 and 3). At salt concentrations above 500 mM, no snRNAs were coimmunoprecipitated (Fig. 3, lane 4). Analysis of the supernatant fractions confirmed the presence of all five snRNA species in all samples (data not shown). The observation that antibodies to Prp31p-HA immunoprecipitate U5 snRNA only at low salt concentrations suggests that Prp31p is associated with this RNA in the context of the U4/U6 · U5 tri-snRNP and is not an integral U5 snRNP protein. The tri-snRNP dissociates to U5 and U4/U6 snRNPs in the presence of high KCl concentrations (>350 mM) (1, 7, 8, 16). Coimmunoprecipitation of the U4 and U6 snRNAs at slightly higher salt concentrations indicates a more stable association of Prp31p with these RNA species.

Levels of U4/U6 are not altered but free U4 snRNA accumulates in a *prp31-1* strain at the nonpermissive temperature. Failure of the tri-snRNP to associate with the assembling spliceosome in inactivated *prp31-1* extracts could indicate a direct requirement for Prp31p in the addition of U4/U6 · U5 to the prespliceosome; alternatively, or in addition, the *prp31-1* mutation may result in a failure of the U4/U6 snRNP to associate with the U5 snRNP and form the U4/U6 · U5 particle or in disruption of the U4/U6 interaction prior to association with U5. To begin to address these different possibilities, nondenaturing gel electrophoresis was utilized to assay amounts of the U4 and U6 snRNAs and the U4/U6 duplex in a *prp31-1* mutant strain. This assay relies on the ability of radiolabeled oligonucleotides complementary to the U4 and U6 snRNAs to anneal to their templates at temperatures below the melting temperature of the U4/U6 complex (53°C) (14). The three oligonucleotide-RNA species are then separated by nondenaturing gel electrophoresis (32).

In wild-type cells that were maintained at 23°C or shifted to 37°C for 4 h, the relative amounts of U4, U6, and U4/U6 snRNAs were unchanged (Fig. 4, *PRP31* lanes). The primary species observed was the U4/U6 complex; some free U6 and very few free U4 snRNAs were detected. In a *prp31-1* strain that was maintained at 23°C, a pattern similar to that seen for

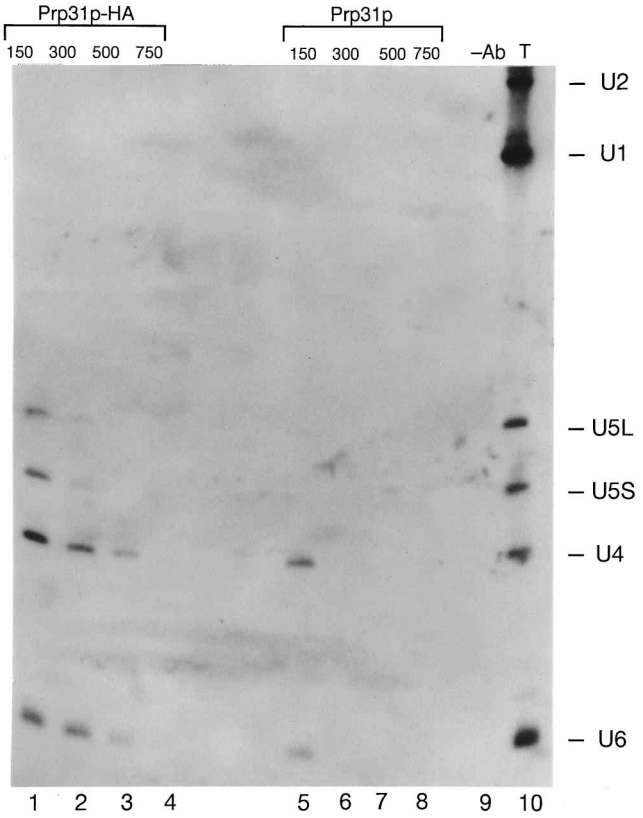

FIG. 3. The U4, U5, and U6 snRNAs are coimmunoprecipitated with Prp31p-HA. Extracts derived from a *PRP31*-HA strain or a strain bearing an untagged allele of the *PRP31* gene were subjected to immunoprecipitation using anti-HA monoclonal antibodies; RNA extracted from immunoprecipitated pellets is shown. Lanes 1 to 4, immunoprecipitation reactions done with Prp31p-HA extracts; lanes 5 to 8, reactions done with extracts containing the untagged Prp31 protein. Immunoprecipitation reactions were performed with increasing concentrations of KCl in the binding and wash buffers, as indicated (millimolar) above each lane. Lane 9, control reaction in which PAS beads not conjugated to antibodies were incubated with extracts containing Prp31p-HA; lane 10, total yeast RNA, to show migration of the snRNAs.

the wild-type strain was observed (Fig. 4, minus lanes). When the mutant strain was shifted to the nonpermissive temperature of 37°C, the U4/U6 complex was still the primary species observed; however, free U4 snRNA was also detected at the earliest time point assayed, by which time unspliced pre-mRNAs accumulate (Fig. 4, second lane, and data not shown). We quantified the amounts of U4, U6, and U4/U6 complexes compared to that of the small nucleolar RNA snR190 at 37 versus 23°C. snR190 plays a role in rRNA processing (62) and thus should not be affected in the *prp31-1* strain. The relative amounts of the U6 snRNA and the U4/U6 complex compared to that of snR190 did not decrease to a significant degree in the *prp31* mutant strain after a shift to the nonpermissive temperature.

Since the levels of the U4/U6 snRNP complexes are not noticeably affected after the temperature shift, the free U4 snRNA that accumulates at 37°C is not likely to be derived entirely from destabilized U4/U6 snRNP complexes. In contrast, heat inactivation of the U4/U6 core snRNP protein Prp3p in extracts of a *prp3-1* Ts^- mutant results in marked decreases in U6 and U4/U6 snRNA levels (4). These differences in snRNA complex stabilities in the different *prp* strains are consistent with a more indirect effect on U4/U6 assembly or stability in a *prp31-1* strain than in a *prp3-1* strain.

U4/U6 · U5 tri-snRNP complexes form in heat-inactivated *prp31-1* extracts. To further examine the role of Prp31p in U4/U6 and tri-snRNP biogenesis and stability, the populations of snRNP complexes in wild-type and *prp31-1* mutant cells were examined by glycerol gradient fractionation of splicing extracts. This procedure effectively separates the U6, U4/U6, U5, and U4/U6 · U5 snRNPs in an extract; RNA blot analysis of gradient fractions can then be utilized to analyze patterns of snRNP formation in extracts incubated under permissive or nonpermissive conditions. Differences in distribution patterns between wild-type and mutant extracts are indicative of defects in particle assembly or stability. Similar studies have indicated that Prp6p and Prp8p are required for tri-snRNP assembly from the U4/U6 and U5 snRNPs, while a mutation in the *PRP4* gene disrupts U4/U6 complexes (15, 23).

In extracts derived from a wild-type strain, all four snRNPs were observed when the extract was maintained at 23°C prior to being loaded (Fig. 5C); similar patterns were detected if the extract was incubated at 37°C (Fig. 5D). Free U6 snRNP migrated near the top of the gradient, followed by the U4/U6 complex. The U5 snRNP tends to spread across a number of fractions in these gradients but was observed as a free particle in the middle fractions. The U4/U6 · U5 tri-snRNP migrates near the bottom of the gradient. Under permissive conditions, *prp31* extracts generated a pattern similar to that seen in wild-type extracts (Fig. 5A). In extracts that were incubated at 37°C prior to loading (Fig. 5B), free U6, U4/U6, and U5 complexes were detected. Tri-snRNPs also formed, although to a lesser extent than in heated wild-type extracts. Under these conditions, splicing in the *prp31* extract was completely inactivated; no spliced products or intermediates were detected (data not shown). This result differs from gradient analyses of *prp3*, *prp4*, *prp6*, and *prp8* heat-inactivated extracts, all of which demonstrate severe particle instability at the nonpermissive temperature; no tri-snRNPs can be detected in any of these extracts at the nonpermissive temperature (4, 15, 23).

Prp31p is present in splicing complexes containing pre-mRNA. The results presented thus far indicate that Prp31p is associated with U4/U6 and U4/U6 · U5 snRNPs but is involved primarily in assembly of the tri-snRNP complex onto the spliceosome rather than in maintenance of snRNP integrity. To further elucidate the role of Prp31p in spliceosome

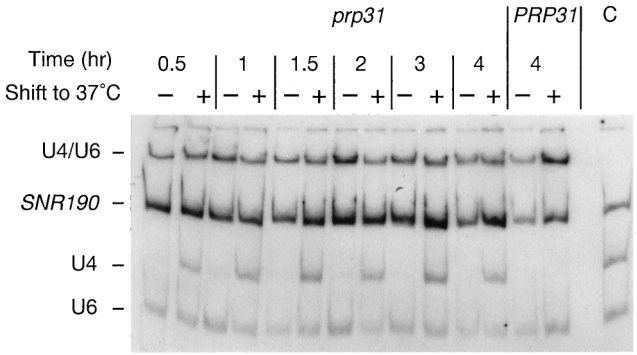

FIG. 4. Free U4 snRNA accumulates rapidly after a *prp31-1* strain is shifted to 37°C, while levels of U6 snRNA and the U4/U6 complex remain unchanged. RNA was isolated from a *prp31-1* strain maintained at 23°C or shifted to 37°C for the times indicated above the lanes. Radiolabeled oligonucleotides complementary to the U4, U6, and snR190 RNAs were annealed to their templates under conditions in which the U4/U6 base-pairing interaction remains stable. Complexes were separated by nondenaturing gel electrophoresis. Lane 15, control reaction in which the sample was heated to 70°C prior to addition of radiolabeled oligonucleotides; this control marks the migration of the free U4 and U6 species.

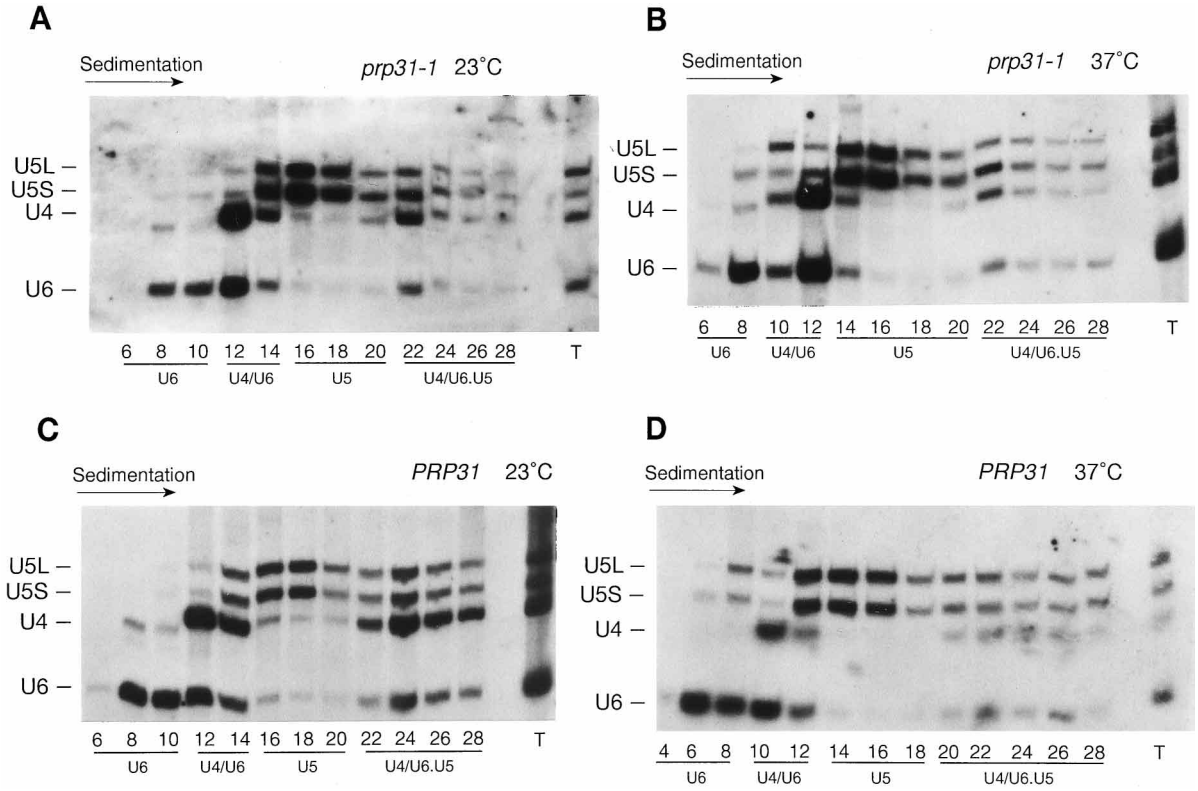

FIG. 5. Tri-snRNPs are stably formed in heat-inactivated *prp31* extracts. Extracts derived from a *prp31* strain (A and B) or a wild-type strain (C and D) were maintained on ice or incubated at 37°C for 30 min. Samples were layered onto 10 to 30% glycerol gradients, and snRNP complexes were separated by centrifugation. Following fractionation, RNA was isolated from even-numbered gradient fractions to assay for the presence of the U6, U5, U4/U6, and U4/U6 · U5 snRNPs. Northern analysis was performed using radiolabeled oligonucleotides complementary to U4, U5, and U6 snRNAs. Lanes T, total yeast RNA.

formation, we examined whether Prp31p is present with pre-mRNA in splicing complexes. To do so, we assayed coimmunoprecipitation of mRNA species with Prp31p. Splicing reactions were performed, using extracts bearing the HA epitope-tagged Prp31 protein as the only source of Prp31p. Following incubation under standard splicing conditions, samples were immunoprecipitated by using anti-HA antibodies coupled to PAS beads. Analysis of radiolabeled mRNA species present in pellet fractions by denaturing gel electrophoresis revealed the presence of pre-mRNA in samples containing tagged protein (Fig. 6, lane 2). No nonspecific coimmunoprecipitation of radiolabeled RNAs was detected in samples to which no antibodies were added (Fig. 6, lane 4). The interaction of Prp31p with the splicing substrate was also observed in the presence of heparin (Fig. 6, lane 6). Heparin treatment destabilizes weak RNA-protein interactions; therefore, this result confirms the specificity of the interaction observed in the immunoprecipitation experiments. Similar experiments were performed using antibodies to the trimethyl guanosine cap present on the U1, U2, U4, and U5 snRNAs; under these conditions, the lariat intron–3′-exon intermediate and the lariat intron product were detected in addition to the pre-mRNA (Fig. 7, lane 9); this result is consistent with the association of at least a subset of the snRNAs with the spliceosome throughout the various cleavage reactions of splicing. Failure to coimmunoprecipitate splicing intermediates with Prp31p-HA may indicate that Prp31p does not remain associated with the spliceosome subsequent to the first cleavage reaction; alternatively, conformational rearrangements might obscure the epitope in the functional spliceosome.

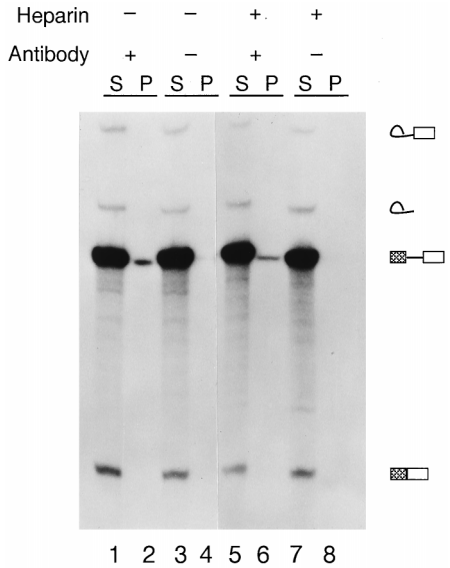

FIG. 6. Pre-mRNA is coimmunoprecipitated with Prp31p-HA. Splicing reactions with extracts derived from a strain bearing the *PRP31*-HA allele were immunoprecipitated with anti-HA monoclonal antibodies. For lanes 5 to 8, heparin was included in the IP stop buffer and the binding buffer. RNA was extracted from supernatant (S) and pellet (P) fractions and analyzed by denaturing gel electrophoresis. Lanes 3, 4, 7, and 8, control reaction mixtures to which PAS beads without antibody were added. In longer exposures, no intron product or lariat–3′-exon splicing intermediates were detected in pellet fractions. ▩—□, pre-mRNA; ⌒—□, lariat intron–3′-exon intermediate; ⌒, lariat intron product; ▩□, spliced mRNA.

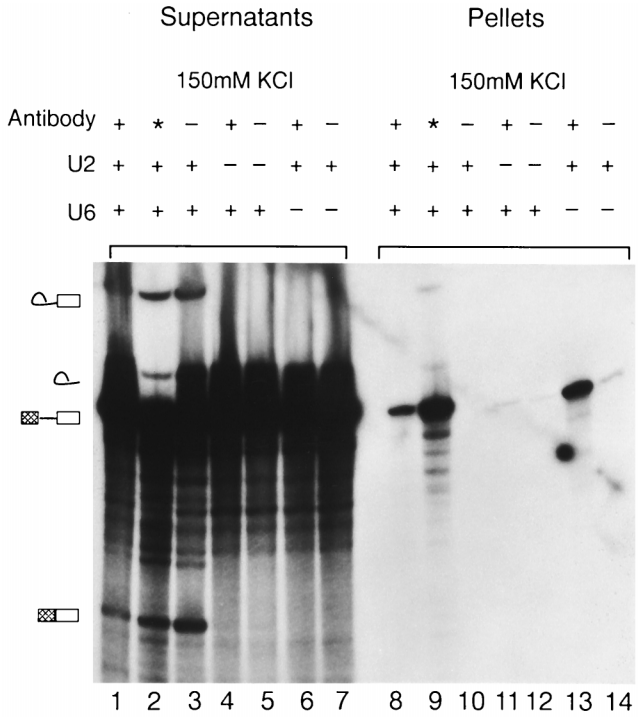

FIG. 7. Coimmunoprecipitation of pre-mRNA with Prp31p requires the presence of U2, but not U6, snRNA. Immunoprecipitation reactions were performed with extracts derived from a *PRP31*-HA strain. For the reactions in lanes 4, 5, 11, and 12, oligonucleotide-directed RNase H cleavage was used to deplete the extract of the U2 snRNA prior to initiation of the splicing reaction. For reactions in lanes 6, 7, 13, and 14, extracts were depleted of the U6 snRNA prior to addition of radiolabeled substrate. Lanes 1 to 7, RNA species present in the supernatant fractions of the immunoprecipitation reactions; as expected, depletion of the U2 or U6 snRNA blocks the formation of splicing intermediates and products (lanes 4 to 7). Lanes 8 to 14, pellet fractions from the same samples; lanes 2 and 9 (asterisks), reaction mixtures subjected to immunoprecipitation with antibodies to the trimethyl guanosine cap present on the U1, U2, U4, and U5 snRNAs; lanes 3, 5, 7, 10, 12, and 14, control reaction mixtures to which no antibodies were added.

Prp31p is present with pre-mRNA in prespliceosomes but not in commitment complexes. As described in previous sections, analysis of splicing complex formation in extracts derived from a *prp31-1* strain indicated that commitment complexes and prespliceosomes were capable of assembling following heat inactivation. These results indicate that Prp31p might be associated with these complexes or, alternatively, that the protein might be present but not required for these early assembly steps. To determine the presence or absence of Prp31p in early splicing complexes, oligonucleotide depletion experiments were utilized to block formation of specific splicing complexes. An oligonucleotide complementary to sequences in the U2 snRNA was added to extracts bearing HA-tagged Prp31p; endogenous RNase H activity is sufficient to direct cleavage under these conditions (52). Prespliceosome complex formation and splicing were completely blocked in the treated extracts (Fig. 7, lanes 4 and 5, and data not shown). Under these conditions, commitment complexes can form (35, 54). Depletion of the U2 snRNA eliminated coimmunoprecipitation of pre-mRNA to background levels (Fig. 7, lane 11); prolonged overexposure of the gels failed to reveal any other immunoprecipitated species. These results suggest that Prp31p is not stably associated with the splicing substrate in the absence of the U2 snRNA. Alternatively, low levels of commitment complexes in the extracts may limit the detectability of immunoprecipitated products, or the HA epitope may be masked under these conditions.

To analyze whether Prp31p is associated with prespliceosomes prior to addition of the U4/U6 · U5 snRNP, oligonucleotide-directed cleavage experiments were performed to deplete extracts of U6 snRNA. Depletion of U6 completely blocked the formation of spliceosomes from prespliceosomes (data not shown), as previously observed (17, 19), and abolished the splicing activity of the extract (Fig. 7, lanes 6 and 7). Under these conditions, however, pre-mRNA was coimmunoprecipitated with Prp31p-HA (Fig. 7, lane 13). The sensitivity of this association to increased salt concentration was assessed; specific coimmunoprecipitation of pre-mRNA was still observed when the concentration of KCl was increased to 300 mM, though the levels of background coimmunoprecipitation were variable (data not shown). These results indicate that the Prp31 protein is present with the splicing substrate in the prespliceosome independently of addition of the U4/U6 · U5 tri-snRNP to form the spliceosome.

DISCUSSION

It has been shown previously that *prp31* mutants are defective in pre-mRNA splicing in vivo and in vitro (38, 60). In this paper, we describe experiments to determine at which steps in spliceosome assembly or splicing Prp31p is required and with which molecules Prp31p is associated. Analysis of splicing complex formation in *prp31* extracts indicates that commitment complexes and prespliceosomes form following heat inactivation, but assembly of the spliceosome is blocked (Fig. 1 and 2). Coimmunoprecipitation assays indicate that Prp31p is associated with U4, U6, and U5 snRNAs in the U4/U6 · U5 tri-snRNP (Fig. 3) and with the prespliceosome (Fig. 6 and 7). The amounts of each of the snRNAs or of the snRNPs are not significantly decreased in heat-inactivated *prp31* extracts (Fig. 4 and 5). These data are consistent with a primary role for Prp31p in tethering the tri-snRNP to the spliceosomal complex, rather than in the formation or maintenance of individual snRNP complexes. The *prp31* mutant is distinct from other yeast mutants defective in assembly of spliceosomes from prespliceosomes. Mature spliceosome complex formation is blocked upon inactivation or depletion of Prp3p, Prp4p, Prp6p, and Prp8p (1, 4, 7, 15). However, unlike the case for *prp31* mutants, the defect in spliceosome assembly in *prp3*, *prp4*, *prp6*, or *prp8* mutant extracts results from a loss of U4/U6 · U5 tri-snRNPs (4, 15, 23) rather than a failure to assemble the tri-snRNPs with the prespliceosome. Thus, Prp31p is the first identified yeast protein necessary for assembly of tri-snRNPs with the prespliceosomes.

The results of coimmunoprecipitation of snRNAs with anti-HA antibodies in a *PRP31*-HA extract indicate that Prp31p is associated with the U4/U6 · U5 tri-snRNP, similarly to Prp6p. For both proteins, U4, U5, and U6 snRNAs are coimmunoprecipitated in low salt concentrations (150 to 300 mM NaCl); U5 snRNA is no longer precipitated above 300 mM NaCl, and U4 and U6 snRNAs are not efficiently precipitated at higher salt concentrations (1, 23). Thus, Prp6p and Prp31p may be tri-snRNP-specific proteins that associate via the U4/U6 moiety. Disruption of the U4/U6 · U5 complex to separate U4/U6 and U5 snRNPs in high salt concentrations might allow some residual coimmunoprecipitation of the U4/U6 snRNP. In contrast, Prp3p and Prp4p are coimmunoprecipitated with U4 and U6 snRNAs at salt concentrations up to 750 mM and thus are presumed to be integral components of the U4/U6 snRNP (4, 7, 44, 61); Prp8p is similarly classified as a

U5-specific snRNP protein (36), and Prp24p is tightly associated with U6 snRNA (56).

Precipitation of pre-mRNA with antibodies to Prp31p-HA indicates that the protein is a component of the spliceosome, at least during the assembly stages. This result is consistent with a role for Prp31p in conversion of the prespliceosome to the spliceosome. That depletion of U2 snRNA prior to immunoprecipitation abolishes the coimmunoprecipitation of pre-mRNA suggests that Prp31p joins the assembling spliceosome subsequent to or concomitantly with prespliceosome formation. Alternatively, the protein may be present in an alternate, inaccessible conformation until the U2 snRNP and associated factors assemble onto the commitment complex.

Coimmunoprecipitation experiments further demonstrate that although Prp31p appears to be associated with the U4/U6 · U5 snRNP (Fig. 3), the U6 snRNA, and therefore the tri-snRNP, is not required for association of Prp31p with pre-mRNA (Fig. 7). These results suggest that Prp31p associates with both the prespliceosome and the tri-snRNP, perhaps serving to recruit the U4/U6 · U5 snRNP onto the prespliceosome, e.g., by dimerizing or by associating with other protein factors present in prespliceosomes or tri-snRNPs. Another possibility is that the *PRP31* protein is a tri-snRNP component that has affinity for the pre-mRNA or some factor present on the prespliceosome. Depletion of the U6 snRNA in extracts might destabilize interaction of Prp31p with snRNP complexes, allowing the protein to dissociate and interact with the splicing substrate in the absence of the U4, U5, and U6 snRNAs. An alternative model, which we think unlikely, is that upon depletion of U6 snRNA, an aberrant U4/U5 particle containing Prp31p is present in the extracts. If such a U4/U5 complex can assemble with the prespliceosome in the absence of U6 snRNA, antibodies to Prp31p-HA would coimmunoprecipitate the pre-mRNA artifactually. Experimental evidence from both mammalian and yeast systems does support the existence of a U4/U5 complex in U6-depleted extracts (10, 30, 55). However, evidence does not support the association of U4, U5, or U6 snRNAs onto the spliceosome in the absence of a tri-snRNP. Depletion of U4 and U6 snRNAs in mammalian systems (10) or of U5 snRNA in mammalian or yeast extracts (10, 30, 55) blocks splicing complex assembly subsequent to formation of the prespliceosome. These data are consistent with our observation that prespliceosomal complexes are formed in U6-depleted extracts, but no aberrantly migrating species of higher molecular weight, representing a U4/U5 complex associated with the prespliceosome, are detected.

The levels of U4, U6, and U4/U6 species are not decreased upon shifting of *prp31* mutant cultures to 37°C. These results differ from those for mutants defective in other known U4/U6, U5, U6, or U4/U6 · U5 snRNP proteins. Upon heat inactivation of *prp3*, *prp4*, *prp6*, *prp24*, or *prp38* mutants, levels of U6 snRNA rapidly decrease 3- to 20-fold; levels of other snRNAs are unchanged (11, 23). Heat inactivation of the *prp8-1* mutant has no effect on snRNA levels, but depletion of Prp8p using a galactose-dependent *GAL::PRP8* strain results in reduction of levels of U4, U5, and U6 snRNAs (11, 15). Inactivation or depletion of these snRNP proteins may affect the structure or stability of the snRNPs, leading to degradation of one or more of the snRNA constituents (11, 15). The failure to observe decreased amounts of U4, U6, or U4/U6 snRNAs in *prp31* mutants at 37°C (Fig. 4) is consistent with there being little or no change in the levels of snRNPs in heat-inactivated *prp31* extracts (Fig. 5).

Although U4, U6, or U4/U6 snRNA levels in the *prp31* mutant at 37°C do not decrease, the amounts of free U4 snRNA increase. Similar results were obtained with strains containing mutations in the *SNR14* gene encoding U4 snRNA or in the *SNR6* gene encoding U6 snRNA. Yeast strains containing a mutation of G to C at position 14 (G14C mutation) in U4 snRNA, which destabilizes U4/U6 interactions, have fivefold more U4 snRNA than wild-type strains (56); the excess U4 snRNA appears to sequester excess free U6 snRNA, resulting in increased levels of the U4/U6 snRNP and decreased levels of free U6 snRNP. Strains bearing an A62G or A79G mutation in U6 snRNA, which hyperstabilizes the U6 3′ stem and thus prevents efficient base-pairing between U4 and U6 snRNAs, accumulate free U4 snRNA (20). Increased levels of U4 snRNA may therefore reflect a response to alterations in the amounts or functionality of U4/U6 and U4/U6 · U5 tri-snRNP complexes.

The biochemical analyses described here indicate that Prp31p interacts stably with both the splicing substrate and the tri-snRNP to promote conversion of the prespliceosome to the active spliceosomal complex. Evidence from metazoan splicing extracts as well as yeast indicates that, in addition to RNA-RNA base-pairing interactions, assembly of the U1 and U2 snRNPs with the pre-mRNA is mediated by multiple protein-protein and protein-RNA interactions (reviewed in references 27, 31, 40, 47, 48, and 58). Much less is known about the mechanism of association of the U4/U6 · U5 tri-snRNP with the prespliceosome prior to formation of the active spliceosome. Are specific associations established between the U4/U6 · U5 tri-snRNP and the prespliceosome before U4/U6 base-pairing is destabilized and before U6 snRNA base-pairs with U2 snRNA and the 5′ splice site? In HeLa cells, five U4/U6 · U5 tri-snRNP-specific proteins have been identified; like Prp31p and Prp6p in yeast, these proteins appear to be necessary for formation of a stable U4/U6 · U5 complex, but their role, if any, in association of the tri-snRNP with splicing complexes has not been determined (8, 57). Likewise, a number of proteins in *S. cerevisiae* have been identified as associated with the U4/U6 · U5 snRNP (18), including one species that migrates similarly to Prp31p in SDS-polyacrylamide gel electrophoresis analyses. However, the function of the majority of these proteins in either assembly or function of the tri-snRNP has not been established. Recently, however, Roscigno and Garcia-Blanco (49) have found that assembly of the U4/U6 · U5 tri-snRNP with prespliceosomes in HeLa nuclear extracts requires SR proteins. Further analysis of the precise function of these SR proteins in mammalian extracts and of Prp31p in yeast should increase our understanding of the interactions required to stably maintain protein-RNA associations that occur during the formation of the spliceosome and the processing of the RNA substrate.

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