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Interactions between Highly Conserved U2 Small Nuclear RNA Structures and Prp5p, Prp9p, Prp11p, and Prp21p Proteins Are Required To Ensure Integrity of the U2 Small Nuclear Ribonucleoprotein in *Saccharomyces cerevisiae*

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Binding of U2 small nuclear ribonucleoprotein (snRNP) to the pre-mRNA is an early and important step in spliceosome assembly. We searched for evidence of cooperative function between yeast U2 small nuclear RNA (snRNA) and several genetically identified splicing (Prp) proteins required for the first chemical step of splicing, using the phenotype of synthetic lethality. We constructed yeast strains with pairwise combinations of 28 different U2 alleles with 10 *prp* mutations and found lethal double-mutant combinations with *prp5*, *-9*, *-11*, and *-21* but not with *prp3*, *-4*, *-8*, or *-19*. Many U2 mutations in highly conserved or invariant RNA structures show no phenotype in a wild-type *PRP* background but render mutant *prp* strains inviable, suggesting that the conserved but dispensable U2 elements are essential for efficient cooperative function with specific Prp proteins. Mutant U2 snRNA fails to accumulate in synthetic lethal strains, demonstrating that interaction between U2 RNA and these four Prp proteins contributes to U2 snRNP assembly or stability. Three of the proteins (Prp9p, Prp11p, and Prp21p) are associated with each other and pre-mRNA in U2-dependent splicing complexes in vitro and bind specifically to synthetic U2 snRNA added to crude splicing extracts depleted of endogenous U2 snRNPs. Taken together, the results suggest that Prp9p, -11p, and -21p are U2 snRNP proteins that interact with a structured region including U2 stem loop IIa and mediate the association of the U2 snRNP with pre-mRNA.

Splicing of nuclear pre-mRNA requires a sophisticated ribonucleoprotein (RNP) complex called the spliceosome. The spliceosome is built on an intron-containing transcript by the sequential binding of small nuclear RNPs (snRNPs) to each other and to specific sites on the transcript, so that the pre-mRNA is properly arranged for splicing (20, 21). Before, during, and after the cleavage-ligation steps of splicing take place, the spliceosome is acted upon by a series of extrinsic factors, some of which bind transiently and trip a limited set of steps in the sequence (15, 29, 48). The events that define the transition from one state to the next during spliceosome assembly and function are both compositional and conformational: snRNPs and extrinsic factors are added and removed, and RNA-RNA interactions between the small nuclear RNAs (snRNAs) or between the snRNAs and the pre-mRNA within the spliceosome may be established and dissolved (42). Splicing factors include a family of proteins with ATP binding domains suggesting a means for driving the process forward and ensuring the accuracy of events (20, 21).

The expectation that alternative splicing is achieved by regulated spliceosome assembly has placed the early steps of splicing complex formation under close scrutiny. Using substrate commitment experiments, several investigations have led to a similar set of conclusions for both systems (27, 32, 38, 49, 50). Substrate (also referred to as template) commitment is defined as an event that leads to the inclusion of highly labeled pre-mRNA substrate molecules in later complexes despite the subsequent addition of a large excess of unlabeled substrate. By this definition, U1 snRNPs stably bind pre-mRNA in a fashion dependent on correct 5′ splice site and either branchpoint (yeast) or polypyrimidine 3′ splice site (mammalian) sequences to form a commitment complex in a reaction requiring neither ATP nor the U2 snRNP (for a review, see reference 42). Following assembly of the commitment complex, U2 snRNP binds near the branchpoint and its association is stabilized in an ATP-dependent step to form the prespliceosome (complex III or B in yeast extracts, A complex in mammalian cells). Spliceosomes are formed when the U4/6.5 multi-snRNP complex binds to the prespliceosome (42). Some complexes observed in vitro are clearly related to splicing, requiring correct signals in pre-mRNA and snRNPs for their formation, but are refractory to the chase (for example, the mammalian -ATP complex [28]) or occur in defiance of an apparent ATP requirement (for example, the U1-4U complex [33]). Whether the commitment phenomena observed in vitro represent commitment during regulation of splicing in vivo is an open question.

In the yeast system, the biochemical approaches to the question of spliceosome assembly have been complemented by the isolation and characterization of temperature-sensitive mutations defective in splicing (the *prp* mutants [21, 45]). Among the elements required for steps leading to the formation of the prespliceosome in yeast extracts are the *PRP5* (46), *PRP9* (1, 31, 46), *PRP11* (10, 46), and *PRP21/SPP91* (2, 11) gene products, as well as parts of U1 snRNA (33) and U2 snRNA (56). The relationship of the functions of these components to each other in the pathway has not been determined. In some cases, the protein sequence provides clues; for example, the Prp5 protein contains an ATP binding domain, suggesting a role in an ATP-dependent step (16). In other cases,

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protein-protein interactions have been detected. Genetic suppression data (11) and results using the yeast two-hybrid system for detecting protein-protein interactions (14, 18) show that *PRP21* interacts with *PRP9* (30, 31). Similarly *PRP11* interacts with *PRP21* and possibly indirectly with *PRP9* through its interaction with *PRP21* (30). Genetic interactions between pairwise combinations of the proteins also suggest functional interactions (46). How these protein-protein interactions contribute to the process of U2 snRNP addition to the assembling spliceosome remains to be determined.

Relationships between the yeast Prp proteins and mammalian splicing factors, snRNP proteins, and proteins associated with purified presplicing complexes have been detected (7, 8, 19). Two mammalian proteins with structural similarity to yeast Prp9p and Prp11p are found associated with a 17S form of the U2 snRNP and are components of the biochemically defined splicing factor SF3a (5, 8, 9). SF3a cannot bind to U2 snRNP in the absence of another biochemically defined splicing factor, SF3b (8). The mammalian homologs to Prp9p and Prp11p are also found associated with purified prespliceosomes (7). Oligonucleotide-directed RNase H degradation of U2 snRNA in extracts shows that binding of these proteins to form the 17S U2 snRNP requires the highly conserved 5′ half of U2 (6).

Genetic studies of yeast U2 have identified RNA elements required for U2 function during splicing, including a site of interaction with the intron branchpoint (44), a conserved internal U2 stem loop IIa (4), and a region required for the second step of splicing (36) that interacts with U6 snRNA (35). Mutations in stem IIa that cause cold-sensitive growth also cause cold-sensitive prespliceosome formation (56), indicating a role for this structure in spliceosome assembly. We have been interested in how the U2 snRNP is incorporated into the spliceosome and are using the genetic system of the yeast *Saccharomyces cerevisiae* to identify proteins or RNAs that influence the function of U2 snRNA. In this report we describe synthetic lethal interactions (20a, 23) between a panel of U2 snRNA mutations and mutations in the yeast *PRP5*, *-9*, *-11*, and *-21* genes. Some of the U2 mutations are in highly conserved residues and have no detectable phenotype in a wild-type background (3) yet are lethal if the strain carries a temperature-sensitive *prp* mutation. Using a glucose-repressible wild-type U2 gene (40, 49), we show that interactions between U2 and Prp5p, -9p, -11p, and -21p are important for U2 snRNP stability in vivo. We also provide biochemical evidence that three of the proteins are associated with pre-mRNA in a complex that requires U2 snRNP for its formation. The same three proteins bind synthetic U2 RNA added to crude splicing extracts depleted of endogenous U2, suggesting they are part of the U2 snRNP.

MATERIALS AND METHODS

Yeast strains. Yeast strains were grown according to standard procedures (51). Strains carrying the *prp* mutations are listed below. These strains were provided by S. Ruby, and their construction has been described elsewhere (46). The strains used have the following genotypes: SRYWTa, *MAT***a** *PRP*$^+$ *ade*$^-$ *his3-Δ200 his4 leu2 trp1*$^-$ *ura3-52*; SRY3-1b, *MAT*α *prp3-1 ade*$^-$ *his3 leu2 tyr1 ura*$^-$; SRY4-1b, *MAT*α *prp4-1 ade*$^-$ *leu2-3,112 lys2 ura3-52*; SRY5-1b, *MAT*α *prp5-1 ade*$^-$ *his3-Δ200 leu2 tyr1 ura3-52*; SRY5-3b, *MAT*α *prp5-3 ade*$^-$ *his3Δ leu2-3,112 tyr1 ura3-52*; SRY8-1b, *MAT*α *prp8-1 ade*$^-$ *his3-Δ200 leu2 trp*$^-$ *tyr1 ura3-52*; SRY9-1d, *MAT*α *prp9-1 ade*$^-$ *his3-Δ200 leu2 tyr1 ura3-52*; SRY9-2f, *MAT*α *prp9-2 ade*$^-$ *his3-Δ200 leu2 trp*$^-$ *tyr1 ura3-52*; SRY11-1d, *MAT*α *prp11-1 ade*$^-$ *his*$^-$ *his4 leu2 tyr1 ura3-52*; SRY19-1b, *MAT*α *prp19-1 ade*$^-$ *his*$^-$ *leu2 trp1 tyr1 ura3-52*; and SRY21-1b, *MAT*α *prp21-1 ade*$^-$ *his*$^-$ *leu2 tyr1 ura3-52*.

Each of these strains was transformed with a *Hin*dIII fragment carrying sequences in the order U2 promoter distal-*URA3-GAL*-U2 promoter proximal such that the fragment is targeted to the U2 locus and replaces the wild-type U2 promoter with a *GAL*-regulated U2 promoter (24, 40). Transformants were selected on synthetic complete medium with galactose as a carbon source lacking uracil (SCGal-Ura) and screened for galactose dependence by testing for failure to grow on synthetic complete with glucose lacking uracil (SCD-Ura), and the integration event was confirmed by Southern blotting. These strains were transformed with a *LEU2*, centromere plasmid carrying the U2 allele to be tested (YCpU2 [25]), selected on SCGal-Ura,-Leu, and assayed for growth on media containing glucose (SCD-Ura or YPD) or galactose (SCGal-Ura or YPGal) at 26°C.

RNA isolation and analysis. Stationary cultures in SCGal-Ura were used to inoculate starter cultures in YPGal medium. Cells were grown in liquid YPGal at 25°C to an optical density at 600 nm (OD_{600}) of 1 to 2 and diluted into liquid YPD to an OD_{600} of 0.1 to 0.3 to repress U2 synthesis. Cells were grown at 25°C for 16 to 20 h to allow loss of the wild-type U2. As wild-type U2 is depleted, growth is arrested in synthetic lethal strains at an OD of about 1 to 2. Variations in the rate of cell growth between different strains during this period correlated with strain background rather than U2 allele. Total RNA was isolated from 50-ml aliquots of culture at an OD of 0.5 to 3.0 essentially as described previously (4). Cell pellets were resuspended in 0.4 ml of AK buffer (AK buffer is 1 g of triisopropylnaphthalene sulfonic acid, 6 g of sodium *p*-aminosalicylate, 1.17 g of sodium chloride, 6 ml of phenol, and double-distilled H_2O to 100 ml). The mixture was transferred to siliconized Eppendorf tubes containing 0.5 ml of phenol prewarmed to 65°C, vortexed for 30 s at high speed, and incubated at 65°C for 30 min with 30-s vortexing at 10-min intervals. The mixture was placed on ice for 2 min and centrifuged for 5 min to separate the phases. The aqueous phase was removed to a new tube and extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform. Sodium acetate (pH 5.2) was added to 0.3 M, and the RNA was precipitated by the addition of 2 volumes of 95% ethanol. Pellets were rinsed in 70% ethanol, dried, and resuspended in water at a nucleic acid concentration of 1 mg/ml.

Primer extensions were performed as described previously (4) in 20-μl reactions with 10 μg of total RNA and 0.2 ng of each end-labeled oligonucleotide primer. Primer extension reactions with primers YSTR and SCR1 were performed simultaneously (Fig. 4B). SCR1 was a gift of T. Powers and is complementary to scR1 RNA, an abundant cytoplasmic RNA not involved in splicing (17). Oligonucleotide primers were labeled at their 5′ ends with [^{32}P]phosphate, using [γ-^{32}P]rATP and polynucleotide kinase. The sequences and annealing temperatures used are as follows: for YSTR, ATTATTTTGGGT GCCAA and 37°C; for SCR1, CCATCCCCGAGGGAACG GCC and 37°C; and U3A+B, CCAAGTTGGATTCAGTGG CTC and 55°C.

In vivo structure probing. In vivo structure probing was performed as described previously (4), using a set of isogenic strains carrying different U2 alleles. HI70 has the genotype *MAT*α *ura3-52 leu2-3,112 his4-619 lys2 snr20*::*URA3* and carries a *LYS2* centromere plasmid with a marked U2 gene to cover the U2 disruption (4). The indicated U2 alleles were introduced on a *LEU2* centromere plasmid, and loss of the marked wild-type U2 gene was selected by plasmid shuffling on

α-aminoadipate medium (12). The sites of modification in U2 RNA were mapped by primer extension (26, 41) with YSTR as described above. A reference U2 sequence ladder was generated by adding 1 μl of a different dideoxynucleoside triphosphate (ddNTP) stock (ddATP or ddGTP, 0.75 mM; ddCTP, 1 mM; ddTTP, 1.5 mM) to each of four standard primer extension reactions using unmodified wild-type RNA.

In vivo splicing extracts and assays. Strain IH1097 (*MATα pep4-3 prb1 prc1 ura3-52 leu2-3,112 trp1*) was transformed with YEp24-PRP9-3'HA (a gift of M. Rosbach), which carries the epitope-tagged *PRP9* gene (1), and whole cell extract was prepared as described by Lin et al. (34) and was assayed as described by McPheeters et al. (37). Actin pre-mRNA substrate, T7 U2 RNA, and T7 rRNA were synthesized in vitro by using T7 RNA polymerase essentially as described by Milligan and Uhlenbeck (39). The template for substrate synthesis is a wild-type actin intron and is described by Zavanelli and Ares (56). The template for T7 U2 RNA was generated by amplifying the wild-type U2 sequence from a plasmid by using the following oligonucleotides: T7 U2 (5'-CAAGCTTAATAC GACTCACTATAGGACGAATCTCTTTGCC-3') and U2-3' (AAAAGAGCGAACGGGAAG-3').

A smaller transcript was consistently observed in addition to the full-length U2. This smaller transcript functioned as efficiently as the wild type in the reconstitution experiment, consistent with previous results (37). The template used to produce a 977-nucleotide runoff transcript of 23S rRNA (containing domains IV and V) was a generous gift of R. Green. Radiolabeled transcripts were purified by denaturing gel electrophoresis, eluted in 0.3 M sodium acetate–0.2% sodium dodecyl sulfate (SDS)–1 mM EDTA with 10 μg of proteinase K per ml, and then subjected to extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation. Unlabeled transcription reactions were treated with DNase extracted with phenol-chloroform-isoamyl alcohol (25:24:1), run over a Sephadex G-50 column twice, precipitated with ethanol, and quantified by A_{260}, assuming that 1 OD unit equals 40 μg of RNA.

Immunoprecipitation of splicing complexes. Immunoprecipitations were performed as outlined by Steitz (53). Monoclonal antibody 12CA5 was purchased from Berkeley Antibody Co. anti-Prp11p serum was a generous gift of T.-H. Chang (10). Anti-Prp21p serum was a generous gift of J. Arenas (2).

Protein A–Sepharose CL-4B (PAS; Sigma) was suspended in NET (50 mM Tris-Cl [pH 7.4], 0.05% Nonidet P-40, 150 mM NaCl) at 100 mg/4 ml, incubated at 4°C with slow rotation for 1 h, and washed three times with 4 ml of NET. A fraction of the prewashed PAS equivalent to a 10- to 20-μl packed volume was suspended in 0.4 ml of NET, and antibodies were bound for 4 to 8 h by rotation at 4°C. PAS-bound antibodies were washed three times with 0.4 ml of NET prior to incubation with extract. PAS-antibody complexes were suspended in 0.4 ml of NET and incubated in siliconized 0.5-ml tubes with rotation for 1 h at 4°C with a maximum of 20 μl of splicing reaction. Immune complexes were collected, washed three times with 0.5 ml of NET-150, and suspended in 100 μl of 0.3 M sodium acetate (pH 5.2)–0.2% SDS–1 mM EDTA with 10 μg of proteinase K per ml for RNA extraction. After incubation for 10 min at 65°C, extraction with phenol (pH 4.0), and precipitation with 2.5 volumes 95% ethanol, the RNA was analyzed by electrophoresis on a 6% acrylamide denaturing gel and visualized by autoradiography.

Oligonucleotide-directed RNase H digestion of U2 snRNPs in extracts. Endogenous U2 RNA was removed from splicing extracts by oligonucleotide-directed RNase H cleavage (32, 37). Reaction mixtures (80 μl in total, 45 μl of extract) were incubated with splicing buffer with 4 mM ATP for 35 min at 30°C in the presence or absence of 450 nM oligonucleotide L15 (CAGATACTACACTTG), which is complementary to positions 43 to 29 of U2. For detection of complexes containing labeled pre-mRNA, the total reaction volume was then brought to 90 μl by the addition of 40 to 100 fmol of labeled pre-mRNA in 10 μl of 1× splicing buffer. Reaction mixtures were then incubated for 15 min more at 23°C, and 20-μl aliquots were immunoprecipitated as described above.

Immunodepletion of Prp11p-containing complexes from extracts. To deplete splicing reactions of Prp11p-containing complexes, 170-μl splicing reactions were performed as described above, and 80-μl aliquots were removed after a 10-min incubation at 23°C, added to PAS-bound antibodies in 0.4 ml of NET prepared as described above, and incubated with rotation 1 h at 4°C. Prp11p-containing complexes were removed by centrifugation, and 100-μl aliquots of the supernatants were removed to new tubes containing PAS-bound second antibodies as indicated. After incubation for an additional hour, immunoprecipitated RNA was isolated and analyzed as described above.

Reconstitution of U2 snRNP-depleted extracts. A splicing reaction mixture (260 μl) was depleted of endogenous U2 snRNP as described above. Aliquots of 50 μl were mixed with 20 μl of T7-transcribed RNA as indicated in 1× splicing buffer, labeled RNAs were added to a final concentration of 10 nM, and unlabeled competitor RNAs were added to 200 nM. Reconstitution reactions were incubated at 23°C for 20 min, and 30-μl aliquots were removed to precipitation reactions as described above except that precipitations were incubated on a rocking platform. Precipitated RNA was isolated and analyzed as described above.

RESULTS

Double-mutant lethal combinations of U2 and *prp* temperature-sensitive alleles. The genetic phenotype of synthetic lethality or enhancement has been used to identify genes whose products work closely with a gene product of interest (for reviews, see references 20a and 23). Our aim was to identify *PRP* gene products that interact with U2 snRNA by using synthetic lethality, looking for double-mutant heat-sensitive *prp*, cold-sensitive U2 cells that cannot survive at any temperature (Fig. 1A). We also wanted the lethality to be conditional, so that we could observe molecular phenotypes in large cultures of cells. We obtained *prp* mutant strains and replaced the promoter of the endogenous chromosomal U2 gene in each with a *GAL*-U2 promoter fusion (24, 40), thereby bringing the endogenous wild-type U2 gene under *GAL* control (Fig. 1B). We introduced plasmids carrying a mutant U2 allele under constitutive control of the natural U2 promoter into these strains. Under these circumstances, cells grown on glucose effectively express only the mutant U2. If the *prp* mutation is synthetic lethal with the U2 allele, the cells will fail to grow on glucose at a temperature permissive for both single mutants alone (26°C) (Fig. 1B). We refer to this as conditional synthetic lethality, and in our case it can be induced by shifting cells from galactose to glucose.

We chose a set of well-studied *PRP* genes (2, 13, 21, 45, 54), all known to encode products required for early steps in spliceosome assembly (*prp3*, *prp4*, *prp5*, *prp8*, *prp9*, *prp11*, *prp19*, and *prp21*). An early step in spliceosome formation also requires U2 RNA stem loop IIa (56) formed by yeast U2 RNA nucleotides 48 to 67 (mammalian nucleotides 47 to 66). The 30 or so different functional U2 alleles that we tested included 5 with cold-sensitive or cold- and heat-sensitive phenotypes and

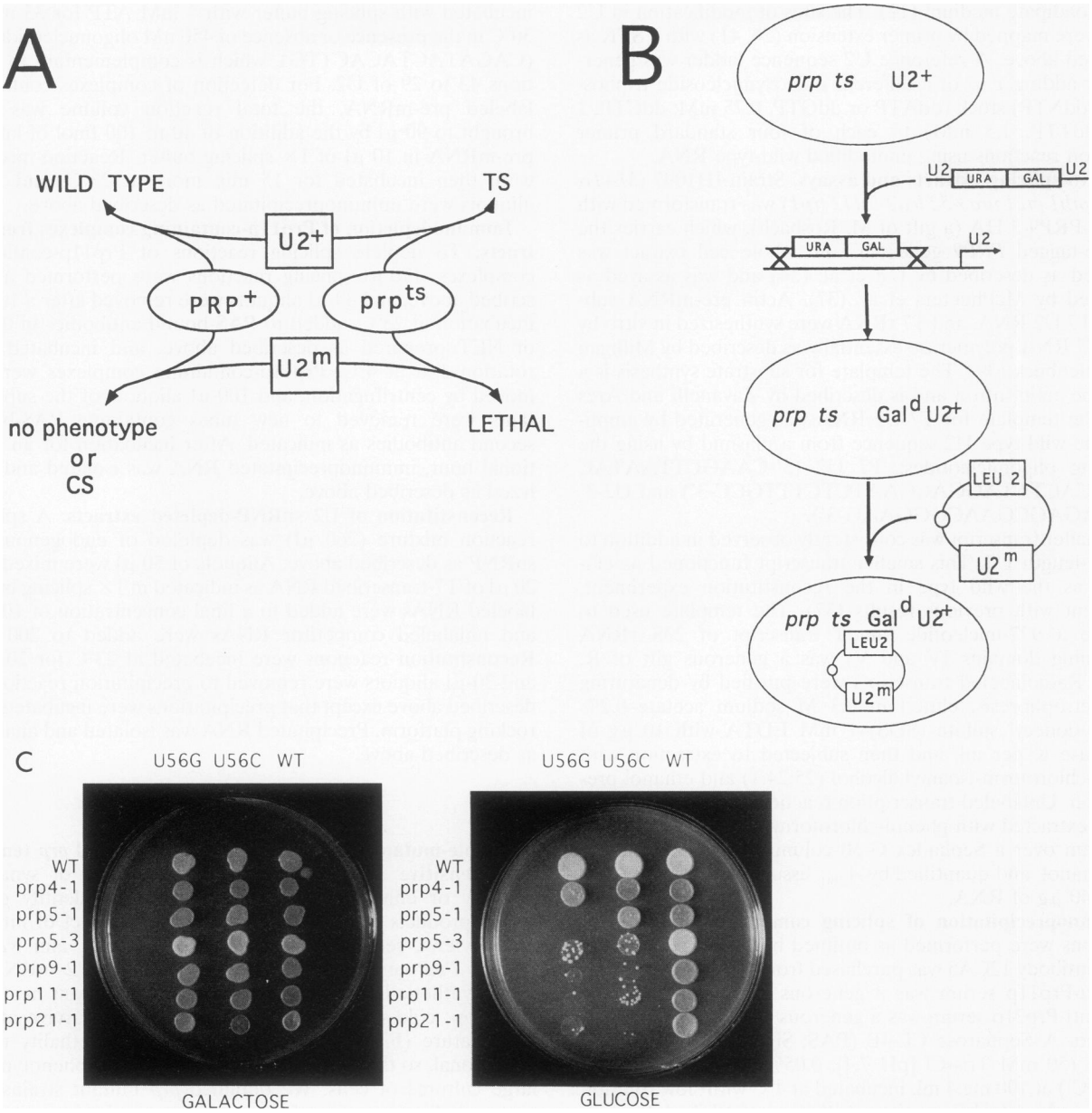


FIG. 1. Use of synthetic lethality to detect factors that interact genetically with U2 RNA. (A) Diagram of the concept of synthetic lethality with U2. Single U2 mutations or splicing protein mutations (prp) that are viable or have conditional phenotypes become lethal if they are combined in the same cell. ts, temperature sensitive. (B) Construction of strains conditional for synthetic lethal interactions. For each of the *prp* mutations tested, a strain was constructed to allow conditional expression of the wild-type U2 gene by bringing it under *GAL* control. Cells were transformed with a centromeric plasmid carrying a U2 gene of interest under control of the U2 promoter. When grown on galactose, strains express both chromosomal (wild-type) and plasmid-borne (mutant) U2 and are viable. When grown on glucose, cells express only plasmid-borne mutant U2, and lethal interactions are revealed. (C) The synthetic lethal phenotype. Cells were spotted on rich medium containing galactose or glucose and incubated at 26°C. Strains carrying a glucose-repressible wild-type (WT) U2 gene, a mutation at the *prp* locus as indicated, and a centromeric plasmid carrying either wild-type U2 or the U2 mutant indicated are shown.

a large number that show no detectable growth phenotypes despite alterations in highly conserved structures (4). We constructed over 250 double-mutant combinations, each carrying a single *prp* temperature-sensitive mutation and a plasmid-borne mutant U2 allele complemented in a galactose-dependent fashion by the *GAL*-controlled wild-type U2 gene. We tested the strains for growth on glucose at temperatures permissive for either single mutant alone (26°C). A sample of these data is shown in Fig. 1C, and the rest of the data are summarized in tabular form next to the secondary structure of this region of yeast U2 (Fig. 2).

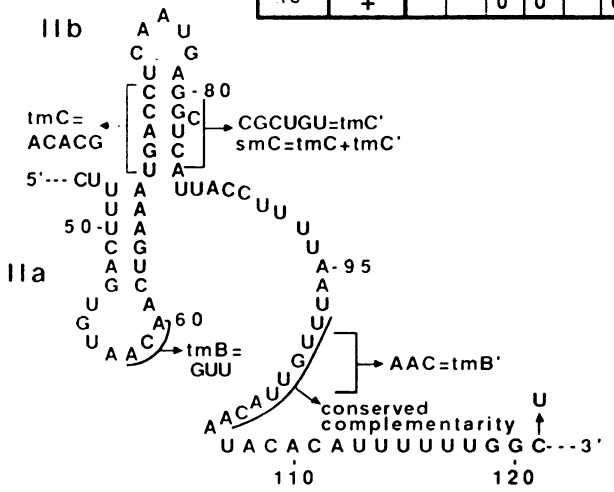
In no case did we observe synthetic lethality with *prp3*, *prp4*, *prp8*, or *prp19* and any U2 mutation, demonstrating that the synthetic lethal phenotype cannot be generated simply by combining any two mutations in the splicing apparatus or by combining any *prp* mutation with a U2 mutation (see also

STEM IIa

	WILD TYPE	3	4	5-1	5-3	8	9	11	19	21
G53C	CS/TS			0	0		0	0		0
G53C C62G	+									
G53A C62U	+				0		0	0		0
G53A	CS			0	0		0	0		0
C62U	CS			0	0		0	0		0
G53C G100A	+			S	0		0	0		0
C62U G100A	+				S		0	0		0
G53A G100A	+				S		0	0		0
U49A	+				S		S	S		S
A66U	+									
U49A A66U	+									
A65G	+			S	0		0	0		0

STEM IIb

	WILD TYPE	3	4	5-1	5-3	8	9	11	19	21
tmC	+				0		0	0		0
tmC'	CS			0	0		0	0		0
smC	+									
ΔC	+			0	0		0	0		0



LOOP IIa

	WILD TYPE	3	4	5-1	5-3	8	9	11	19	21
U56C	+				S		0	0		0
U56G	+			0	0		0	0		0
A57G	+									
A57C	+			S	S		0	0		0
C59U	+									
tmB	+			0	0		0	0		0

OTHER

	WILD TYPE	3	4	5-1	5-3	8	9	11	19	21
dlBD	+						0	S		0
dlBD +III	+						0	S		0
C121U	+									
tmB'	+									
G100A	+									

FIG. 2. Synthetic lethal interactions between U2 and *prp* mutations. U2 mutants tested are divided into four groups based on the region of U2 altered in the mutant. At the center is the secondary structure model of nucleotides 46 to 121 of yeast U2 RNA (4). The first column indicates the U2 mutant tested; the second column indicates the phenotype of that mutation in a wild-type (WT) background. CS, cold sensitive; TS, temperature sensitive; +, no phenotype. The first row indicates the *prp* mutation tested. Inviable double mutants are indicated by 0, double mutants with slower than wild-type growth are indicated by S, and combinations for which no additional synthetic phenotype was observed are indicated by blank spaces. dlBD corresponds to a deletion of nucleotides 123 to 1081 (3, 25), and dlBD+III is the same as dlBD except that nucleotides 633 to 650 have been included to restore a stem-loop that is structurally equivalent to metazoan stem-loop III (4).

reference 46). In contrast, a subset of U2 alleles, most of which have no phenotype on their own, fail to grow in combination with mutations in *prp5*, *prp9*, *prp11*, and *prp21* (Fig. 2). At this level of analysis, we conclude that a discrete subset of Prp proteins (Prp5p, Prp9p, Prp11p, and Prp21p) function closely with U2 snRNA during splicing. For the others (Prp3p, Prp4p, Prp8p, and Prp19p), we can make no conclusion; either they are also in close functional association with U2 and we have not paired appropriate alleles of these genes to reveal an interaction, or these gene products do not function closely with U2.

The majority of U2 alleles that we tested display one of two patterns of lethality with the *prp* mutations: either they show no lethality at all, or they are lethal in combination with all members of the group *prp9*, *prp11*, and *prp21* (Fig. 2). Many U2 alleles are also synthetic lethal with *prp5*, in addition to *prp9*, *-11*, *-21*. The extreme overlap in pattern suggests that these splicing proteins make many of the same demands on U2 RNA structure for their function. A formal genetic interpretation of this result is that these gene products interact with U2 as a unit (for example as a protein complex) or interact separately (presumably sequentially) in very similar ways. Interaction as a unit is supported by evidence that *PRP5*, *PRP9*, *PRP11*, and *PRP21* show synthetic lethal interactions with one another (46), and a suppressor of *prp9-1* called *SPP91* (11) is an allele of *PRP21* (2). Results from the two-hybrid system have also detected protein-protein interactions between Prp21p and both Prp9p and Prp11p (30, 31). Differences in the pattern of synthetic lethality with *prp5* may indicate that its function with U2 has different requirements than those of *prp9*, *prp11*, or *prp21*.

U2 RNA structures required for function with mutant Prp proteins. The allele-specific nature of the synthetic lethal interactions reveals structural requirements for U2 function with specific *prp* gene products. The integrity of stem IIa is important, as shown by the numerous single base changes in

stem IIa that show synthetic lethality or synthetic slow growth (Fig. 2). Many of these are in the 53-62 base pair and confer cold-sensitive growth or lethality that is suppressible by compensatory mutation that restores Watson-Crick base pairing in a wild-type background (4). In the *prp9*, *prp11*, or *prp21* mutant background, the G53C C62G compensatory U2 double mutant is viable, but synthetic lethality is observed with the G53A C62U compensatory mutant, demonstrating that not all base pairs are equivalent at this place in the structure and that the stability or primary sequence of stem IIa must also be important in the *prp* mutant background (Fig. 2). The stem IIa mutants are cold sensitive as a result of formation of a competing alternative structure; cold sensitivity can be suppressed (in a wild-type *PRP* background) without restoration of correct pairing in stem IIa if the alternative folding is inhibited by mutation of U2 G100 to A (57). This intragenic suppressor of stem IIa cold sensitivity is inadequate for suppression of stem IIa synthetic lethality with *prp9*, *-11*, or *-21* (Fig. 2), reaffirming the conclusion that the structure or primary sequence of stem IIa is important in a *prp* mutant background. The G100A mutation suppresses *prp5* synthetic lethality for two of the three point mutations in stem IIa (Fig. 2, G53A G100A and C62U G100A, but not G53C G100A), indicating that mutant Prp5p will function with a mismatched stem IIa, provided that alternative structures that interfere with stem IIa folding are removed. Compared to the other *prp* mutations, *prp5* seems more tolerant of changes in U2, either because its function is less compromised or because its demands on U2 structure are qualitatively different.

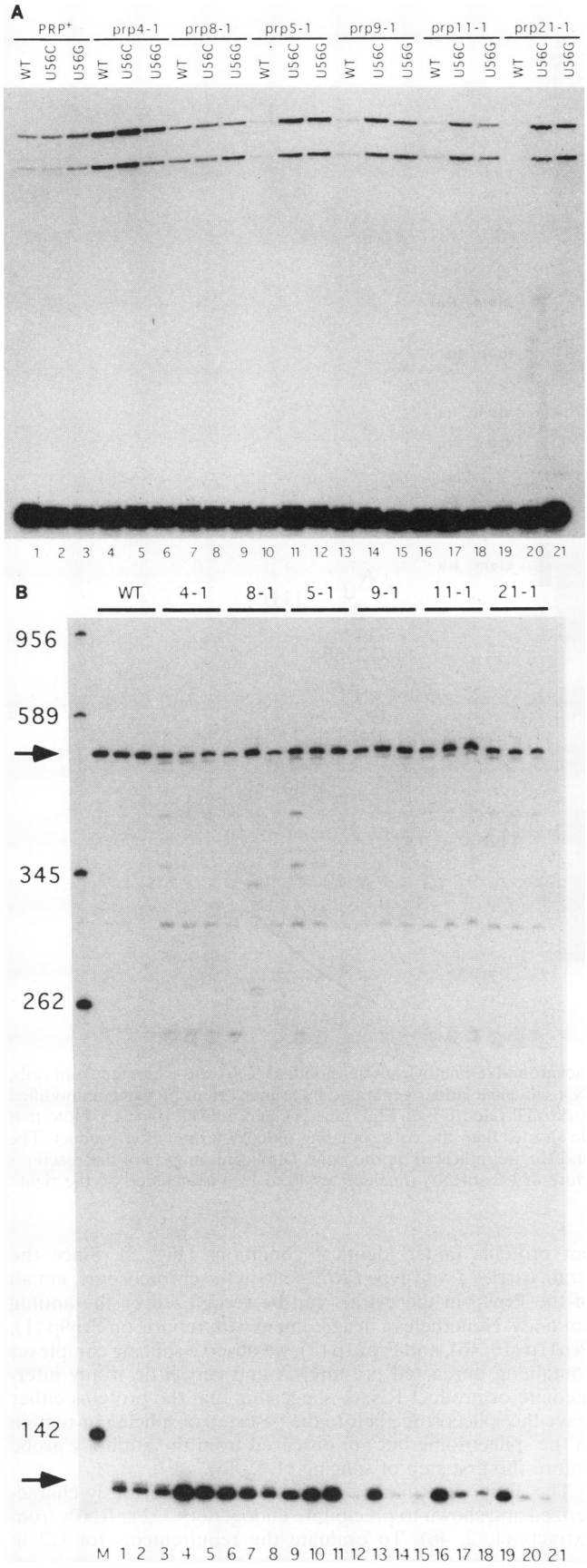
Certain mutations in loop IIa also interfere with the function of this group of Prp proteins. Mutation of the invariant U2 nucleotide U-56 to either C or G has no apparent phenotype in a wild-type background (4) but is synthetic lethal with *prp9*, *-11*, or *-21* (Fig. 1C and 2). Growth of the U56C *prp5-1* double mutant is only slightly growth impaired (data not shown), whereas the U56C *prp5-3* double mutant grows very slowly. Both the U56G *prp5-1* and U56G *prp5-1* and U56G *prp5-3* double mutants are inviable. Two loop IIa mutations that are consistent with observed phylogenetic variation, C59U (found in mammals and plants [3, 22]) and A57G (found only in *Tetrahymena thermophila* [43]), show no synthetic lethal defect. The other single mutation in the loop, A57C, is lethal with *prp9*, *-11*, and *-21* and shows some degree of growth inhibition with *prp5*. The triple mutation in the loop (tmB) is also synthetic lethal. No synthetic lethality has been observed with mutations in the conserved complementarity to the loop IIa (residues 98 to 105; mutant G100A or tmB′; Fig. 2).

The structure of stem loop IIb is also necessary for function with mutant *prp* gene products. This element is phylogenetically conserved but dispensable in a wild-type *PRP* background, in which its deletion produces no apparent growth defect (4). Mutations in stem IIb (tmC and tmC′), as well as complete deletion of the stem, nucleotides 69 to 85 (ΔC), are synthetic lethal with the group *prp5*, *-9*, *-11*, and *-21* (Fig. 2). The compensatory combination of tmC and tmC′ (smC) is not synthetic lethal with these *prp* mutations, demonstrating that it is the structure of stem IIb, rather than the primary sequence, that is important for cooperative function with the *prp* gene products. We also tested a set of other mutations, including the large internal deletion (nucleotides 123 to 1081) spanning the yeast equivalent of stem III (dlBD [3, 25]) and a derivative of this mini-U2 into which just the stem III-homologous sequence (nucleotides 633 to 650) has been placed (dlBD+III [4]). Neither of these is synthetic lethal with *prp5*, but both are synthetic lethal with *prp9* and *-21*, and growth is severely inhibited in *prp11*. A single base change downstream from the Sm site (C121U) shows no synthetic lethality (Fig. 2). Five mutations in U2 stem I in a region that interacts with U6 (U23G, U23C, C22G, C22A, and A27C U28C [35]) were also tested with the *prp5-3* mutant. Strains with all of these mutations are aphenotypic in a wild-type background except for the A27C U28C mutant, which grows slowly at 25°C (35). No additional growth inhibition or lethal phenotype was observed for any of these combinations (data not shown), supporting the interpretation that the synthetic lethal interactions are restricted to distinct functional domains of U2 RNA.

Two independently isolated alleles of *prp5* and two of *prp9* are available, and we have tested them to see if they differ in their requirements for U2 function. The two alleles of *prp9* give identical patterns of synthetic lethality (not shown), consistent with the conclusion (31) that these two mutations are in the same functional domain of the Prp9p protein. The *prp5-1* allele shows fewer synthetic lethal interactions than the *prp5-3* allele, with no additional interactions not shown by the *prp5-3* allele. This is consistent with a quantitative effect, especially considering that the temperature-sensitive growth phenotype of *prp5-1* is leakier than that of *prp5-3*. Until more about functional domains of the Prp5 protein is known, we cannot exclude the possibility that these effects are due to qualitative differences in the functions of the mutant proteins. We conclude that four splicing proteins, those encoded by the *PRP5*, *-9*, *-11*, and *-21* genes, are individually in close functional association with U2 RNA (see also reference 46) and that this cooperative function is exquisitely sensitive to alterations in the structured region of U2 encompassing stem loops IIa and IIb.

Splicing is inhibited in cells carrying lethal double mutations. Genetic results alone are inadequate to understand the nature of functional relationships between gene products at the cell and molecular levels. To demonstrate that the synthetic lethal phenotype is associated with an increased splicing defect in vivo, we measured ratios of spliced to unspliced U3 in our *GAL*-dependent synthetic lethal strains after a shift to glucose at permissive temperatures (Fig. 3A). Many of the U2 mutations that we tested confer no splicing defect at 30°C (4), and many *prp* mutations have only subtle splicing defects at 23°C. In all cases of synthetic lethality, unspliced pre-U3 accumulates more readily in the double mutant than in either a single *prp* mutant with wild-type U2 or a strain carrying mutant U2 and wild-type *PRP* alleles. Note that although the *prp4* strains accumulate substantial levels of pre-U3 in combination with wild-type U2, these levels do not increase with mutant U2, and *prp4* is not synthetic lethal. Synthetic lethal combinations with *prp5*, *-9*, *-11*, or *-21* accumulate unspliced U3 after the shift to glucose. Even the nonlethal but mildly growth impaired combination of U2 U56C with *prp5-1* shows splicing inhibition comparable to the lethal combinations (Fig. 3A, lane 11). We conclude that the double-mutant combinations that lead to synthetic lethality also specifically result in the inhibition of splicing in vivo.

Mutant U2 snRNA does not accumulate in cells carrying a *prp5*, *-9*, *-11*, or *-21* mutation. The inhibition of splicing was observed after repression of wild-type U2 RNA synthesis. One possible explanation for splicing inhibition might be that mutant U2 fails to accumulate efficiently in strains harboring mutant Prp proteins. To test this, we measured accumulation of mutant U2 snRNA in our conditional synthetic lethal strains by primer extension (Fig. 3B). In all cases, the failure of mutant U2 snRNA to accumulate correlates precisely with synthetic lethality, suggesting that cells may die as a result of the absence of U2 snRNPs. In the case of the double mutant U56C *prp5-1* U2 RNA, stability is not lost (Fig. 3B, lane 11) and growth is only slightly impaired (Fig. 1C and data not



shown). For the U56C *prp5-3* double mutant, U2 RNA is destabilized (not shown) and growth is more severely inhibited (Fig. 1C). Splicing is inhibited for both *prp5* alleles in combination with the mutant U2 (Fig. 3A, lane 11, and data not shown), demonstrating that physical loss of U2 snRNP is not necessary to observe splicing inhibition. We conclude that the wild-type Prp5p, -9p, -11p, and -21p are all required for the stable accumulation of mutant U2 snRNA and may contribute to the stability of wild-type U2 in vivo. Additional experiments monitoring levels of U1, U4, U5, and U6 snRNAs in the cell indicate that only the U2 level is affected in the synthetic lethal cases (data not shown).

Mutant U2 RNA displaying synthetic lethal interactions is not grossly misfolded. Since many mutations that disrupt stem IIa are synthetic lethal (Fig. 2), we would expect loop mutations that disrupt the stem to cause synthetic lethality as well. To refine our understanding of the effect of some of the U2 mutations on U2 structure as an aid in interpreting the synthetic lethal alleles, we performed structure probing on mutant U2 snRNAs in vivo (Fig. 4), using chemical modification by dimethyl sulfate (4). U56G and tmB cause an increase in the reactivity of adjacent stem nucleotides (Fig. 4), introducing the possibility that failure of these mutants to function with altered *prp* gene products may be indirectly mediated by their impact on stem IIa structure. In the case of U56C and A57C, however, structure probing reveals no such perturbation, suggesting that these residues contribute to a functional interaction through their impact on primary sequence or subtle features of loop structure that evade detection by the probe. Stem IIb mutations, including the complete deletion ΔC, do not disrupt stem IIa (not shown), suggesting that they may not cause the synthetic lethal defect indirectly by disrupting stem IIa. We conclude that gross misfolding is not a consistent feature of synthetic lethal U2 snRNA mutations and that the primary sequence of U2 snRNA in at least some regions contributes to the functional interactions defined by synthetic lethality either through non-Watson-Crick interactions within U2 snRNA or by interaction with other splicing factors. Because mutant U2 RNA fails to accumulate in most cases of synthetic lethality, we are unable to probe the structure of mutant U2 RNA in combination with the mutant Prp protein.

Immunoprecipitation of pre-mRNA by anti-Prp9p, anti-Prp11p, or anti-Prp21p antibodies is U2 snRNP dependent. We wanted to determine whether the different *PRP* gene

FIG. 3. Splicing defects and reduced levels of U2 snRNA in strains induced for synthetic lethality. (A) Inhibition of pre-U3 splicing in RNA was isolated following a shift to glucose, and the levels of spliced and unspliced U3 were measured by primer extension using a labeled oligonucleotide complementary to the second exon of the U3A and U3B genes. The two upper bands correspond to pre-U3A and pre-U3B, the spliced product is the major band at the bottom of the gel. Three U2 alleles are shown for each *prp* allele shown. The amount of sample in each lane has been adjusted so that all contain equal amounts of spliced U3. WT, wild type. (B) U2 snRNA levels in strains induced for synthetic lethality. RNA was isolated from cells following growth in glucose, and the levels of U2 snRNA were analyzed by primer extension using an end-labeled oligonucleotide complementary to U2 snRNA (YSTR), indicated by the lower arrow. An end-labeled oligonucleotide complementary to scR1, a cytoplasmic RNA not involved in splicing, was included in each reaction as an internal control for RNA amounts (upper arrow). The *prp* alleles tested are indicated at the top, and the U2 alleles are as follows: wild type (WT; lanes 1, 4, 7, 10, 13, 16, and 19), U56C (lanes 2, 5, 11, 14, 17, and 20), and U56G (lanes 3, 6, 12, 15, 18, and 21). Marker sizes are indicated in nucleotides.

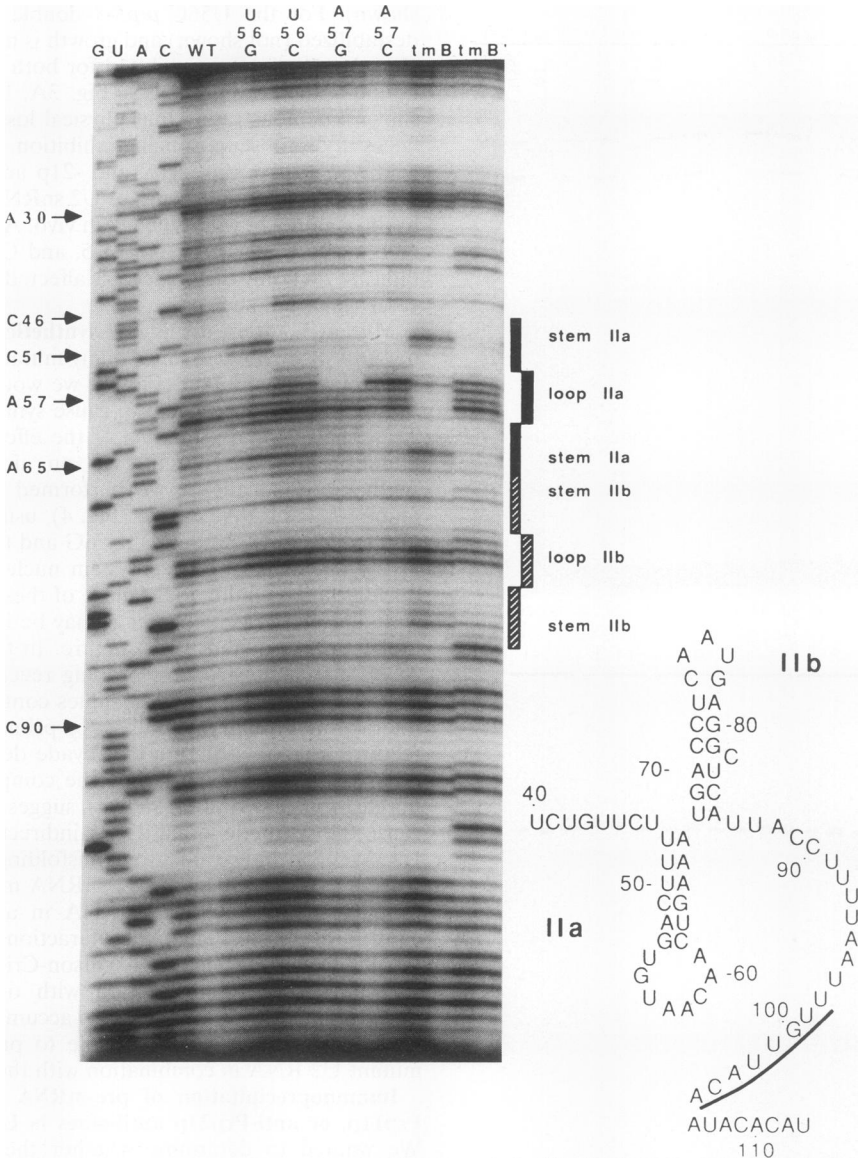


FIG. 4. Secondary structure of U2 snRNA loop IIa mutants in a PRP$^+$ background. Dimethyl sulfate-modified RNA was extracted from cells, and sites of modification were mapped by primer extension relative to an RNA sequence ladder generated by primer extension using unmodified wild-type RNA and ddNTPs. Reaction mixtures contained ddCTP (lane G), ddATP (lane U), ddTTP (lane A), and ddGTP (lane C). Note that dimethyl sulfate stops caused by the modified nucleotide are one nucleotide shorter than the corresponding dideoxy termination product. The position of selected dideoxy stops are indicated on the left; stem-loops IIa and IIb are indicated on the right. Duplicate samples of the reactions from the modified RNA are loaded in adjoining lanes. The secondary structure of U2 snRNA (nucleotides 40 to 113) is included on the right.

products that we identified by genetic interactions might be physically associated with one another in a splicing complex. Each of the proteins Prp5p, -9p, -11p, and -21p is individually required for the formation of the prespliceosome (Prp5p [46], Prp9p [1], Prp11p [10, 51], and Prp21p [2]). Little is known about participation of the yeast U2 snRNP in complexes prior to its association with the prespliceosome. We obtained anti-Prp11p (10) and anti-Prp21p antibodies (2) and a *PRP9* gene tagged with an epitope (hemagglutinin) recognized by a commercially available monoclonal antibody (12CA5) (1). We introduced the tagged *PRP9* gene on a high-copy-number plasmid into a wild-type yeast strain, prepared splicing extracts, and used the antibodies to assay the formation of splicing complexes containing labeled splicing substrate and the different proteins under identical conditions (Fig. 5). Since the strain carries a wild-type *PRP9* gene in the chromosome, not all of the Prp9p in the extract can be recognized by the antitag antibody. Nonetheless, in agreement with reports on Prp9p (1), Prp11p (10, 46), and Prp21p (2), we observe splicing complexes containing unreacted pre-mRNA and very little if any intermediate or product RNAs, suggesting that the proteins either leave the spliceosome before the first step of splicing or remain in the spliceosome but are obscured from the antibody probe before the first step of splicing (1, 2, 46).

The antibodies that we used have been previously characterized and shown to precipitate endogenous U2 snRNPs from extracts (1, 2, 46). To evaluate the requirements for U2 in formation of pre-mRNA containing complexes previously de-

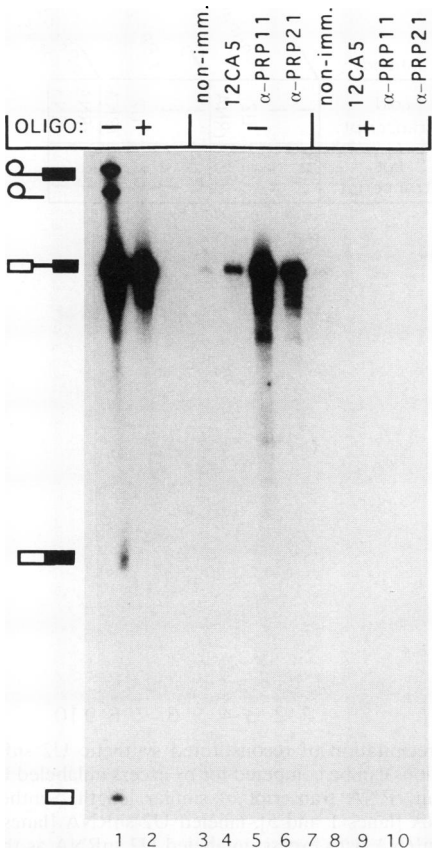


FIG. 5. Immunoprecipitation of complexes containing labeled pre-mRNA and different Prp proteins from in vitro splicing reactions. Shown is precipitation of U2 snRNA-dependent complexes containing labeled pre-mRNA and different Prp proteins. Labeled pre-mRNA was added to splicing reactions pretreated with no oligonucleotide (OLIGO) (lane 1) or with oligonucleotide L15 complementary to U2 snRNA (lane 2). Immunoprecipitations from the mock-treated splicing reaction (lanes 3 to 6) or the reaction mixture treated with oligonucleotide L15 (lanes 7 to 10) are shown. Following 15 min of incubation under splicing conditions, reaction mixtures were immunoprecipitated with nonimmune (non-imm.) serum (lanes 3 and 7), 12CA5 (lanes 4, 8), anti-Prp11p serum (α-PRP11; lanes 5 and 9), or anti-Prp21p serum (α-PRP21; lanes 6 and 10). Aliquots of the splicing reaction mixtures prior to immunoprecipitation without (lane 1) or with (lane 2) oligonucleotide are shown. Symbols at the left represent splicing substrate, intermediates, and products.

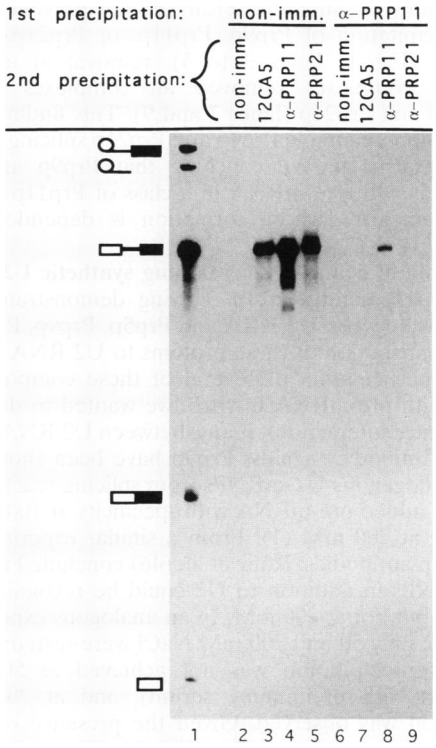


FIG. 6. Pre-mRNA complexes containing Prp9p and Prp21p also contain Prp11p. Splicing reaction mixtures were cleared of immunoprecipitable complexes by using nonimmune (non-imm.) serum (lanes 2 to 5) or an anti-Prp11p antibody (α-PRP11; lanes 6 to 9). Pre-mRNA complexes containing Prp11p were removed from the extract, and a second precipitation was performed with nonimmune serum (lanes 2 and 6), 12CA5 (lanes 3 and 7), or an anti-Prp11p (α-PRP11; lanes 4 and 8) or anti-Prp21p (α-PRP21; lanes 5 and 9) antibody. Symbols at the left represent splicing substrate, intermediates, and products.

scribed, we assessed pre-mRNA–Prp complex formation in extracts depleted of U2 snRNP. When U2 is destroyed by oligonucleotide-directed RNase H digestion (32, 37), complexes containing pre-mRNA and any of the proteins are not detected (Fig. 5). This finding is in agreement with results for Prp9p (1) and demonstrates that association of Prp11p or Prp21p with pre-mRNA requires intact U2 snRNP. We conclude that formation of immunoprecipitable complexes containing Prp9p, -11p, or -21p and pre-mRNA requires intact U2 snRNA. Additional data for mutant pre-mRNA substrates indicates that the formation of the complexes that we observe is dependent on a correct branchpoint signal in pre-mRNA (not shown), in agreement with work of others (2, 46). Taken together, the data indicate that in the presence of ATP, all three proteins bind the pre-mRNA either in conjunction with or as part of the U2 snRNP. We have not assayed complex formation in ATP-depleted extracts; it is possible that one or more of the proteins interact with the pre-mRNA in the absence of ATP as reported previously (46).

Immunoprecipitable pre-mRNA complexes that contain Prp9 or Prp21p also contain Prp11p. The data presented above indicate that splicing complexes containing each of the three proteins are similar in that they contain unreacted pre-mRNA and require U2 snRNP for their formation. The data do not show whether they are in the same complex together. In addition, the genetic results (Fig. 2) do not distinguish between models involving sequential or simultaneous interaction of the proteins with U2. Although genetic data (11, 46) and results from the two-hybrid system (30, 31) suggest that proteins of this group interact, we wanted to determine if the three proteins are in the same complex at the same time with pre-mRNA. We performed a double-immunoprecipitation experiment (Fig. 6) in which radioactive pre-mRNA was added to a splicing reaction. After incubation to allow splicing complex formation, the splicing reaction was chilled and diluted, and anti-Prp11p antibody was added in excess to remove all formed splicing complexes containing Prp11p. The supernatant from which all immunoprecipitable Prp11p-containing splicing complexes had been removed was then challenged with a second antibody against Prp9p(tag) or Prp21p in order to determine whether any additional splicing complexes containing Prp9p or Prp21p but not Prp11p could be detected. Although control pretreatment of the reaction

mixture with a nonimmune serum did not prevent the subsequent precipitation of Prp9p, Prp11p, or Prp21p-containing complexes (Fig. 6, lanes 3 to 5), removal of all Prp11p-containing complexes removed all complexes containing Prp9p(tag) and Prp21p (lanes 7 and 9). This finding indicates that immunoprecipitable Prp9p and Prp21p splicing complexes all contain Prp11p. We conclude that Prp9p and Prp21p interact only with pre-mRNA in a class of Prp11p-containing splicing complexes whose formation is dependent on U2 snRNP.

Formation of complexes containing synthetic U2 RNA and Prp9p, Prp11p, and Prp21p. Having demonstrated genetic interactions between U2 RNA and Prp5p, Prp9p, Prp11p, and Prp21p, contribution of these proteins to U2 RNA stability in vivo, and participation of several of these components in a complex with pre-mRNA in vitro, we wanted to detect more direct physical interactions, if any, between U2 RNA and these proteins. Antibodies against Prp9p have been shown to precipitate endogenous U2 snRNPs from splicing reactions in the absence of added pre-mRNA with specificity at 100 mM NaCl but poorly at 200 mM (1). From a similar experiment using anti-Prp11p antibodies, Ruby et al. (46) concluded that one or more snRNPs in addition to U2 could be recognized at 150 mM NaCl but not at 200 mM. In an analogous experiment on Prp21p (2), only 50 and 200 mM NaCl were tested; specificity of snRNP precipitation was not achieved at 50 mM (by comparison with preimmune serum), and at 200 mM no precipitation was observed. Given the presence of all three proteins in a common complex (Fig. 6) and the salt-sensitive nature of the association of Prp9p with U2 (1), we attempted to reconstitute U2-protein complexes by using synthetic U2 RNA, with the expectation that salt concentrations lower than 200 mM might be necessary for their detection. Addition of synthetic U2 RNA to yeast splicing extracts depleted of endogenous U2 RNA can reconstitute splicing (37), arguing that any important functional interactions between Prp proteins and synthetic U2 must occur in such a reconstituted system.

We mixed yeast splicing extracts depleted of endogenous U2 RNA with different synthetic RNAs and immunoprecipitated them with antibodies against Prp9p(tag), Prp11p, and Prp21p (Fig. 7). Synthetic U2 snRNA was coprecipitated by anti-Prp11p (Fig. 7, lane 6), whereas a transcript of similar length from *Escherichia coli* 23S rRNA was not (lane 5). Precipitation of synthetic U2 RNA by anti-Prp11p can be competed for by excess unlabeled synthetic U2 RNA (lane 7) but not by an excess of the rRNA transcript (lane 8). Anti-Prp21p and anti-Prp9p(tag) also specifically precipitate synthetic U2 RNA in the presence of excess nonspecific competitor (lanes 9 and 10), whereas control nonimmune serum did not (lane 4). We conclude that the Prp9p, -11p, and -21p specifically bind synthetic U2 RNA after incubation in splicing extracts depleted of endogenous U2 and that this association does not require the addition of pre-mRNA. The binding of these proteins to the RNA may be mediated through other proteins or snRNPs present in the extract. These antibodies have been shown to precipitate other snRNAs along with U2 snRNA from untreated splicing extracts (2, 46). Our experiment does not eliminate the possibility that association between synthetic U2 snRNA and Prp9p, Prp11p, or Prp21p requires other factors.

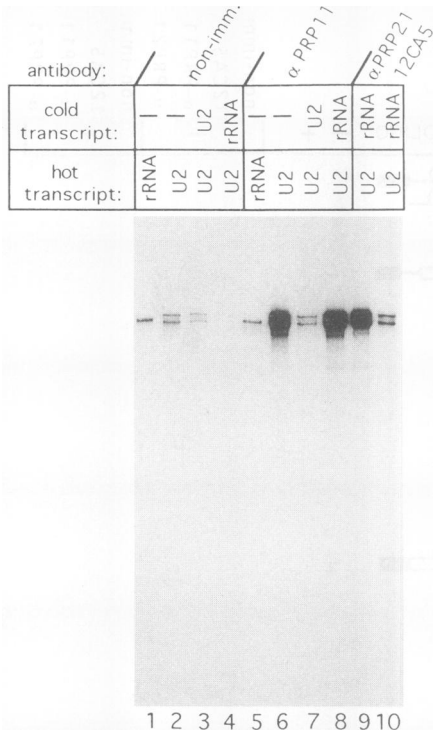


FIG. 7. Precipitation of reconstituted synthetic U2 snRNA from splicing reactions can be competed for by excess unlabeled U2 snRNA but not by an rRNA transcript of similar length. Synthetic RNAs (labeled rRNA [lanes 1 and 5], labeled U2 snRNA [lanes 2 and 6], labeled U2 snRNA with excess unlabeled U2 snRNA as the competitor [lanes 3 and 7], or labeled U2 snRNA with excess unlabeled rRNA as the competitor [lanes 4 and 8 to 10]) were added to extracts which had been depleted of the endogenous U2 snRNA. Reaction mixtures were incubated under splicing conditions followed by precipitation with nonimmune (non-imm.) serum (lanes 1 to 4), anti-Prp11p (αPRP11; lanes 5 to 8), anti-Prp21p (αPRP21; lane 9), or 12CA5 (lane 10). The PAS binds a small amount of RNA nonspecifically, resulting in a lower background binding of the labeled (hot) transcript in the presence of unlabeled (cold) competitor (compare lanes 1 and 2 with lanes 3 and 4). Background levels of binding for lanes 8 to 10 are best determined by comparison with lane 4. Precipitation with 12CA5 was consistently less efficient in part because the extracts contain both tagged and untagged Prp9p.

DISCUSSION

We have used the genetic phenomenon of synthetic lethality, engineered as a conditional phenotype, to identify which of several well-characterized splicing proteins might function closely with U2 snRNA in splicing (Fig. 1). We constructed strains representing all combinations of 27 different U2 alleles with 10 different *prp* mutations and found three main patterns of synthetic lethality. U2 mutations were synthetic lethal with either (i) none of the tested *prp* mutations, (ii) the set containing *prp9*, *-11*, and *-21*, or (iii) the set containing *prp5*, *-9*, *-11*, and *-21* (Fig. 2). This finding indicates that these proteins interact functionally with U2 snRNA (see also reference 46) through discrete U2 RNA structures. In vivo, synthetic lethality is accompanied by failure of mutant U2 to accumulate, indicating that the mutant Prp proteins are unable to carry out a function required for U2 snRNP assembly with the mutant U2 RNA (Fig. 3B). Some of the U2 RNAs that display synthetic lethality are not detectably misfolded, indicating that subtle contributions to structure can have important consequences for cooperative function with Prp proteins (Fig. 4). Using yeast splicing extracts containing wild-type components, we show that three of the proteins are in the same complex

with pre-mRNA (Fig. 6) and that formation of this complex requires intact U2 snRNP (Fig. 5). These three proteins can also be incorporated into complexes containing synthetic U2 RNA in the absence of added pre-mRNA (Fig. 7).

U2 snRNA function is overspecified. The observation that silent U2 mutations become lethal when *PRP* function is compromised exposes the functional importance of phylogenetically conserved but clinically dispensable structures of yeast U2 snRNA (4). The apparent dispensability of highly conserved U2 RNA structures in yeast was initially surprising; however, viewing U2 RNA structure through the lens of a *prp* mutation reveals that the conserved structural elements of U2 are critical (Fig. 2) and contribute to the stability and function of the RNA (Fig. 3B), in part through snRNP assembly or maintenance. The ability of mutant U2 to work adequately in a wild-type background but not a *prp* mutant background (Fig. 2) argues that the functional interaction of U2 with Prp proteins is overspecified or robust in a wild-type yeast cell. The most subtle of the synthetic lethal U2 interactions that we observe are those involving the single base changes in the loop IIa. Structure probing experiments (Fig. 4) indicate that the folded structure of the bulk of mutant U2 in the cell is unperturbed, suggesting that these residues make a direct contribution to the interaction between U2 RNA and the Prp proteins. Stem IIb mutations can improve the stability of stem IIa (57), and it seems possible that mutations in stem IIb can cause synthetic lethality indirectly by affecting the structure of the adjacent stem IIa, although structure probing of these mutants (not shown) does not support this idea. It is possible that the efficient function of U2 with the Prp5p, -9p, -11p, and -21p in vivo may require stem loop IIb directly.

Deletion of the large internal region of U2 that contains a structure homologous to mammalian stem III is also synthetic lethal. We noted that the accumulation of the internal deletion (dlBD) U2 was reduced (25) and reported that addition of stem III sequences (dlBD+III) did not rescue the poor accumulation (4). The synthetic lethality in the case of these constructs could either be direct, if the internal sequences were required to make contacts with the protein(s), indirect through effects on folding of adjacent RNA required for the direct interactions, or indirect through additional reduction of the already low levels of stable U2 snRNP by other independent mechanisms. Since the stability of the dlBD and dlBD+III U2 RNAs is known to be compromised (4, 25) and there is loss of stable U2 RNA in other synthetic lethal combinations (Fig. 3B), we favor the hypothesis of an indirect mechanism that further reduces U2 snRNP stability. Surprisingly, one U2 mutation, C121U just downstream of the Sm site, does slightly affect U2 RNA accumulation in a wild-type *PRP* background (4); the loss of stability in this case is insufficient to cause synthetic lethality with mutant Prp9p, -11p, or -21p (Fig. 2). The sequence of this region influences the function of human-yeast U2 chimeras in yeast cells (52), possibly through an effect on stability. Since a synthetic stability defect (Fig. 3B) is observed with U2 mutations that compromise stability no more than C121U does, it seems likely that multiple regions of U2 contribute to interactions that maintain U2 snRNP stability through independent means.

Because synthetic lethality may be generated by the contribution of several factors, literal interpretation of allele-specific synthetic lethal effects is dangerous. Allele specificity of an interaction might mean that the Prp protein in question requires a direct physical interaction with the region of U2 altered in the mutant, or it could mean that overall U2 activity may vary in different U2 and *prp* mutants, and if the activity falls below a certain level, the cell dies. In the former situation, function is lost qualitatively. In the latter example, the product of two closely acting or codependent functions is quantitatively below a threshold required for function. In either case, the added severity of the phenotype observed in the double mutants compared with either single mutant is striking and provides a rationale for why highly conserved U2 RNA elements may be tolerant to mutation in spite of their universal presence: wild-type U2 is more than adequate to perform the job, provided that it has the assistance of specific proteins.

U2 function is influenced by a set of proteins that function together. The astonishing degree of correspondence between U2 alleles that interact negatively with the set of Prp proteins including Prp9, -11, and -21 argues that all of these proteins require nearly the same U2 structures to carry out their function. The simplest interpretation is that the proteins interact with U2 simultaneously as a complex. This interpretation is supported by genetic interactions between different members of this group of proteins. A suppressor of *prp9* (*SPP91* [11]) was isolated genetically and is the same as *PRP21* (2). Prp9-Prp21 fusion proteins function in the two-hybrid system, showing that there are protein-protein interactions between them (31). Protein-protein interaction studies with the two-hybrid system also identify a region of interaction between Prp11p and Prp21p but do not reveal a region of direct interaction between Prp9p and Prp11p; rather, Prp9p and Prp11p may interact indirectly through Prp21p (30). Synthetic lethal interactions have also been reported to occur between every pairwise combination of the group *prp5*, *-9*, *-11*, and *-21* (46). Overexpression suppression (46, 55a) and overexpression lethality (55a) have also been observed with specific pairwise combinations.

In spite of all of the genetic data, direct biochemistry to demonstrate a stable complex between these proteins is limited. An elaborate regimen for complementing *prp11*-inactivated extracts with in vitro-synthesized Prp11p hinted that Prp11p might be part of a complex (10). Heat-inactivated extracts from temperature-sensitive *prp9* and *prp11* strains do not complement each other well for splicing in vitro, although each can be complemented by a heat-inactivated *prp5* extract (46), suggesting that Prp9p and Prp11p may be part of the same functional unit in vitro, but that Prp5p activity is more diffusible. The ability of anti-Prp11p antibodies to remove all Prp9p and Prp21p protein associated with pre-mRNA (Fig. 6) indicates that neither Prp9p nor Prp21p can form stable splicing complexes without Prp11p. Recently a weak Prp11p interaction with pre-mRNA in an ATP-depleted extract has been reported (46). Since stable U2 snRNP addition is an ATP-dependent step (32, 33, 50), it was suggested that Prp11p associates with the commitment complex prior to U2 snRNP addition (46). We have also observed ATP-independent coimmunoprecipitation of pre-mRNA with anti-Prp11p as well as anti-Prp9p(tag) and anti-Prp21p. In addition, U2 snRNP has been reported to associate with pre-mRNA in extracts depleted of ATP in both the mammalian (28) and mutant yeast (33) extracts. Unfortunately, unless ATP-independent complexes can be chased into the functional splicing pathway, their relevance to splicing is uncertain. In any event, our U2 depletion experiments do not exclude the possibility that U2 snRNP is required in the presence of ATP to stabilize a preexisting ATP-independent interaction between Prp11p and pre-mRNA.

Our data provide a rationale for the requirement of several elements in the stable addition of U2 snRNP to pre-mRNA during spliceosome assembly. Stem loop IIa is required for this step (56) and for cooperative function with Prp5p, -9p, -11p, and -21p (Fig. 2), each of which is independently required for

the same step (1, 2, 31, 46). The association of Prp9p and Prp11p with endogenous U2 snRNA has been demonstrated (1, 46); however, reconstitution of these interactions in the absence of added pre-mRNA suggests that these proteins as well as Prp21p associate with U2 snRNA prior to the association of U2 snRNP with the pre-mRNA. Since intact U2 snRNA is required for association of all three proteins with pre-mRNA in the presence of ATP (1) (Fig. 5), and conversely each of the proteins is required for the association of U2 snRNP with the pre-mRNA (1, 2, 31, 46), the simplest explanation for the genetic and biochemical interdependence is that all of the components are part of the same functional unit, the U2 snRNP. The contribution of the interactions to U2 RNA stability (Fig. 3B) provides critical evidence that these associations contribute to the integrity of U2 snRNP in vivo. In the case of Prp5p, a nucleotide binding motif and similarity to helicases argues for additional functions with the U2 snRNP (16) but does not exclude a structural role as well.

snRNP proteins or splicing factors? In mammalian systems, the U2 snRNP as isolated from the cell exists in two forms that differ (at least) in their association with a complex of proteins (6; see also reference 55) that include a proposed Prp9p homolog (8) and a Prp11p homolog (7). The association of these mammalian proteins with U2 is salt labile and reversible (6, 8, 9) and requires the 5′ half of U2 RNA for binding to the snRNP (6). In addition, the proteins are required for stable U2 snRNP binding to the assembling spliceosome, a property expected of U2 snRNP proteins (5, 8, 9). Given this, the question arises as to whether Prp9p, Prp11p, and Prp21p and even Prp5p should be considered snRNPs or splicing factors, or whether it is useful to make such distinctions. By the definition of salt-stable association with the core snRNP particles (in mammals [6, 9]; in *S. cerevisiae* for at least Prp9p [1]), they are clearly extrinsic factors. On the other hand, they associate specifically with U2 snRNP in the absence of added pre-mRNA (1, 6, 9) and contribute to snRNP stability in vivo (Fig. 3B), as does, for example, a yeast Sm protein (47). Because in yeast cells they become undetectable in pre-mRNA-containing complexes after a point prior to the first substrate cleavage-ligation reaction, it has been suggested that they become buried, or their association with the spliceosome is transient (1, 2, 46) (Fig. 5). Transient association is reminiscent of other splicing factors such as Prp2p (29), Prp16p (48), and Prp22p (15), as well as the U1 and U4 snRNPs (20, 21). In the end, the distinction between intrinsic snRNP proteins and extrinsic splicing factors may become blurred. Genetic interactions such as synthetic lethality and the molecular phenotypes underlying them provide an additional context in which the function of factors that cooperate with snRNAs during splicing may be understood.

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