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Genetic Analysis Reveals a Role for the C Terminus of the *Saccharomyces cerevisiae* GTPase Snu114 During Spliceosome Activation

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ABSTRACT

Snu114 is the only GTPase required for mRNA splicing. As a homolog of elongation factor G, it contains three domains (III–V) predicted to undergo a large rearrangement following GTP hydrolysis. To assess the functional importance of the domains of Snu114, we used random mutagenesis to create conditionally lethal alleles. We identified three main classes: (1) mutations that are predicted to affect GTP binding and hydrolysis, (2) mutations that are clustered in 10- to 20-amino-acid stretches in each of domains III–V, and (3) mutations that result in deletion of up to 70 amino acids from the C terminus. Representative mutations from each of these classes blocked the first step of splicing *in vivo* and *in vitro*. The growth defects caused by most alleles were synthetically exacerbated by mutations in *PRP8*, a U5 snRNP protein that physically interacts with Snu114, as well as in genes involved in snRNP biogenesis, including *SAD1* and *BRR1*. The allele *snu114-60*, which truncates the C terminus, was synthetically lethal with factors required for activation of the spliceosome, including the DExD/H-box ATPases *BRR2* and *PRP28*. We propose that GTP hydrolysis results in a rearrangement between Prp8 and the C terminus of Snu114 that leads to release of U1 and U4, thus activating the spliceosome for catalysis.

PRE-mRNA splicing is catalyzed by the spliceosome, a large dynamic complex composed of five small nuclear RNAs (snRNAs) and >80 proteins (Burge *et al.* 1998; Jurica and Moore 2003). The chemistry of splicing comprises two sequential transesterification reactions (Moore *et al.* 1993). In the first reaction, the 5′ splice site is cleaved and a branched lariat structure is formed within the intron. In the second reaction, the 3′ splice site is cleaved and the two exons are joined together. During the splicing cycle, the RNA and protein components of the spliceosome undergo numerous rearrangements, which must be highly coordinated to ensure fidelity of the process (Staley and Guthrie 1998). Most of these rearrangements appear to be energy dependent and are correlated with the activity of individual ATPases of the DExD/H-box family. Eight known DExD/H-box proteins are required for the splicing cycle, and mutations in these proteins inhibit the ATP-dependent steps of splicing (Staley and Guthrie 1998). Additionally, splicing requires one GTPase, Snu114, which is an essential protein in *Saccharomyces cerevisiae* (Fabrizio *et al.* 1997). Notably, Snu114 is homologous to the ribosomal translocase elongation factor G (EF-G in prokaryotes/EF2 in eukaryotes), leading to the hypothesis that Snu114 may similarly use the energy of GTP hydrolysis to drive rearrangements of the spliceosome (Fabrizio *et al.* 1997).

Snu114 is packaged with other proteins and the U5 snRNA to form the U5 small ribonucleoprotein particle (snRNP). Prior to formation of the spliceosome, U5 snRNP interacts with the U4/U6 di-snRNP, in which U4 and U6 snRNAs are extensively base paired, thus forming U4/U6·U5 tri-snRNP (reviewed in Burge *et al.* 1998). According to the canonical model of spliceosome assembly, the tri-snRNP is then recruited to the pre-spliceosome, in which U1 snRNA is base paired with the 5′ splice site and U2 snRNA is base paired with the branchpoint sequence, an intronic consensus sequence near the 3′ splice site. Although the addition of tri-snRNP forms the complete spliceosome, this complex is catalytically inert. Activation requires that the U1/5′ splice site interaction and the base pairing between U4 and U6 be disrupted, such that U1 and U4 are no longer stably associated with the spliceosome. In contrast to the stepwise pathway of spliceosome assembly, recent evidence suggests that a holospliceosome containing all five snRNPs interacts as a complex with each intron (Stevens *et al.* 2002). Nonetheless, ordered rearrangements of the snRNPs must occur prior to catalysis.

Rearrangements that occur during the early stages of spliceosome activation are regulated by several components of the U5 snRNP (Brow 2002). The Prp28 ATPase is required to unwind the U1/5′ splice site duplex, possibly by destabilizing protein components of U1 snRNP (Staley and Guthrie 1999; J. Y. Chen *et al.* 2001),

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and the Brr2 ATPase is required to unwind the U4/U6 duplex (Laggerbauer *et al.* 1998; Raghunathan and Guthrie 1998a; Kim and Rossi 1999). Presumably, the activities of Prp28 and Brr2 must be tightly regulated to ensure that catalytic activation does not occur prematurely. Genetic studies have suggested that the activities of these ATPases are modulated by Prp8, which is a large (280 kDa), well-conserved U5 snRNP protein (Kuhn *et al.* 1999; Collins and Guthrie 2000; Kuhn and Brow 2000). Prp8 is believed to inhibit the activities of Prp28 and Brr2 until spliceosome formation has occurred (Kuhn *et al.* 2002). The mechanism by which Prp8 inhibits the ATPases is as yet unknown.

A strong physical interaction between Prp8 and Snu114 suggests that Snu114 may also play a regulatory role during spliceosome activation. Treatment of U5 snRNP from human cell extract with high concentrations of chaotropic salts disrupts the complex, but Prp8 (U5-220kD in human) and Snu114 (U5-116kD in human) remain associated as a heterodimer (Achsel *et al.* 1998). Indeed, deletion of the N-terminal 120 amino acids of Snu114, which compose the only domain that is not found in EF-G, causes a temperature-sensitive block to growth and inhibits the release of U1 and U4 from the spliceosome (Bartels *et al.* 2002). Similarly, a mutation in Snu114 that is predicted to convert GTP binding to XTP binding inhibits U1 and U4 release and also decreases cellular levels of U5 and tri-snRNP (Bartels *et al.* 2003). While the N-terminal and GTPase domains have been implicated in spliceosome activation, previous studies have not addressed a function for the other domains of Snu114.

We used a two-step strategy to elucidate the timing and mechanism of Snu114 activity. First, we generated conditionally lethal alleles of Snu114 by random mutagenesis. We identified mutations in all domains of the protein, including clusters of mutations in domains III–V and mutations within the GTPase domain. We also found that deletion of the C-terminal 70 amino acids (*snu114-60*) causes a growth defect at 16° and 37°. Second, we analyzed synthetic interactions between *snu114* alleles and mutants of other proteins that function at distinct stages of splicing. We found strong genetic interactions between the *snu114* alleles and mutations in factors involved in snRNP formation and spliceosome activation. In particular, synthetically lethal interactions with *snu114-60* demonstrate a critical function for the C terminus of the protein during spliceosome activation.

MATERIALS AND METHODS

Strains and plasmids: Yeast strains are listed in Table 1 and plasmids are listed in Table 2. All strains are isogenic with BY4743 (Giaever *et al.* 2002), which is an S288C derivative, unless otherwise noted. Heterozygous diploids with *KanMX* deletions of *SNU114*, *SUB2*, *PRP22*, *PRP43*, *BRR2*, *PRP28*, and *SNU66* and haploids with *KanMX* deletions of *ISY1*, *SNT209*, and *BRR1* were obtained from Research Genetics (Huntsville, AL) (Giaever *et al.* 2002). A *PRP8* deletion strain was created by transforming a wild-type diploid with the *PRP8::LYS2 Sac*I/*Apa*I fragment from pJU224 (Umen and Guthrie 1996); integrants were confirmed by PCR. *sad1-1* (BSY387) (Lygerou *et al.* 1999), *prp2-1* (SS304), and *prp19-1* (ts87) (Vijayraghavan *et al.* 1989) strains were backcrossed twice to *snu114::KanMX* strains; *prp5-1* (SPJ 5.41) (Vijayraghavan *et al.* 1989) was crossed once to a *snu114::KanMX* strain. yTB136 was derived from GLS618 (Rader and Guthrie 2002), and yTV161 was derived from yS79 (*MAT*α sister of yS78; Wang and Guthrie 1998). yTB2 was created by sporulating YPF5 (Fabrizio *et al.* 1997).

*Eco*RI/*Bgl*II sites (in boldface type) were inserted immediately following the AUG start codon of SNU114 by PCR ampli-

TABLE 1

Yeast strains used in this study

Strain	Genotype
yTB2	*MAT***a** *trp1-Δ1 his3Δ ura3-52 lys2-801 ade2-101 snu114::HIS3* pTB1
yTB13	*MAT***a** *lys2Δ trp1Δ snu114::KanMX* pTB1
yTB23	*MAT***a** *lys2Δ snu114::KanMX* pTB1
yTB100	*MAT***a** *lys2Δ snu66::KanMX*
yTB102	*MAT***a** *lys2Δ snu66::KanMX snu114::KanMX* pTB1
yTB103	*MAT***a** *lys2Δ brr2::KanMX snu114::KanMX* pTB1 pPR151
yTB105	*MAT***a** *lys2Δ brr2::KanMX snu114::KanMX* pTB1 pTB150
yTB106	*MAT*α *lys2Δ met15Δ prp28::KanMX snu114::KanMX* pTB1 pPR9
yTB107	*MAT*α *lys2Δ met15Δ prp28::KanMX snu114::KanMX* pTB1 pPR8
yTB108	*MAT***a** *lys2Δ trp1Δ prp8::LYS2 snu114::KanMX* pTB1 pSN25
yTB111	*MAT*α *lys2Δ trp1Δ snu114::KanMX isy1::KanMX* pTB1
yTB117	*MAT*α *HIS3 prp5-1 snu114::KanMX* pTB1
yTB118	*MAT*α *HIS3 snu114::KanMX* pTB1
yTB128	*MAT*α *met15Δ*
yTB133	*MAT*α *lys2Δ brr1::KanMX snu114::KanMX* pTB1
yTB134	*MAT***a** *trp1Δ prp22::KanMX snu114::KanMX* pTB1 pTB122
yTB135	*MAT***a** *trp1Δ lys2Δ prp43::KanMX snu114::KanMX* pTB1 pTB123
yTB136	*MAT***a** *lys2Δ met15Δ prp24::KanMX snu114::KanMX* pTB1 pPR097
yTB139	*MAT***a** *lys2Δ snt309::KanMX snu114::KanMX* pTB1
yTB142	*MAT*α *met15Δ sad1-1 snu114::KanMX* pTB1
yTB143	*MAT*α *met15Δ snu114::KanMX* pTB1
yTB144	*MAT*α *lys2Δ prp19-1 snu114::KanMX* pTB1
yTB145	*MAT*α *lys2Δ snu114::KanMX* pTB1
yTB146	*MAT*α *lys2Δ prp2-1 snu114::KanMX* pTB1
yTB148	*MAT***a** *lys2Δ met15Δ sub2::KanMX snu114::KanMX* pTB1 pCG466
yTB163	*MAT*α *met15Δ snu114-40*
yTB165	*MAT*α *met15Δ snu114-60*
yTB171	*MAT*α *met15Δ snu114-12*
yTV161	*MAT***a** *trp1 lys2 prp16::LYS snu114::KanMX* pSB2 pTB1

All strains are *his3Δ leu2Δ ura3Δ* unless otherwise noted.

TABLE 2

Plasmids used in this study

Name	Genotype	Source/reference
pPR8	pSE362/*PRP28*	P. Raghunathan
pPR9	pSE362/*prp28-1*	P. Raghunathan
pPR150	pSE362/*BRR2-pya*	Raghunathan and Guthrie (1998a)
pPR151	pSE362/*brr2-1-pya*	Raghunathan and Guthrie (1998a)
pSN25	YCp50/*PRP8*	S. Noble
pJU204	pSE362/*PRP8-HA3*	Umen and Guthrie (1995)
pAK338	pSE362/*prp8-1-HA3*	A. Kutach
pJU206	pSE362/*prp8-101-HA3*	Umen and Guthrie (1995)
pCC18	pSE362/*prp8-brr-HA3*	C. Collins, S. Noble
pCC11	pRS424/*PRP8*	C. Collins
pCC121	pRS424/*prp8-201*	C. Collins, D. Brow
pPR97	pSE360/*PRP24*	Rader and Guthrie (2002)
pPR113	pSE362/*PRP24*	Rader and Guthrie (2002)
pSR53	pSE362/*prp24-RRM3sub*	Rader and Guthrie (2002)
pSR70	pSE362/*prp24-RRM4sub*	Rader and Guthrie (2002)
pSR39	pSE362/*prp24Δ10*	Rader and Guthrie (2002)
pCG466	pRS316/*SUB2*	Kistler and Guthrie (2001)
pAK354	pRS313/*SUB2*	Kistler and Guthrie (2001)
pAK356	pRS313/*sub2-1*	Kistler and Guthrie (2001)
pAK355	pRS313/*sub2-5*	Kistler and Guthrie (2001)
pSB2	pSE360/*PRP16*	Burgess and Guthrie (1993)
pSB58	pSE358/*PRP16*	Burgess and Guthrie (1993)
BHM108	pSE358/*prp16-101*	H. Madhani
BHM109	pSE358/*prp16-1*	H. Madhani
BHM110	pSE358/*prp16-2*	H. Madhani
BHM115	pSE358/*prp16-302*	H. Madhani
pTB1	pRS316/*SNU114*	Fabrizio *et al.* (1997)
pTB2	pRS314/*SNU114*	This study
pTB3	pRS316/*SNU114* + *Eco*RI/*Bgl*II sites	This study
pTB4	pRS314/*SNU114* + *Eco*RI/*Bgl*II sites	This study
pTB19	pRS314/myc-*SNU114*	This study
pTB75	pRS314/myc-*snu114-60*	This study
pTB115	pSE358/*PRP22*	Schwer and Meszaros (2000)
pTB116	pSE358/*prp22-H606A*	Schwer and Meszaros (2000)
pTB117	pSE358/*prp22-R805A*	Schwer and Meszaros (2000)
pTB118	pSE358/*PRP43*	Martin *et al.* (2002)
pTB119	pSE358/*prp43-H218A*	Martin *et al.* (2002)
pTB120	pSE358/*prp43-G429A*	Martin *et al.* (2002)
pTB121	pSE358/*prp43(91-732)*	Martin *et al.* (2002)
pTB122	pSE360/*PRP22*	Schwer and Meszaros (2000)
pTB123	pSE360/*PRP43*	Martin *et al.* (2002)

pRS plasmids are described by Sikorski and Hieter (1989) and pSE plasmids are described by Elledge and Davis (1988).

fying pTB1 (pRS316/SNU114) (Fabrizio *et al.* 1997) with the primer pairs oTB1 (5′-GGGAACAAAAGCTGGGTACCGGGC-3′)/oTB2 (5′-GGAAGC**GAATTC**CATTTTGCTATGTTAGGA GCTATTG-3′) and oTB3 (5′-CCGACC**GAATTCAGATCT**G AAGGTGACGATTTATTCGATGA-3′)/oTB4 (5′-ATCCTCT CCGGAATGTTAGCCAT-3′). The oTB1/oTB2 PCR product was digested with *Kpn*I and *Eco*RI and inserted into the same sites of pRS316. The resulting plasmid and the oTB3/oTB4 PCR product were digested with *Eco*RI and *Bam*HI and ligated together. The 4.73-kb *Bsp*EI-*Nsi*I fragment of the resulting plasmid was ligated with the 3.62-kb *Bsp*EI/*Nsi*I fragment of pTB1 to create pTB3. The *SNU114*-containing *Xho*I/*Sac*I fragments of pTB1 and pTB3 were inserted into the same sites in pRS314 to create pTB2 and pTB4, respectively.

A single myc epitope was placed immediately after the start codon of SNU114 by inserting the annealed oligos oKD140 (5′-AATTCCCAGAACAAAAATTGATTTCTGAAGAAGATTT GAATA-3′) and oKD141 (5′-GATCTATTCAAATCTTCTTCA GAAATCAATTTTTGTTCTGGG-3′), which have overhanging *Eco*RI/*Bgl*II sites, into the same sites of pTB4. The resulting plasmid was named pTB19. pTB19 was transformed into yTB2, the plasmid pTB1 was lost by passage on 5-FOA media, and the presence of the myc epitope was confirmed by Western blotting with the 9E10 antibody (BAbCO). The yeast strain carrying pTB19 grew at the same rate as the strain with pTB1 at 16°, 25°, 30°, and 37°.

The *snu114-50* mutations E910G and C928R were separated by digesting pRS314/*snu114-50* and pTB19 with *Pst*I and *Nde*I and inserting the 356-bp fragment from each plasmid into the 7.89-kb fragment of the other plasmid.

To remove the myc tag and/or the restriction sites from the N terminus of *snu114* alleles, the following restriction enzymes were used to clone the *snu114* mutations into pTB2: *Bsp*EI and *Bst*BI (*snu114-12* and *snu114-14*), *Bst*BI and *Sac*I (*snu114-30* and *snu114-50*), *Bst*BI and *Pst*I (*snu114-40*), and *Pst*I and *Sac*I (*snu114-60*). Plasmids pRS314/*SNU114* and pRS314/*snu114-12*, *-14*, *-15*, *-30*, *-40*, *-50*, and *-60* were named pTB95-102. The *snu114* alleles were moved from pRS314 (pTB95-102) to pRS315 (pTB106-113) by transforming the PCR-amplified *LEU2* marker from pRS315 with *Hin*dIII-cut pRS314/*snu114* plasmids into a wild-type strain. Plasmids were recovered from LEU2+ transformants.

Mutagenesis of *SNU114*: *SNU114* was PCR amplified in two fragments. Fragment 1 was amplified with oTB7 (5′-CTTGCC AACGGCTGACGATTGC-3′; 67 bp upstream of start AUG) and oTB8 (5′-CAAACAGTCCATATACAGCTCTCC-3′; 1965 bp downstream of start AUG). Fragment 2 was amplified with oTB9 (5′-CCGCGACGTTGTACTCTGTAAAG-3′; 1720 bp downstream of start AUG) and oTB10 (5′-CCAGTGAATTG TAATACGACTCAC-3′; 3270 bp downstream of start AUG). Mutagenesis was performed under standard PCR conditions, using the natural error rate of Taq DNA polymerase. PCR reactions (100 μl volume) contained 1× Taq buffer (Roche), 200 μM each dNTP, 400 nM each oligo, 2.5 units of Taq (Roche), and 10 ng plasmid DNA. The PCR cycling parameters were the following: 2 min at 94°; 10 cycles of 1 min at 94°, 1 min at 55°, 2 min at 72°; and 4 min at 72°. After every 10 cycles of PCR amplification, reactions were diluted 1:100. Two separate sets of reactions were performed (set A/B and set C). For set A, a total of 20 PCR cycles were performed, and an additional 10 cycles gave set B. For set C, a total of 40 cycles using Taq were performed, followed by an additional 10 cycles using Expand Taq. At least two independent PCR reactions were performed for each set. pTB19 (pRS314/*myc-SNU114*) was the template for sets A and B and pTB4 (pRS314/*SNU114*) was the template for set C. Fragment 1 PCR products were transformed with the *Eco*RI/*Bst*BI-cut vector of pTB19 or pTB4, and fragment 2 PCR products were transformed with the *Bst*BI/*Nde*I-cut vector of pTB19 or pTB4. PCR products and gapped vectors were transformed into yTB2. Transformants were selected on SD-TRP media at 25° and replica plated to 5-FOA media at 25°. Lethality on 5-FOA was 8–13% for fragment 1 and 2–4% for fragment 2, depending on the number of PCR cycles. Strains were then tested for conditional lethality by replica plating to YPD and incubating at 16° for 3 days, 25° for 1–2 days, and 37° for 1 day. Approximately 25,000 colonies were screened for each fragment. Plasmids from strains that were growth impaired at 37° or 16° were recovered and transformed into yTB2 and yTB13. Plasmids that conferred conditional growth defects were sequenced by the Biomolecular Resource Center DNA sequencing facility at the University of California, San Francisco. The NCBI BLAST alignment server (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) was used to identify mutations in the recovered *snu114* alleles.

The mutations K146I and T147N were created using the QuikChange site-directed mutagenesis method (Stratagene, La Jolla, CA) with the template pTB2. The mutations P216N and H218R were created using site-directed mutagenesis of the template pTB19. Mutations were confirmed by sequencing and were subcloned into fresh plasmids.

Integration of *snu114* alleles: An integrating *snu114-12* plasmid (pTB126) was made by inserting the 3.3-kb *Pvu*II fragment of pTB107 into the *Pvu*II-cut vector of pRS306. Integrating *snu114-40* and *snu114-60* plasmids (pTB130 and pTB132) were created by ligating the 4.25-kb *Nhe*I/*Ngo*MIV fragment of pTB126 with the 3.63-kb *Nhe*I/*Ngo*MIV fragments of pTB111 and pTB113. Integrating plasmids pTB126, pTB130, and pTB132 were linearized with *Nhe*I (pTB126) or *Eco*47III (pTB130/132), transformed into yTB128, and selected on SD-URA media at 25°. Following growth on 5-FOA, transformants were streaked to YPD and grown at 16°, 25°, and 37° to select for thermal-sensitive (ts) or ts/cold-sensitive (cs) integrants.

Primer extensions: For primer extensions, the following strains were grown in liquid YPD media to OD 0.5–1.0: yTB128 (*SNU114*), yTB165 (*snu114-60*), and yTB171 (*snu114-12*), all grown at 25°; and yTB23 carrying either pTB106 (*SNU114*) or pTB111 (*snu114-40*), grown at 30°. Cells were spun down, resuspended in YPD media preincubated at 37° or 16°, and grown in water baths at either 37° or 16°; 10-ml aliquots were removed at the indicated times. Cultures were diluted during the time course to maintain an OD between 0.5 and 1.0. *prp16-2* [yS79 (Wang and Guthrie 1998) + BHM110] was shifted to 37° for 3 hr as a control for a mutant that blocks the second step of splicing. RNA was isolated (Schmitt *et al.* 1990), and primer extension was performed as described (Boorstein and Craig 1989). Ten micrograms of RNA was used per reaction. The following oligos were used: U3, 5′-CCAAGTTGG ATTCAGTGGCTC-3′; RPS17/RP51, 5′-CTTAGAAGCACGC TTGACGG-3′; PGK1, 5′-ATCTTGGGTGGTGTTCC-3′; and U14, 5′-ACGATGGGTTCGTAAGCGTACTCCTACCGT-3′. Data were quantitated by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

***In vitro* splicing assays:** Liquid cultures of yTB23 in which pTB1 had been replaced by pTB106 (*SNU114*), pTB107 (*snu114-12*), pTB111 (*snu114-40*), or pTB113 (*snu114-60*) were grown at 30° to OD 1.2–1.4. Splicing extracts were prepared and actin pre-mRNA was spliced as described (Umen and Guthrie 1995). Extracts were preincubated for 20 min at 37° or on ice in the presence of splicing buffer components (2.5 mM MgCl2, 60 mM potassium phosphate pH 7, 3% PEG 8000) prior to addition of radiolabeled actin and ATP (final concentrations of 0.5 nM and 2 mM, respectively). Splicing reactions were performed at 25°.

Testing genetic interactions: To test genetic interactions, we created strains deleted for *SNU114* (*snu114::KanMX*) in combination with deletion or mutation of a second gene. Deletions were covered by wild-type plasmids marked with *URA3*. Plasmids containing mutant alleles of *snu114* (and, in some cases, of a second gene) were transformed, and the ability to lose the *URA3*-marked wild-type plasmid(s) on 5-FOA was tested.

The following strains (mutant allele/wild-type allele) were transformed with pTB106-113: yTB102/yTB23 (*snu66*Δ), yTB103/yTB105 (*brr2-1*), yTB106/yTB107 (*prp28-1*), yTB117/yTB118 (*prp5-1*), yTB133/yTB23 (*brr1D*), yTB139/yTB23 (*snt309*Δ), yTB142/yTB143 (*sad1-1*), yTB144/yTB145 (*prp19-1*), and yTB146/yTB145 (*prp2-1*). The strains yTB111/yTB15 (*isy1*Δ) were transformed with pTB95-102. yTB108 (*prp8*Δ) was transformed with combinations of either pTB95-102 and pJU204, pAK338, pJU206, and pCC18 or pTB106-113 and pCC11 and pCC121. yTB134 (*prp22*Δ) was transformed with pTB106-113 and pTB115-117. yTB135 (*prp43*Δ) was transformed with pTB106-113 and pTB118-121. yTB136 (*prp24*Δ) was transformed with pTB106-113 and pPR113, pSR53, pSR70, and pSR39. yTB148 (*sub2*Δ) was transformed with pTB106-113 and pAK354-356. yTV161 (*prp16*Δ) was transformed with pTB106-113 and pSB58, BHM108-110, and BHM115. Additionally, *snu114*Δ*N* (Bartels *et al.* 2002) was cloned into pRS315 and transformed into yTB23, yTB102, yTB103, and yTB105-107. In all cases, corresponding empty vectors were also transformed as negative controls. Transformants were selected on the appropriate selective media at 25°. Between 4 and 14 transformants were streaked onto 5-FOA-containing media and incubated at 25° for up to 6 days. If no colonies grew on 5-FOA, the combination of alleles was considered synthetically

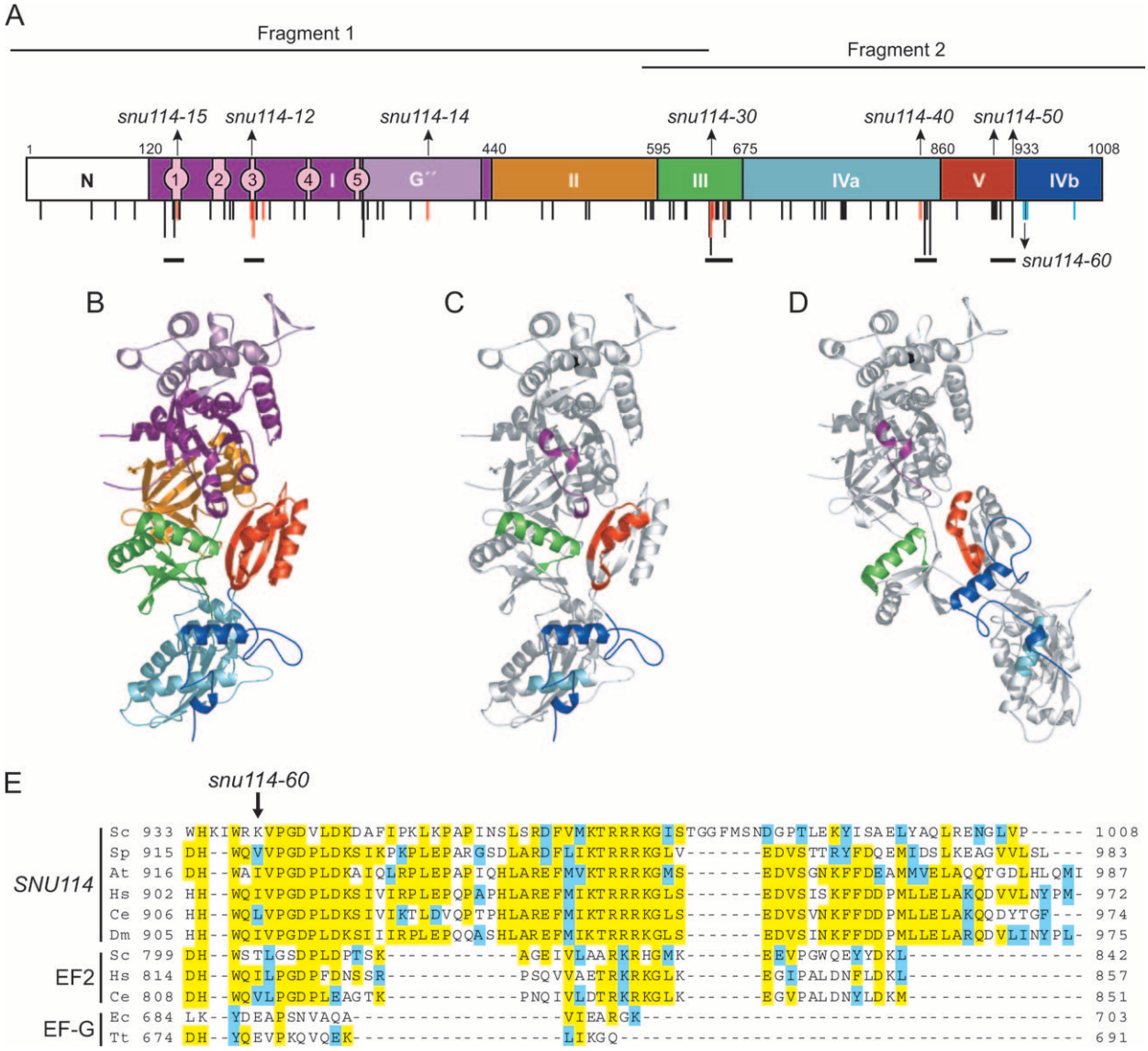

FIGURE 1.—Mutagenesis of *SNU114* and mapping mutations to the predicted three-dimensional structure. (A) Linear diagram of the domains of Snu114, as defined by homology to EF2. Domains are labeled by Roman numerals. The domain N is not found in EF2. The GTPase motifs G1–G5 are found within domain I and are labeled 1–5. G′′ is an insertion within domain I unique to EF2 and Snu114. Fragments 1 and 2 represent the portions of the gene that were PCR amplified to screen for conditionally lethal mutations. Each mutation arising from PCR mutagenesis is depicted as a vertical bar below the position of the affected amino acid. The length of the vertical bars represents the number of times an amino acid was identified in our screen (one, two, or three times). Single point mutations that were found to cause a growth phenotype are red. Frameshift and nonsense mutations that result in early truncation of the protein are blue. Clusters of mutations are underlined with a black bar. Arrows indicate the positions of mutations studied in this work. (B) A model of the structure of Snu114, as determined by comparison with the structure of *S. cerevisiae* EF2 using the program MODELLER. Domains are colored as in A. (C) The clusters of mutations underlined in A are shown in color. Of the two clusters identified in domain I, the G1 motif is the top purple segment, and the G3 motif is the bottom purple segment. The *snu114-14* mutation is black. The region of domain IVb that is deleted in *snu114-60* is shown in dark blue. (D) A model of Snu114 based on the structure of EF2 bound to the translocation inhibitor sordarin. Clusters of mutations are colored as in C. (E) ClustalW alignment of domain IVb of Snu114 orthologs, EF2, and EF-G from *S. cerevisiae* (Sc), *S. pombe* (Sp), *A. thaliana* (At), *H. sapiens* (Hs), *C. elegans* (Ce), *D. melanogaster* (Dm), *T. thermophilus* (Tt), and *E. coli* (Ec). Identical residues are yellow and similar residues are blue. The arrow indicates the *snu114-60* truncation.

lethal. Viable strains were streaked to YPD media at 25°. Liquid cultures of each strain in duplicate were grown overnight at 25° and diluted to OD 0.1. Tenfold serial dilutions were grown on YPD plates at 16° for 8 days and at 25°, 30°, and 37° for up to 4 days. For *prp8* strains, six 5-fold serial dilutions were made; the middle three dilutions are shown in Figure 7. For weak genetic interactions, we cannot distinguish between synthetic enhancement and additive enhancement.

A high number of transformants of *snu114-12*, *snu114-14*, and *snu114-15* in combination with *prp28-1* or *snu66*Δ (and *snu114-50* with *snu66*Δ) either were not viable on 5-FOA or produced very few colonies. For example, for the combination

TABLE 3

***SNU114* alleles generated by PCR mutagenesis of domains N, I, and II (fragment 1)**

Name	Growth 37°	Growth 16°	G1 (aa 143–149)	G3 (aa 216–221)	Other
1A-4	+	+++	L149S		N135S, I191S
1A-6	+	+++	H143R	H218R	
1A-9	+	+++	S144P		F298L
1A-11[a]	−/+	+++			V227D
1A-79[a,b]	+	+			L381P
1C-4	−/+	+++	T147A		Y178C
1C-5	−/+	+++			L199P, F320L, L435P
1C-6	−/+	+++			S196P, K325E
1C-8	−	+++		P216N	S256G, H334Y
1C-11	+	+++			T321A, F592C
1C-12	−/+	+++			F19L, N135D, L425P, I488M, D529N, D532N
1C-13	−/+	+++		H218R	E67V, F265L
1C-16	−/+	+++			D233G, L589P
1C-20	−/+	+++		F221S	S339F
1C-23	−/+	+++	S144L		T107A, D497G
1C-28	−/+	+++	S148P		T88A, T321A, E365G, L611S

Growth at 37° and 16° was scored, as compared to wild type (+++). Mutations in the G1 and G3 motifs are listed under the appropriate heading, while remaining mutations are listed as "other."

[a] Alleles containing a single mutation.

[b] 1A-79 is the allele *snu114-14*.

of *snu114-12* and *snu66*Δ, 4/14 transformants were not viable on 5-FOA, and 9/14 transformants produced only a small number of colonies on 5-FOA. To avoid the possibility that the viable colonies represented suppressors or revertants, we crossed integrated *snu114-12* (yTB171), *snu114-40* (yTB163), *snu114-60* (yTB165), and *SNU114* (yTB28) to *snu66::KanMX* (yTB100) and *prp28-1* (yEJS51) (Strauss and Guthrie 1991) strains and dissected tetrads. Genotypes of the spores were determined by replica plating to G418-containing media (for *snu66::KanMX*) and to YPD plates that were incubated at 16° and 37° (for the other ts/cs mutations).

Sequence alignment and structure modeling: An alignment between Snu114 and Eft1 (*S. cerevisiae* EF2) was created using ClustalW (Thompson *et al.* 1994) and modified by hand, and the structure of Snu114 was modeled on the structure of apo-EF2 (PDB code 1N0V) and sordarin-bound EF2 (PDB code 1N0U) using the program MODELLER (Sali and Blundell 1993). Structures were visualized with PyMOL (DeLano 2002). Coordinates of the models are available upon request.

The alignment of domain IVb was shaded using BOXSHADE (version 3.2; K. Hofmann and M. Baron). Swiss-Prot accession numbers are as follows: Snu114 from *S. cerevisiae* (P36048), *Schizosaccharomyces pombe* (O94316), *Arabidopsis thaliana* (Q9LNC5), *Homo sapiens* (Q15029), *Caenorhabditis elegans* (Q23463), *Drosophila melanogaster* (Q9VAX8); EF2 from *S. cerevisiae* (P32324), *H. sapiens* (P13639), *C. elegans* (P29691); EF-G from *Thermus thermophilus* (P13551), *Escherichia coli* (P02996).

RESULTS

Modeling the structure of Snu114: To analyze how mutations in Snu114 might affect its function, we used the program MODELLER (Sali and Blundell 1993) to model the structure of Snu114 onto the crystal structure of *S. cerevisiae* EF2 (Jorgensen *et al.* 2003), which was possible because of the high sequence similarity between the two proteins (26% identity, 46% similarity). By homology with EF2, Snu114 contains five structural domains (Figure 1A), as well as a 120 -amino-acid N-terminal extension that is not conserved in EF2 and thus could not be modeled. The C terminus of the protein (IVb) folds back onto domain IVa and so is considered part of domain IV. For clarity, we refer to the two portions of domain IV as IVa and IVb. Figure 1, B and C, show the structure of Snu114 modeled onto nucleotide-free EF2, and Figure 1D shows the structure of Snu114 modeled onto EF2 bound to the translation inhibitor sordarin, which is believed to block EF2 on the ribosome in a post-translocation state (Jorgensen *et al.* 2003). The two structures demonstrate the flexibility of the protein. In particular, domains I and II appear as a rigid body, while domain III rotates around a linker between domains II and III, and domains IV and V rotate as a rigid unit.

Screen for conditionally lethal alleles of *SNU114*: To generate conditionally lethal alleles of *SNU114*, we used the error-prone polymerase Taq to amplify the gene in two pieces: fragment 1 spans domains N, I, and II, and fragment 2 spans domains III–V (Figure 1A). Each fragment was transformed in combination with an appropriately gapped plasmid containing *SNU114* and the *TRP1* marker into a yeast strain in which the chromosomal copy of *SNU114* was deleted and wild-type *SNU114* was present on a counterselectable *URA3*-marked plasmid (Muhlrad *et al.* 1992). For each fragment, we screened ~25,000 transformants. When colonies were replica plated to 5-FOA-containing media to select against the wild-type *SNU114*

TABLE 4

***SNU114* alleles generated by PCR mutagenesis of domains III–V (fragment 2)**

Name	Alleles used in this study	Growth 37°	Growth 16°	Domain III (aa 646–664)	Domain IVa (aa 842–851)	Domain V (aa 909–928)	Domain IVb (truncation)	Other
2A-1[a]		−/+	+++	G648V				
2A-4		++	+++	D658G		D917G		
2A-5[a]	*snu114-30*	−/+	+++	G646R				
2A-9[a]		−/+	+++	G646R				
2A-11[a]		+	+++	L659P				
2A-13		−	−/+	S662G	C846R, L851R			
2A-15		−/+	+++		C846R			N623S
2A-16	*snu114-50*	−/+	+++			E910G, C928R		
2A-17		−/+	+++	G646E	L851Q			
2A-18	*snu114-62*	+	+++				G986fs	V940A
2A-20		−/+	+++		V848I	S911P		F768S
2A-24		+	+++	L659R				N743S
2B-107[a]	*myc-snu114-60*[b]	−/+	−				K939ns	
2B-119	*snu114-61*	++	++				G942fs	D585G, N683S
2C-2		−/+	+++	D653V, A664T				I795T, V876A
2C-3		−/+	+++	L645P				T753I
2C-7		−	+++			C928R		N770D, N772D
2C-9		−	+++		L851P	G913R		
2C-11	*snu114-40*	−/+	+++		M842R			
2C-12		−/+	+++					L607P, L622P, Q721R, I750V
2C-13		+	+++	L645P				E811K
2C-14		−/+	+++	Y651C	C846R			
2C-16		−	+++			I909T		A712P, P812L, K819I

Growth at 37° and 16° was scored, as compared to wild type (+++). Mutations found in small clusters in domain III (aa 646–664), domain IVa (aa 842–851), and domain V (aa 909–928) are listed under the appropriate heading, while the remaining mutations are listed as "other." Three alleles with truncations of domain IVb were found: fs, frameshift mutation; ns, nonsense mutation.

[a] Alleles containing a single mutation.

[b] *myc-snu114-60* should be compared to *snu114-60* (Table 5).

plasmid, >87% of the colonies were viable. We then screened for colonies that were unable to grow at 37° or 16°. From the transformation of mutagenized fragment 1, we isolated 37 ts strains and one strain that was both cs and ts. Mutagenesis of fragment 2 yielded 54 ts and three cs/ts strains. Plasmids were recovered from 20 ts strains for each of the fragments and from all of the cs/ts strains and were retransformed into the starting strain. Five of the plasmids from fragment 1 did not retest and were discarded. The remaining plasmids were sequenced.

The sequenced alleles contained an average of two mutations per gene, with a range of one to six mutations (Tables 3 and 4). Despite the presence of multiple mutations per allele, many of the mutations clustered within small stretches of highly conserved amino acids, highlighting regions that are important for Snu114 function (Figure 1A). Individual amino acids within these clusters were often mutated in multiple independent clones.

Fragment 1 mutations: Domain I contains the conserved motifs, G1–G5, which are present in all GTPases and are necessary for GTP binding and hydrolysis (Bourne *et al.* 1991). Of the 16 alleles from fragment 1 that were sequenced, 6 contain a mutation in the G1 motif, and 4 contain a mutation in the G3 motif. Concomitant with the PCR mutagenesis, we designed two mutations in the G1 motif of *SNU114* that are expected to decrease nucleotide binding (Ogg *et al.* 1998); these mutations, K146I (*snu114-15*) and T147N, confer a ts growth phenotype (Table 5). To study single point mutations in the G3 motif of *SNU114* that are predicted to affect GTP hydrolysis, we created the individual mutations P216N (*snu114-12*) and H218R, which had arisen in combination with other mutations during PCR mutagenesis. Each of these mutations alone causes a ts growth defect (Table 5).

While the structure of the G domain of most GTPases is similar, an insertion termed the G′′ domain is found in EF2 and Snu114, but not in the other translational GTPases (Aevarsson 1995). Although no function has been assigned to this domain, it has been postulated to act as a guanine exchange factor (GEF), since EF2 is

TABLE 5

Alleles resulting from site-directed mutagenesis or cloning

Alleles used in this study	Mutation	Motif	Growth 37°	Growth 16°
	T147N[a]	G1	−/+	+++
snu114-15	K146I[a]	G1	−	+++
snu114-12	P216N[b]	G3	−/+	+++
	H218R[c]	G3	+	+++
	E910G[d]		+++	+++
	C928R[d]		+++	+++
snu114-60	K939ns[e]		++	++

Growth was scored at 37° and 16°, as compared to wild type (+++).

[a] Site-directed mutation based on Ogg *et al.* (1998).

[b] Site-directed mutation based on allele 1C-8 (Table 3).

[c] Site-directed mutation based on alleles 1A-6 and 1C-13 (Table 3).

[d] Separation of mutations in *snu114-50* by cloning (Table 4).

[e] Removal of myc tag from *myc-snu114-60* (Table 4).

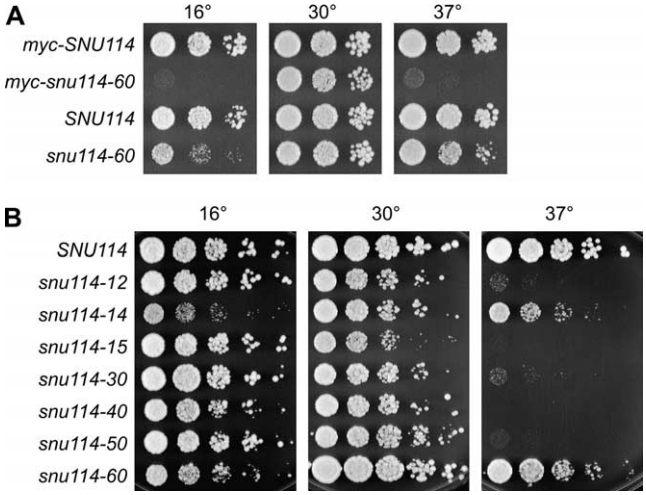

Figure 2.—*snu114* alleles exhibit conditional growth defects at 16° and 37°. (A) Growth of serial dilutions of strains carrying wild-type *SNU114* and *snu114-60*, with and without the myc epitope. (B) Growth of serial dilutions of *snu114* alleles, present as low-copy plasmids with no epitope tag. Cells were spotted onto YPD media and grown at 16° for 8 days, 30° for 2 days, and 37° for 2 days.

not known to have an extrinsic GEF (Aevarsson *et al.* 1994; Czworkowski *et al.* 1994). In Snu114, a single mutation in this domain, L381P (*snu114-14*), causes slow growth and a weak ts/cs phenotype.

Fragment 2 mutations: Over half of the mutations identified in domains III–V are found within three 10- to 20-amino-acid stretches, which are underlined in Figure 1A and highlighted in Figure 1, C and D. The 23 sequenced fragment 2 alleles contain a total of 49 mutations. Thirteen of the mutations fall within a short stretch of domain III (residues 645–664), 8 mutations are within a small region of domain IVa (residues 842–851), and 7 mutations are clustered in domain V (residues 909–928). While a majority of the alleles that arose from mutagenesis of fragment 2 contain multiple mutations, 6 alleles contain a single point mutation. Four of these mutations, including *snu114-30*, are in the domain III cluster, and one, *snu114-40*, is in the domain IVa cluster. That single-amino-acid changes cause a conditional growth defect emphasizes the functional significance of these regions. Furthermore, in comparing Snu114, EF2, and EF-G, the amino acid cluster in domain III contains the most conserved stretch of amino acids outside of domain I (Cammarano *et al.* 1992). As shown in Figure 1, C and D, the clusters in domain III and V are in close proximity to each other and to the G3 motif. The cluster of residues in domain IVa forms a helix that faces domain IVb.

In addition to the mutations in domains III, IVa, and V, an additional class of mutations was found in domain IVb. Domain IVb is larger in Snu114 than in its homologs: the domain is 76 residues in Snu114, but only 44 residues in yeast EF2 and 20 residues in *E. coli* EF-G (Figure 1E). In comparison with EF2, Snu114 domain IVb has several insertions as well as a C-terminal extension. Only three sets of mutations were found in this domain, and all result in early truncation of the protein. The weak ts allele *snu114-62* causes a deletion of 23 amino acids, while the ts/cs alleles *snu114-60* and *snu114-61* result in the deletion of 70 and 68 amino acids, respectively (Table 4).

The severity of the growth defect of the C-terminal deletions was affected by the presence of a myc epitope that had been placed at the N terminus of the gene prior to mutagenesis. Although *myc-snu114-60* and *snu114-61* have similar deletions, the growth defect of *myc-snu114-60* is much stronger. The allele *snu114-61* also contains mutations in domains II and IV and does not contain the myc epitope, perhaps due to homologous recombination with chromosomal *SNU114*. We tested whether any of these differences could suppress the growth defect of *myc-snu114-60* and found that removing the myc epitope from this allele greatly reduces the severity of the growth defect at both high and low temperatures (Figure 2A). The presence or absence of the myc epitope did not affect the growth phenotype of the other *snu114* alleles that were further characterized.

Where possible, we focused subsequent experiments on a set of alleles with single mutations in each of the domains that were identified above as important. For the work described below, we used the alleles *snu114-15* (G1 motif), *snu114-12* (G3 motif), *snu114-14* (G′′ domain), *snu114-30* (domain III), *snu114-40* (domain IVa), *snu114-50* [domain V; *snu114-50* contains two mutations, because the single mutations did not cause a growth phenotype (Table 5)], and *snu114-60* (truncation of domain IVb; see Tables 3 and 4). All alleles are ts. *snu114-14* and *snu114-60* are only weakly ts as well as weakly cs (Figure 2B).

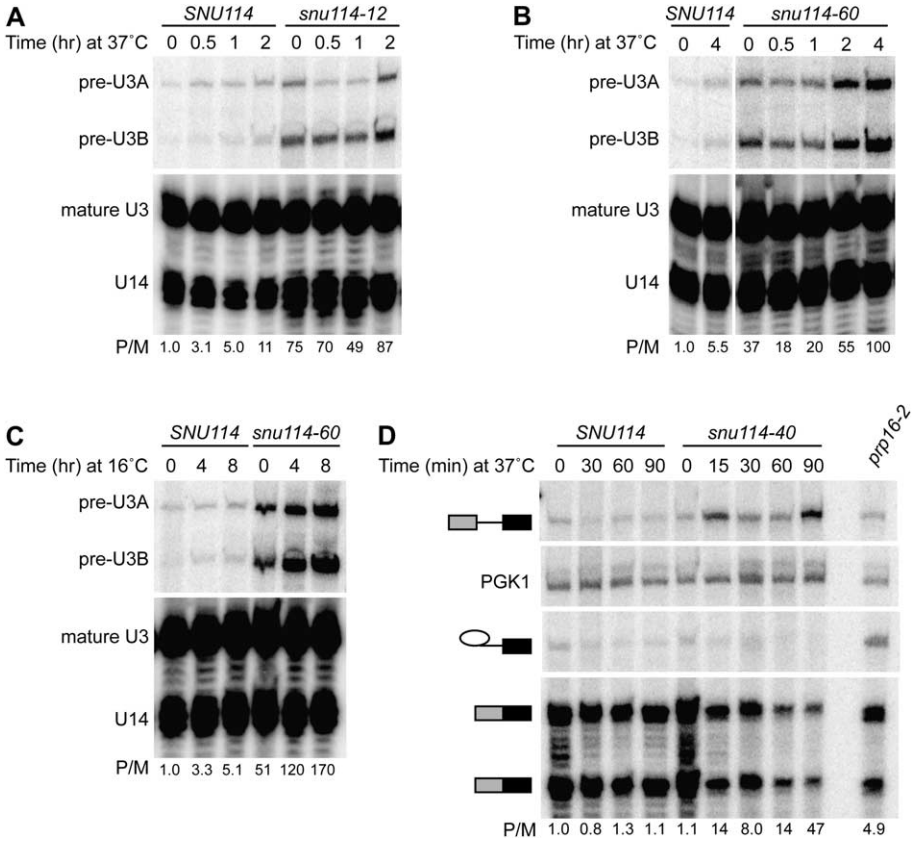

FIGURE 3.—*snu114* mutations block the first step of splicing *in vivo*. (A–C) Primer extensions of U3 RNA from wild-type, *snu114-12* (A), and *snu114-60* (B and C) cells shifted to either 37° (A and B) or 16° (C). The positions of pre-U3A, pre-U3B, and mature U3 are indicated. The snoRNA U14 was used as a loading control. The top portions of the gels are shown at a darker exposure than the bottom portions. (D) Primer extension of *RPS17/RP51* RNA from wild-type and *snu114-40* cells shifted to 37°. RNA from *prp16-2* cells shifted to 37° for 3 hr was included in the right lane as a standard for a second-step mutant that causes an increase in the level of lariat intermediate. The two *RPS17* mRNA species arise because the oligo hybridizes to both the *RPS17A* and *RPS17B* transcripts. *PGK1* was used as a loading control. The positions of the unspliced pre-mRNA, lariat intermediate, and spliced mRNA are shown schematically. P/M is the ratio of pre-mRNA to mature RNA. In A–C, pre-U3B was used for P. In D, the two mRNA species were summed for M. For A–D, ratios were normalized such that P/M for wild type at time 0 = 1.0.

***snu114* mutants exhibit *in vivo* and *in vitro* splicing defects:** We tested whether a subset of the *snu114* mutations inhibit splicing. To monitor *in vivo* splicing, we purified RNA from cells that had been shifted to the nonpermissive temperature for various times and performed primer extensions on the intron-containing transcripts U3 and *RPS17/RP51*. U3 is a nucleolar snoRNA, and *RPS17* encodes a ribosomal protein. Mutations that block the first chemical step of splicing are expected to increase the level of precursor mRNA, while mutations that affect the second step of splicing should cause an accumulation of lariat intermediate. Strains containing the integrated *snu114-12* allele grow slowly even at 25° and show a constitutive accumulation of U3 precursor (Figure 3A). The level of pre-U3 in *snu114-60* cells, which are both ts and cs, increases following a shift to either 37° or 16° (Figure 3, B and C). Primer extension of the less stable *RPS17* transcript reveals that shifting *snu114-40* to 37° causes a rapid increase in pre-mRNA, as well as a rapid and dramatic decrease in the level of mature mRNA (Figure 3D). While primer extension with U3 allows only the differentiation of precursor from mature RNA, the lariat intermediate of *RPS17* can be resolved. In contrast to the second-step mutant *prp16-2* (BURGESS and GUTHRIE 1993), none of the *snu114* mutants that we tested accumulate lariat intermediate (Figure 3D and data not shown). Together, pre-mRNA accumulation and a lack of lariat intermediate accumulation show that the *snu114* mutants block splicing at or before the first chemical step.

The data from *in vitro* splicing in extracts made from *snu114* mutant strains grown at the permissive temperature were consistent with the *in vivo* splicing data. To monitor splicing *in vitro*, radiolabeled actin pre-mRNA was incubated with extracts that had been preincubated either at 37° or on ice. The splicing intermediates and products were then separated by PAGE and visualized by phosphorimaging. The *snu114-12* extract splices poorly even at 25°, consistent with a constitutive defect (Figure 4A). Splicing in both *snu114-40* and *snu114-60* extracts can be inactivated by preincubation at high temperature (Figure 4, B and C). While the kinetics of splicing in *snu114-60* extract at 16° *in vitro* are slow, they are not strongly inhibited (data not shown). For all *snu114* alleles, the levels of all splicing intermediates are decreased in the mutant extracts, indicating an early block to splicing.

Genetic interactions of *snu114* mutants: Inhibition of the first step of splicing can arise for many reasons, including defects in snRNP levels, defects in U1 or U2 addition, defects in tri-snRNP addition, and defects in activation of the spliceosome. Synthetic enhancement has proven to be a powerful tool for elucidating functions of and interactions between factors of large multicomponent complexes (GUARENTE 1993; DOYE and HURT 1995). To gain an understanding of the timing of

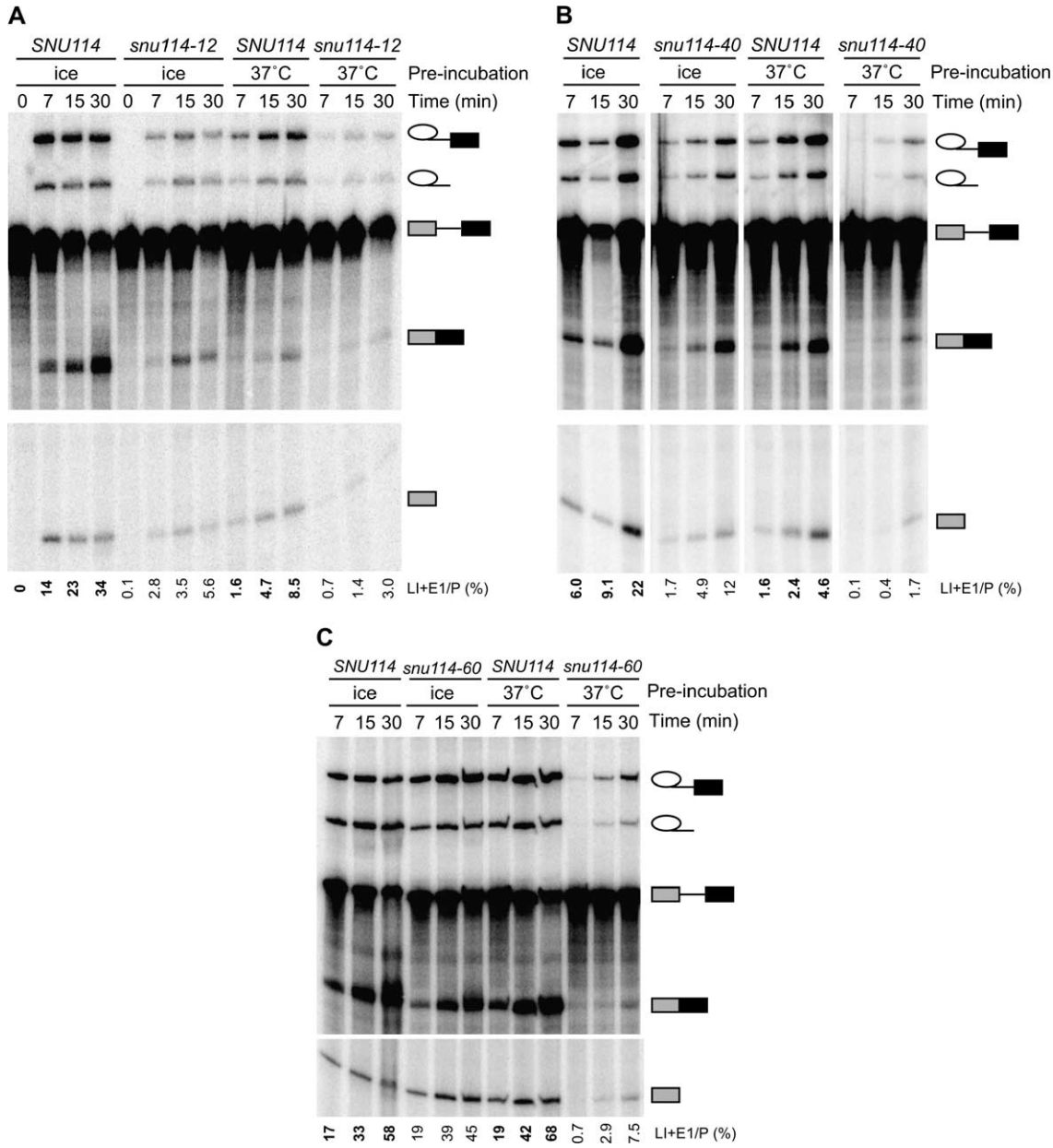

FIGURE 4.—Mutations in *SNU114* inhibit splicing *in vitro*. Extracts from wild-type, *snu114-12* (A), *snu114-40* (B), and *snu114-60* (C) strains were preincubated either on ice or at 37° for 20 min. Splicing was monitored by adding ATP and ^{32}P-labeled actin pre-mRNA and incubating at 25° for the time listed. The positions of the lariat intermediate, lariat, pre-mRNA, spliced mRNA, and exon 1 are indicated schematically on the right in A–C. The products of the first-step reaction are expressed as a percentage of the lariat intermediate (LI) + exon 1 (E1) compared to precursor (P).

Snu114 function(s), we examined genetic interactions between the *snu114* alleles and mutations or deletions of 16 splicing factors known to be required at different stages of splicing (Figure 5). We tested each combination for viability and for conditional growth defects. Taking into consideration changes in growth rates at 16°, 25°, 30°, and 37°, the overall strength of synthetic enhancement for each interaction was rated on a scale of 0 to 5, where 0 indicates no interaction and 5 indicates a synthetically lethal interaction (Table 6). Interactions that were synthetically lethal or synthetically sick are summarized in Figure 5 (solid stars and shaded stars, respectively).

Interactions with ATPases: Each of the eight spliceosomal DExD/H box ATPases acts at a clearly defined and distinct stage of splicing (STALEY and GUTHRIE 1998). Strikingly, mutations in the ATPases Prp28 and Brr2, which are needed for activation of the spliceosome, exhibit strong genetic interactions with *snu114* mutations. Although *snu114-60* has only a weak conditional growth defect, it is inviable in combination with *prp28-1*, a cs mutation of Prp28 that disrupts the ex-

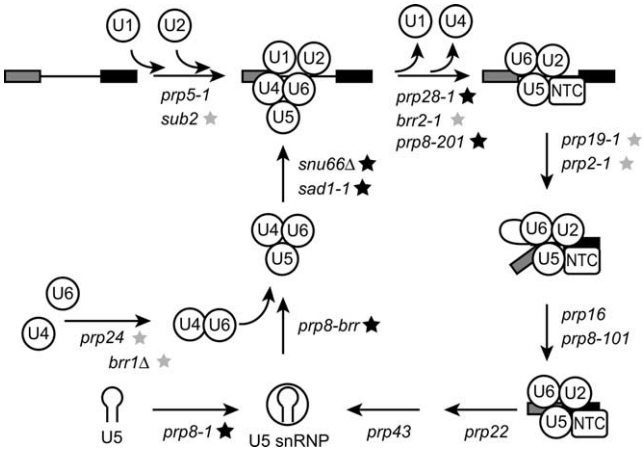

FIGURE 5.—*snu114* alleles are synthetically lethal with splicing factors that act prior to the first step of catalysis. The place at which each tested mutation blocks splicing in the canonical splicing cycle is depicted. In cases where multiple alleles of the same gene are thought to affect the same step, only the gene name (and not the allele) is listed. Mutations that are synthetically lethal or synthetically sick with at least one *snu114* allele are symbolized by solid and shaded stars, respectively.

change of U1 for U6 at the 5′ splice site (STALEY and GUTHRIE 1999) (Figure 6A). *prp28-1* is also synthetically sick in combination with mutations in domain I (*snu114-12*, *snu114-14*, and *snu114-15*) (data not shown). *brr2-1*, a cs mutation that decreases the U4/U6 unwinding activity of Brr2 (RAGHUNATHAN and GUTHRIE 1998a), is synthetically lethal with *snu114-60* at 16° and 37° (Figures 6A and 7B). In contrast to *prp28-1*, *brr2-1* interacts strongly only with *snu114-60*, although *snu114-14* and *snu114-40* enhance the cs growth defect (Figure 7B and data not shown).

snu114-60 is not synthetically lethal with any of the other ATPase mutations that were tested (Figure 6A and data not shown). The growth defects of the other *snu114* alleles are moderately enhanced by mutations in Sub2, which functions during a U2 snRNP addition (KISTLER and GUTHRIE 2001; LIBRI *et al.* 2001; ZHANG and GREEN 2001); in Prp2, which acts immediately before the first chemical step (KIM and LIN 1996); and in Prp22, which helps to disassemble the postcatalytic spliceosome (COMPANY *et al.* 1991). Mutations in Prp5, Prp16, and Prp43, which are involved in U2 addition, second-step catalysis, and spliceosome recycling, respectively, exhibit no genetic interactions with *snu114* alleles (SCHWER and GUTHRIE 1991; RUBY *et al.* 1993; ARENAS and ABELSON 1997). Together, the pattern of genetic interactions with the spliceosomal ATPases suggests that Snu114, and especially domain IVb, is specifically involved in spliceosome activation.

Interactions with *PRP8* alleles: Distinct mutations of the core U5 protein Prp8 affect several stages of spliceosome assembly and both steps of splicing. While none of the *snu114* mutations have strong genetic interactions with *prp8-101*, which inhibits the second step of splicing (UMEN and GUTHRIE 1995), many of the alleles have synthetic interactions with *prp8* mutations that cause earlier splicing defects (Figures 6B and 7A). The mutations *prp8-1* and *prp8-brr* impair the formation of U5 and tri-snRNP (BROWN and BEGGS 1992; COLLINS 2001) and exhibit strong genetic interactions with *snu114* mutations. Notably, *prp8-1* and *prp8-brr* are synthetically lethal with *snu114-40* and *snu114-60*, indicating that Snu114 domain IV is important for interacting with Prp8 during snRNP formation. Additionally, many of the *snu114* mutations are synthetically sick or lethal in combination with *prp8-201*, an allele that affects spliceosome activation (KUHN *et al.* 1999).

Interactions with factors involved in tri-snRNP addition, snRNP biogenesis, and snRNP recycling: Because *snu114* mutations cause a block prior to the first step of splicing, we also tested genetic interactions with factors that are involved in the addition of tri-snRNP to the spliceosome and in snRNP biogenesis and recycling. In mammalian extract, depletion of either of the tri-snRNP proteins Snu66 or Sad1 inhibits tri-snRNP addition (MAKAROVA *et al.* 2001). In yeast, deletion of *SNU66* causes a cs growth defect and inhibits the first step of splicing (GOTTSCHALK *et al.* 1999; STEVENS *et al.* 2001), and the ts allele *sad1-1* blocks splicing and decreases the formation of U4/U6 di-snRNP (LYGEROU *et al.* 1999). Deletion of *SNU66* is synthetically lethal with *snu114-60* and enhances the growth defects of *snu114-12*, *-14*, *-15*, and *-50* (Figures 6A and 8 and data not shown). All of the *snu114* alleles are synthetically lethal or sick in combination with *sad1-1*.

The recycling factor Prp24 assists in the base pairing of U4 and U6 snRNAs (RAGHUNATHAN and GUTHRIE 1998b). It has also been proposed to influence spliceosome activation, as alleles of *prp24* are synthetically lethal with *prp28-1* and with U4-cs1, a mutation in U4 that blocks spliceosome activation (STRAUSS and GUTHRIE 1991; KUHN and BROW 2000). The ts mutation *prp24-RRM3sub*, which is believed to disrupt the ability of Prp24 to bind RNA (VIDAVER *et al.* 1999), enhances the growth defect of *snu114-60*. However, none of the other *snu114* mutations exhibit genetic interactions with *prp24* mutations.

Deletion of the nonessential gene *BRR1* causes a cs growth defect, affects the processing of newly transcribed snRNAs, and causes a decrease in snRNA and snRNP levels (NOBLE and GUTHRIE 1996; INADA 2004). The deletion of *BRR1* is synthetically lethal with *snu114-60* at 16° and 37°, and the combination of *brr1Δ* with the other *snu114* mutations impairs growth at 30° (Figure 7C and data not shown).

Interactions with the NTC: Finally, we tested components of the prp-*n*ine*t*een *c*omplex (NTC), a complex that interacts with the spliceosome immediately prior to the first step of catalysis (TARN *et al.* 1993; CHAN *et al.* 2003). *prp19-1*, a ts mutation that blocks the first step of splicing (VIJAYRAGHAVAN *et al.* 1989), enhances the

TABLE 6

Synthetic interactions between *snu114* alleles and alleles of other splicing factors

			snu114 allele						
Allele	**Activity**	**Particle**	*-12*	*-14*	*-15*	*-30*	*-40*	*-50*	*-60*
prp5-1	ATPase		0	0	0	0	0	0	0
sub2-1	ATPase		1	1	1	1	1	1	0
sub2-5			1	0	3	1	2	2	0
prp24-RRM3sub			0	0	0	0	0	0	3
prp24-RRM4sub			0	0	0	0	0	0	1
prp24-CT10			0	0	0	0	0	0	1
brr1Δ			2	2	2	2	2	2	4
prp8-1		U5/tri-snRNP	3	2	3	3	4	4	5
prp8-brr			3	0	3	0	5	1	5
prp8-201			2	1	2	0	3	2	5
prp8-101			1	1	1	0	0	0	2
sad1-1		Tri-snRNP	5	5	5	4	5	4	5
snu66Δ		Tri-snRNP	3	3	3	0	0	3	5
prp28-1	ATPase	U5 snRNP	3	3	3	0	0	0	5
brr2-1	ATPase	U5/tri-snRNP	0	1	0	0	2	0	4
prp19-1		NTC	3	3	3	3	1	2	3
isy1Δ		NTC	0	0	0	0	0	0	0
snt309Δ		NTC	0	0	0	0	0	0	0
prp2-1	ATPase		2	2	2	2	2	2	0
prp16-1	ATPase		0	0	0	0	0	0	0
prp16-2			0	0	0	0	0	0	0
prp16-101			0	0	0	0	0	0	0
prp16-302			0	0	0	0	0	0	0
prp22-R805A	ATPase		1	1	1	1	1	1	0
prp22-H606A			0	0	0	0	0	0	0
prp43-H218A	ATPase		0	0	0	0	0	0	0
prp43-G429A			0	0	0	0	0	0	0
prp43(91-732)			0	0	0	0	0	0	0

The strength of synthetic enhancement, based on changes in growth rate at 16°, 25°, 30°, and 37°, was rated on a scale of 0 (no interaction) to 5 (synthetic lethality). Boxes are shaded according to strength of interaction.

growth defects of all of the *snu114* alleles. Notably, Prp19 is an E3 ubiquitin ligase, and the *prp19-1* mutation decreases its enzymatic activity (Ohi *et al.* 2003). Deletions of the nonessential NTC proteins *ISY1* and *SNT309* (C. H. Chen *et al.* 1998, 2001; Dix *et al.* 1999) do not interact genetically with the *snu114* alleles.

DISCUSSION

Snu114 is a GTPase with homology to the ribosomal translocase EF-G, suggesting that it may mediate conformational rearrangements in the spliceosome. On the basis of comparison with EF-G, Snu114 can be divided into five structural domains (I–V), as well as a nonconserved N-terminal domain. In this study, we screened for conditionally lethal alleles of Snu114. We recovered a large number of mutations within the GTPase domain and in three small clusters of amino acids in domains III–V. Additionally, we found that *snu114-60*, which causes an almost complete deletion of the C-terminal domain IVb, causes a conditional growth defect. Domain IVb is larger in Snu114 than in its ribosomal homo-

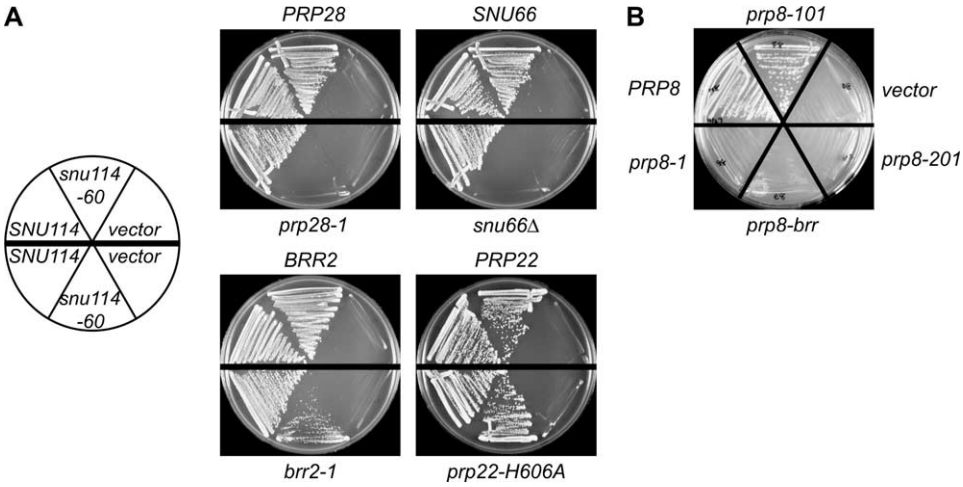

FIGURE 6.—Synthetic lethal interactions of *snu114-60*. The following strains were streaked onto 5-FOA media and grown at 25° for 3 days: (A) *snu114*Δ strains carrying *SNU114* on a *URA3*-marked plasmid, in combination with either a wild-type or a mutant copy of the indicated gene, which had been transformed with *SNU114*, *snu114-60*, or an empty *LEU2*-marked vector; and (B) a Δ*snu114* Δ*prp8* strain containing *SNU114* and *PRP8* on URA3-marked plasmids that was transformed with *snu114-60* and the indicated *prp8* allele or empty vector. B is a composite taken from different plates.

logs, suggesting that this domain may be important for an interaction specific to splicing. *snu114-60* is synthetically lethal with mutations in *prp8* and in other factors involved in activation of the spliceosome. We propose that domain IVb interacts with Prp8 to influence the activities of the DExD/H box ATPases Prp28 and Brr2 during spliceosome activation.

Structural interpretations: Studies of EF-G and EF2 indicate that GTP hydrolysis causes a substantial movement of domain IV with respect to domains I and II (STARK *et al.* 2000; JORGENSEN *et al.* 2003) (see Figure 1, C and D). This conformational rearrangement of EF-G/EF2 is predicted to drive movement of tRNA within the ribosome (RODNINA *et al.* 2000). By analogy, GTP hydrolysis by Snu114 may cause rearrangements of the spliceosome.

All members of the GTPase superfamily share a similar architecture of the G domain and contain conserved

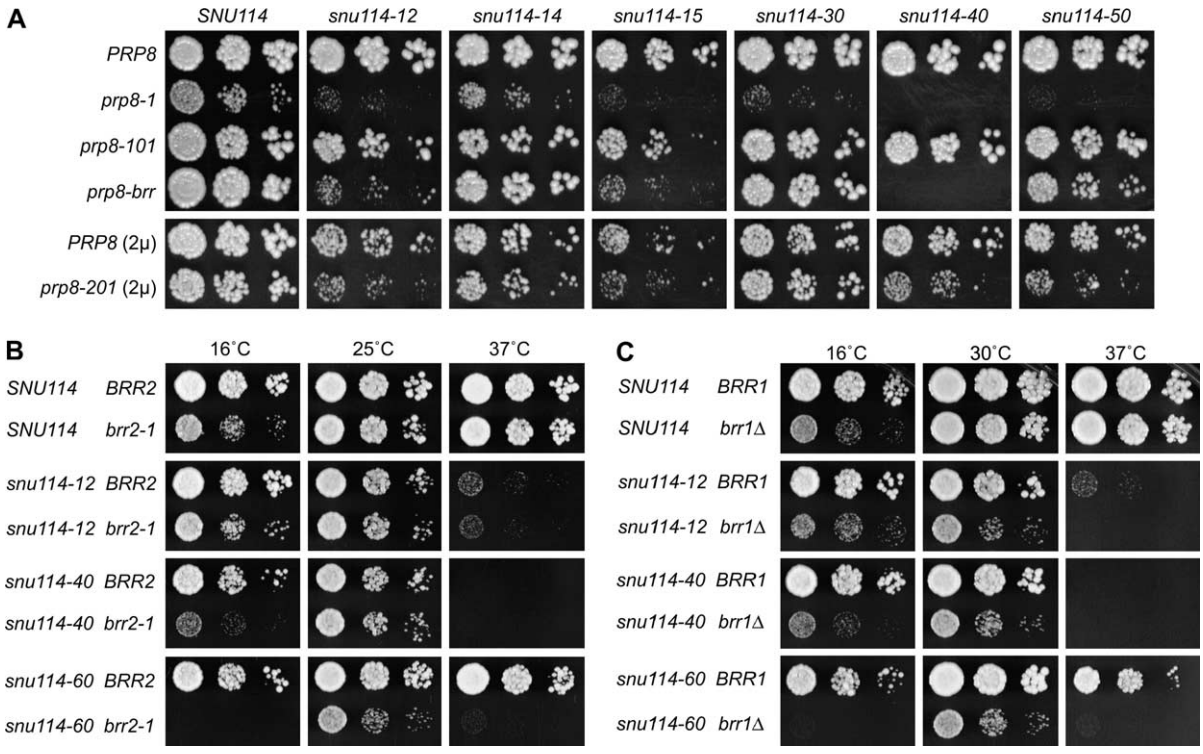

FIGURE 7.—Genetic interactions of *snu114* alleles with *prp8* alleles, *brr2-1*, and *brr1*Δ. (A) Serial dilutions of *snu114*Δ *prp8*Δ cells carrying the indicated *snu114* and *prp8* alleles were grown at 30° on YPD for 3 days. *prp8-1*, *prp8-101*, and *prp8-brr* were present on low-copy (CEN) plasmids; *prp8-201* was present on a high-copy (2μ) plasmid. (B) Serial dilutions of *snu114*Δ *brr2*Δ cells carrying the indicated *snu114* alleles and either *BRR2* or *brr2-1*. (C) Serial dilutions of *snu114*Δ *brr1*Δ cells and *snu114*Δ *BRR1* cells carrying the indicated *SNU114* alleles. In B and C, serial dilutions were grown on YPD for 8 days at 16° and for 2 days at 25°, 30°, and 37°.

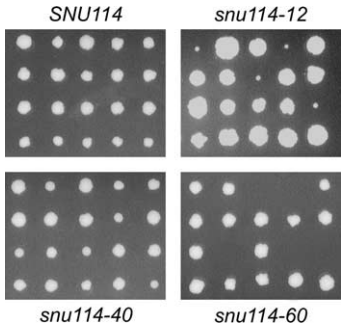

FIGURE 8.—Genetic interactions between *snu66*Δ and *snu114* mutations. Five tetrads grown on YPD and dissected from diploids generated by crossing a *snu66*Δ strain to *snu114* mutants are shown. Tetrads dissected from the cross of *snu114-12* with *snu66*Δ were photographed after 8 days of growth at 25°, while the other tetrads are shown at day 4. *snu114-60 snu66*Δ spores were not viable even after 8 days.

motifs (G1–G5) that interact with GTP/GDP (BOURNE *et al.* 1991; SPRANG 1997). Our screen for conditionally lethal alleles of Snu114 identified mutations in the G1 and G3 motifs, which are predicted to cause defects in GTP binding and hydrolysis (SIGAL *et al.* 1986; ANBORGH *et al.* 1989; ZEIDLER *et al.* 1995). In multidomain G proteins, the GTP/GDP binding status is communicated to other domains via rearrangements of the G2 and G3 motifs (SPRANG 1997). The clusters of mutations that we identified in domains III and V are located near points of contact with the G3 motif (Figure 1, C and D). Although the G2 motif is disordered in all EF-G/EF2 structures, comparison with the structure of the GTPase elongation factor Tu (EF-Tu) suggests that it is also close to the domain III cluster (LAURBERG *et al.* 2000). Therefore, the mutations that we found in domains III and V may impair a conformational rearrangement of the protein that normally results from GTP hydrolysis.

Domain IV of EF-G is necessary for transmitting a rearrangement within the protein, arising from GTP hydrolysis, into a conformational change of the ribosome, as deletion of IVa or IVa + IVb decreases translocation by ~2000-fold without affecting GTP hydrolysis (RODNINA *et al.* 1997; MARTEMYANOV and GUDKOV 1999; SAVELSBERGH *et al.* 2000). Notably, deletion of six amino acids in EF-G that correspond to the cluster of mutant amino acids that we identified in Snu114 domain IVa, including *snu114-40*, causes a 300-fold reduction in translocation of the ribosome (SAVELSBERGH *et al.* 2000). Thus, the mutations in Snu114 domain IVa may prevent the transmission of a conformational rearrangement that normally results from GTP hydrolysis or nucleotide exchange. Studies of EF-G/EF2 have not directly addressed the role of domain IVb alone (although this domain is only 20 amino acids in *E. coli*). On the basis of our finding that deletion of domain IVb (*snu114-60*) causes a conditional growth defect and a splicing defect, we predict that domain IVb could be an interaction domain that communicates conformational changes of Snu114 to other splicing factors.

The βαββαβ structure of domains III, IVa, and V of EF-G is characteristic of the RNA recognition motif (RRM) (AEVARSSON *et al.* 1994; MURZIN 1995; LAURBERG *et al.* 2000). In RRM domains, hydrophobic residues in two of the β-sheets interact directly with RNA (VARANI and NAGAI 1998). In our mutagenesis of Snu114, we found very few mutations in residues that would be predicted to interact with RNA; thus, our data do not provide evidence for a direct interaction between Snu114 and RNA through the RRM motifs. However, it is possible that we failed to obtain such mutations because they cause lethality or because our screen was not saturating.

Biochemistry and genetics suggest that Snu114 functions during spliceosome activation: We have found that mutations in Snu114 block splicing prior to the first chemical step both *in vivo* and *in vitro*. A first-step block to splicing could reflect defects at a number of stages, including snRNP biogenesis and stability, tri-snRNP addition to the spliceosome, and spliceosome activation. In fact, we found that *snu114* mutants exhibit genetic interactions with splicing mutants that affect each of these stages (Figure 5). However, *snu114* mutants do not interact genetically with any factors that function after the first step of catalysis, in agreement with the biochemical data showing a first-step block.

A number of proteins that we tested affect multiple stages of splicing. For example, Prp24 and Sad1 are both implicated in tri-snRNP addition/reorganization as well as in formation of U4/U6 snRNP (RAGHUNATHAN and GUTHRIE 1998b; LYGEROU *et al.* 1999; KUHN and BROW 2000; MAKAROVA *et al.* 2001). The snRNP biogenesis factor *BRR1* exhibits genetic interactions not only with snRNP biogenesis factors, but also with factors that affect spliceosome assembly. Interestingly, *brr1*Δ and the *snu114* alleles are synthetically lethal with many of the same mutations, including *prp24-RRM3sub*, *sub2* mutations, *sad1-1*, and *snu66*Δ (INADA 2004). The genetic interactions observed between *snu114* mutations and *prp24-RRM3sub*, *sad1-1*, and *brr1*Δ could arise because (a) all of the proteins are involved in the addition and rearrangement of tri-snRNP, (b) the function of Snu114 during spliceosome activation is particularly sensitive to low levels of snRNPs, or (c) Snu114 is also necessary for snRNP biogenesis. However, *snu114-60*, which has the strongest interactions with *prp24*, *sad1*, and *brr1* mutants, contains wild-type snRNP levels (T. J. BRENNER and C. GUTHRIE, unpublished data).

Snu114 alleles exhibit strong genetic interactions with factors involved in spliceosome activation, including *prp28-1*, *brr2-1*, and *prp8-201* (GUTHRIE 1998a; KUHN *et al.* 1999; RAGHUNATHAN and STALEY and GUTHRIE 1999). We also found strong genetic interactions with deletion of *SNU66* and mutation of *SAD1*, factors in-

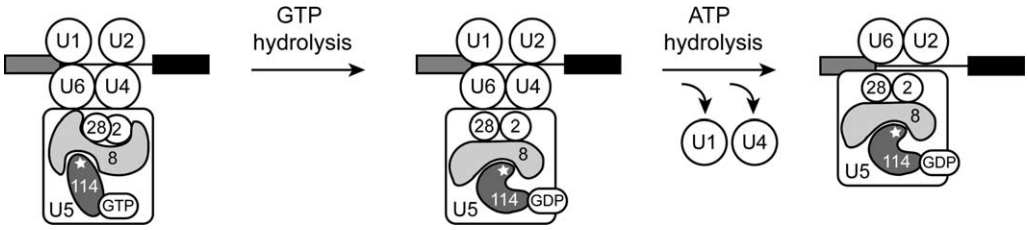

Figure 9.—Model for Snu114 activity during spliceosome activation. We propose that Snu114 is bound to GTP when tri-snRNP first interacts with the spliceosome. Proper interaction between tri-snRNP and the spliceosome induces GTP hydrolysis, causing a conformational rearrangement of Snu114. This alters the interaction between domain IV of Snu114 (marked by a star) and Prp8, changing the conformation of Prp8. This triggers the activity of the ATPases Prp28 and Brr2, leading to the release of U1 and U4. 114, Snu114; 8, Prp8; 2, Brr2; 28, Prp28.

volved in tri-snRNP addition (Makarova *et al.* 2001). All of these genetic interactions may be related, as mutations that block spliceosome activation, including *prp28-1* and *brr2-1*, decrease the interaction of tri-snRNP with the spliceosome (Raghunathan and Guthrie 1998a; Staley and Guthrie 1999), indicating that rearrangements within the tri-snRNP are necessary for its stable association with the spliceosome. Together, these genetic interactions strongly implicate Snu114 in spliceosome activation.

The N- and C-terminal domains of Snu114 are involved in similar functions: Snu114 contains a 120-amino-acid N-terminal domain that is not found in EF-G/EF2. Previous studies showed that deletion of this domain (*snu114ΔN*) causes a ts growth defect and a block to the release of U1 and U4 from the spliceosome (Bartels *et al.* 2002). Similar to the biochemical phenotype of *snu114ΔN*, genetic interactions suggest that *snu114-60* causes defects in spliceosome activation. Furthermore, we found that the presence of a 15-amino -acid epitope tag at the N terminus of Snu114 is synthetically lethal with *snu114-60* at 16° and 37° (Figure 2A), which suggests that both the N-terminal domain and domain IVb are necessary for the same process. If the N and C termini were involved in the same function, we predicted that they would exhibit the same genetic interactions. Similarly to *snu114-60*, *snu114ΔN* is synthetically lethal with *prp28-1* and *snu66Δ* and sick with *brr2-1* (T. J. Brenner and C. Guthrie, unpublished data). We conclude that both domains are necessary for tri-snRNP addition/spliceosome activation. It is possible that the N-terminal domain is necessary for the activation of GTPase, while the C-terminal domain is necessary to transmit a conformational change driven by GTP hydrolysis. Alternatively, it is possible that both domains must interact with other factors, such as Prp8, for a conformational change in Snu114 to be transmitted.

A model for the activity of Snu114: Snu114 and Prp8 physically interact with each other, and both proteins can be crosslinked to U5 snRNA (Achsel *et al.* 1998; Dix *et al.* 1998). Thus, interactions between Snu114, Prp8, and U5 snRNA form the core of U5 snRNP. Domain IVa/IVb of Snu114 may be particularly important for this interaction, as *snu114-40* and *snu114-60* are synthetically lethal with *prp8-1* and *prp8-brr*.

Synthetic lethality between *snu114-60* and mutations in factors involved in spliceosome activation indicates a pivotal role for domain IVb in this process. Previous genetic data have suggested that an allosteric interaction between Prp8 and the ATPases Prp28 and Brr2 regulates the timing of spliceosome activation (Kuhn and Brow 2000; Kuhn *et al.* 2002). The mutations *prp28-1* and *brr2-1* are synthetically lethal with the U4-cs1 mutation, which increases the base pairing between U4 and U6 snRNAs and inhibits the release of U1 and U4 from the spliceosome (Li and Brow 1996; Kuhn *et al.* 1999). A large number of *prp8* alleles, including *prp8-201*, can suppress the cold sensitivity of U4-cs1, and a subset of these *prp8* alleles can suppress *prp28-1* or *brr2-1*. Thus, it has been hypothesized that Prp8 inhibits the activity of the ATPases until spliceosome formation has occurred, while certain mutations of *prp8* may relax this inhibition.

On the basis of the strong genetic interactions between *SNU114* and *PRP8*, *PRP28*, and *BRR2*, we suggest that Snu114 may regulate Prp8's inhibition of Prp28 and Brr2 (Figure 9). We hypothesize that Snu114 is bound to GTP when tri-snRNP binds to the spliceosome. GTP hydrolysis could be triggered by interactions with U1 snRNP, since Snu114 and the U1 proteins Prp39 and Prp40 interact with neighboring regions of Prp8, according to yeast two-hybrid analyses (Abovich and Rosbash 1997; Dix *et al.* 1998; van Nues and Beggs 2001; Grainger and Beggs 2005). Thus, successful interaction with the spliceosome would induce GTP hydrolysis by Snu114 and a concomitant structural rearrangement of domains III–V with relation to the N terminus. In particular, this may modify the interaction between domain IVb and Prp8, which in turn could alter the conformation of Prp8 and lead to the unwinding of the U1/5′ splice site duplex by Prp28 and the U4/U6 duplex by Brr2. It is possible that Prp8 sequesters the RNA helices, and an altered conformation of Prp8 would allow Brr2 and Prp28 access to their substrates. Alternatively, Prp8 could inhibit the ATPase activity of Prp28 and Brr2 via direct physical interactions with the DExD/H-box proteins, and altering the conformation of Prp8 could relieve the inhibition by severing these associations (Kuhn and Brow 2000; Kuhn *et al.* 2002).

Previous biochemical studies support the model that Snu114 hydrolyzes GTP during spliceosome activation.

A mutation that is expected to convert the nucleotide specificity of Snu114 from GTP to XTP causes a temperature-sensitive block to U1 and U4 release, which can be partially overcome by addition of XTP (Bartels *et al.* 2003). Nonhydrolyzable XTP did not stimulate snRNA release, suggesting that GTP hydrolysis, and not just GTP binding, is important (Bartels *et al.* 2003).

For most GTPases, GTPase-activating proteins (GAPs) are necessary to stimulate GTP hydrolysis. The ribosome itself serves as the GAP for EF-G and EF-Tu, which delivers aminoacyl tRNA to the ribosome (Mohr *et al.* 2002). In the case of EF-Tu, a cognate codon:anticodon interaction is required to trigger GTP hydrolysis (Rodnina *et al.* 2005). Because the structure of EF-Tu bound to aminoacyl-tRNA resembles EF-G (Nissen *et al.* 1995), and because EF-G and Snu114 are homologous, it is tempting to speculate that interactions between spliceosomal components and Snu114 domain IV, which would correspond to the anticodon arm of tRNA, may be necessary to trigger GTPase activity. Just as GTP hydrolysis by EF-Tu functions as a checkpoint to ensure that the appropriate aminoacyl tRNA is retained in the ribosome, GTP hydrolysis by Snu114 after addition of tri-snRNP could serve as a checkpoint to ensure proper formation of the spliceosome. It will be informative to investigate what components of the spliceosome function as a GAP for Snu114. Additionally, we are interested in testing whether mutations in domain IV of Snu114 directly affect GTP binding and hydrolysis.

A link to ubiquitination: Strong genetic interactions between Snu114 and proteins that are involved in ubiquitin metabolism suggest that a cycle of ubiquitination could affect splicing. In a large-scale proteomics study, Snu114 was one of only three splicing proteins, including Sad1, that were found to be ubiquitinated (Peng *et al.* 2003). Interestingly, Sad1, which has strong genetic interactions with Snu114, contains a ubiquitin hydrolase domain (Costanzo *et al.* 2000). Prp19, which we also found to interact genetically with Snu114, is a member of the U-box family of E3 ubiquitin ligases (Hatakeyama *et al.* 2001). The *prp19-1* mutation specifically disrupts the fold of the U-box domain and greatly decreases ubiquitin ligase activity *in vitro* (Ohi *et al.* 2003). Finally, the Snu114-interacting protein Prp8 contains a Jab/MPN domain, which is typically associated with ubiquitin removal (Maytal-Kivity *et al.* 2002; Verma *et al.* 2002). Thus, the genetic interactions with *sad1-1*, *prp19-1*, and *prp8* mutants could reflect the requirement for a cycle of ubiquitination and deubiquitination of Snu114. Currently, the timing of Snu114 ubiquitination is not known. One possibility is that after U5 snRNP is released from the postcatalytic spliceosome in complex with the NTC (Makarov *et al.* 2002), ubiquitination of Snu114 by Prp19 induces dissociation of the two particles. Ubiquitin removal by Sad1 during the following round of spliceosome assembly could promote tri-snRNP binding or spliceosome activation. The particularly strong genetic interactions between *sad1-1* and *snu114* mutations suggests that deubiquitination of Snu114 is essential for splicing. Ubiquitination of Snu114 may also affect its interactions with Prp8, as Prp8 is predicted to bind ubiquitin via its Jab/MPN domain. We are most interested in determining when during splicing Snu114 is ubiquitinated and how this is affected by mutations in *PRP19*, *SAD1*, and *PRP8*.

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LITERATURE CITED

Abovich, N., and M. Rosbash, 1997 Cross-intron bridging interactions in the yeast commitment complex are conserved in mammals. Cell **89:** 403–412.

Achsel, T., K. Ahrens, H. Brahms, S. Teigelkamp and R. Luhrmann, 1998 The human U5–220kD protein (hPrp8) forms a stable RNA-free complex with several U5-specific proteins, including an RNA unwindase, a homologue of ribosomal elongation factor EF-2, and a novel WD-40 protein. Mol. Cell. Biol. **18:** 6756–6766.

Aevarsson, A., 1995 Structure-based sequence alignment of elongation factors Tu and G with related GTPases involved in translation. J. Mol. Evol. **41:** 1096–1104.

Aevarsson, A., E. Brazhnikov, M. Garber, J. Zheltonosova, Y. Chirgadze *et al.*, 1994 Three-dimensional structure of the ribosomal translocase: elongation factor G from Thermus thermophilus. EMBO J. **13:** 3669–3677.

Anborgh, P. H., R. H. Cool, M. Jacquet, G. Parlato and A. Parmeggiani, 1989 Structure-function relationships of the GTP-binding domain of elongation factor Tu, pp. 67–75 in *The Guanine-Nucleotide Binding Proteins: Common Structural and Functional Properties,* edited by L. Bosch, B. Kraal and A. Parmeggiani. Plenum Press, New York.

Arenas, J. E., and J. N. Abelson, 1997 Prp43: an RNA helicase-like factor involved in spliceosome disassembly. Proc. Natl. Acad. Sci. USA **94:** 11798–11802.

Bartels, C., C. Klatt, R. Luhrmann and P. Fabrizio, 2002 The ribosomal translocase homologue Snu114p is involved in unwinding U4/U6 RNA during activation of the spliceosome. EMBO Rep. **3:** 875–880.

Bartels, C., H. Urlaub, R. Luhrmann and P. Fabrizio, 2003 Mutagenesis suggests several roles of Snu114p in pre-mRNA splicing. J. Biol. Chem. **278:** 28324–28334.

Boorstein, W. R., and E. A. Craig, 1989 Primer extension analysis of RNA. Methods Enzymol. **180:** 347–369.

Bourne, H. R., D. A. Sanders and F. McCormick, 1991 The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:** 117–127.

Brow, D. A., 2002 Allosteric cascade of spliceosome activation. Annu. Rev. Genet. **36:** 333–360.

Brown, J. D., and J. D. Beggs, 1992 Roles of PRP8 protein in the assembly of splicing complexes. EMBO J. **11:** 3721–3729.

Burge, C. B., T. H. Tuschl and P. A. Sharp, 1998 Splicing of precursors to mRNAs by the spliceosomes, pp. 525–560 in *RNA World II,* edited by R. F. Gesteland, T. R. Cech and J. F. Atkins. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Burgess, S. M., and C. Guthrie, 1993 A mechanism to enhance mRNA splicing fidelity: the RNA-dependent ATPase Prp16 governs usage of a discard pathway for aberrant lariat intermediates. Cell **73:** 1377–1391.

Cammarano, P., P. Palm, R. Creti, E. Ceccarelli, A. M. Sanangel-

antoni *et al.*, 1992 Early evolutionary relationships among known life forms inferred from elongation factor EF-2/EF-G sequences: phylogenetic coherence and structure of the archaeal domain. J. Mol. Evol. **34:** 396–405.

Chan, S. P., D. I. Kao, W. Y. Tsai and S. C. Cheng, 2003 The Prp19p-associated complex in spliceosome activation. Science **302:** 279–282.

Chen, C. H., W. Y. Tsai, H. R. Chen, C. H. Wang and S. C. Cheng, 2001 Identification and characterization of two novel components of the Prp19p-associated complex, Ntc30p and Ntc20p. J. Biol. Chem. **276:** 488–494.

Chen, H. R., S. P. Jan, T. Y. Tsao, Y. J. Sheu, J. Banroques *et al.*, 1998 Snt309p, a component of the Prp19p-associated complex that interacts with Prp19p and associates with the spliceosome simultaneously with or immediately after dissociation of U4 in the same manner as Prp19p. Mol. Cell. Biol. **18:** 2196–2204.

Chen, J. Y., L. Stands, J. P. Staley, R. R. Jackups, Jr., L. J. Latus *et al.*, 2001 Specific alterations of U1-C protein or U1 small nuclear RNA can eliminate the requirement of Prp28p, an essential DEAD box splicing factor. Mol. Cell **7:** 227–232.

Collins, C. A., 2001 Genetic and biochemical analysis of interactions involving Prp8 and RNA at the catalytic core of the spliceosome. Ph.D. Thesis, Department of Biochemistry and Biophysics, University of California, San Francisco.

Collins, C. A., and C. Guthrie, 2000 The question remains: Is the spliceosome a ribozyme? Nat. Struct. Biol. **7:** 850–854.

Company, M., J. Arenas and J. Abelson, 1991 Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. Nature **349:** 487–493.

Costanzo, M. C., J. D. Hogan, M. E. Cusick, B. P. Davis, A. M. Fancher *et al.*, 2000 The yeast proteome database (YPD) and Caenorhabditis elegans proteome database (WormPD): comprehensive resources for the organization and comparison of model organism protein information. Nucleic Acids Res. **28:** 73–76.

Czworkowski, J., J. Wang, T. A. Steitz and P. B. Moore, 1994 The crystal structure of elongation factor G complexed with GDP, at 2.7 A resolution. EMBO J. **13:** 3661–3668.

DeLano, W. L., 2002 The PyMOL molecular graphics system (http://www.pymol.org).

Dix, I., C. S. Russell, R. T. O'Keefe, A. J. Newman and J. D. Beggs, 1998 Protein-RNA interactions in the U5 snRNP of *Saccharomyces cerevisiae*. RNA **4:** 1239–1250.

Dix, I., C. Russell, S. B. Yehuda, M. Kupiec and J. D. Beggs, 1999 The identification and characterization of a novel splicing protein, Isy1p, of Saccharomyces cerevisiae. RNA **5:** 360–368.

Doye, V., and E. C. Hurt, 1995 Genetic approaches to nuclear pore structure and function. Trends Genet. **11:** 235–241.

Elledge, S. J., and R. W. Davis, 1988 A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in Saccharomyces cerevisiae. Gene **70:** 303–312.

Fabrizio, P., B. Laggerbauer, J. Lauber, W. S. Lane and R. Luhrmann, 1997 An evolutionarily conserved U5 snRNP-specific protein is a GTP-binding factor closely related to the ribosomal translocase EF-2. EMBO J. **16:** 4092–4106.

Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles *et al.*, 2002 Functional profiling of the Saccharomyces cerevisiae genome. Nature **418:** 387–391.

Gottschalk, A., G. Neubauer, J. Banroques, M. Mann, R. Luhrmann *et al.*, 1999 Identification by mass spectrometry and functional analysis of novel proteins of the yeast [U4/U6.U5] tri-snRNP. EMBO J. **18:** 4535–4548.

Grainger, R. J., and J. D. Beggs, 2005 Prp8 protein: at the heart of the spliceosome. RNA **11:** 533–557.

Guarente, L., 1993 Synthetic enhancement in gene interaction: a genetic tool come of age. Trends Genet. **9:** 362–366.

Hatakeyama, S., M. Yada, M. Matsumoto, N. Ishida and K. I. Nakayama, 2001 U box proteins as a new family of ubiquitin-protein ligases. J. Biol. Chem. **276:** 33111–33120.

Inada, M., 2004 Genetic, biochemical and genomic analyses of RNP biogenesis in *S. cerevisiae*. Ph.D. Thesis, Department of Biochemistry and Biophysics, University of California, San Francisco.

Jorgensen, R., P. A. Ortiz, A. Carr-Schmid, P. Nissen, T. G. Kinzy *et al.*, 2003 Two crystal structures demonstrate large conformational changes in the eukaryotic ribosomal translocase. Nat. Struct. Biol. **10:** 379–385.

Jurica, M. S., and M. J. Moore, 2003 Pre-mRNA splicing: awash in a sea of proteins. Mol. Cell **12:** 5–14.

Kim, D. H., and J. J. Rossi, 1999 The first ATPase domain of the yeast 246-kDa protein is required for in vivo unwinding of the U4/U6 duplex. RNA **5:** 959–971.

Kim, S. H., and R. J. Lin, 1996 Spliceosome activation by PRP2 ATPase prior to the first transesterification reaction of pre-mRNA splicing. Mol. Cell. Biol. **16:** 6810–6819.

Kistler, A. L., and C. Guthrie, 2001 Deletion of MUD2, the yeast homolog of U2AF65, can bypass the requirement for sub2, an essential spliceosomal ATPase. Genes Dev. **15:** 42–49.

Kuhn, A. N., and D. A. Brow, 2000 Suppressors of a cold-sensitive mutation in yeast U4 RNA define five domains in the splicing factor Prp8 that influence spliceosome activation. Genetics **155:** 1667–1682.

Kuhn, A. N., Z. Li and D. A. Brow, 1999 Splicing factor Prp8 governs U4/U6 RNA unwinding during activation of the spliceosome. Mol. Cell **3:** 65–75.

Kuhn, A. N., E. M. Reichl and D. A. Brow, 2002 Distinct domains of splicing factor Prp8 mediate different aspects of spliceosome activation. Proc. Natl. Acad. Sci. USA **99:** 9145–9149.

Laggerbauer, B., T. Achsel and R. Luhrmann, 1998 The human U5–200kD DEXH-box protein unwinds U4/U6 RNA duplices in vitro. Proc. Natl. Acad. Sci. USA **95:** 4188–4192.

Laurberg, M., O. Kristensen, K. Martemyanov, A. T. Gudkov, I. Nagaev *et al.*, 2000 Structure of a mutant EF-G reveals domain III and possibly the fusidic acid binding site. J. Mol. Biol. **303:** 593–603.

Li, Z., and D. A. Brow, 1996 A spontaneous duplication in U6 spliceosomal RNA uncouples the early and late functions of the ACAGA element in vivo. RNA **2:** 879–894.

Libri, D., N. Graziani, C. Saguez and J. Boulay, 2001 Multiple roles for the yeast SUB2/yUAP56 gene in splicing. Genes Dev. **15:** 36–41.

Lygerou, Z., G. Christophides and B. Seraphin, 1999 A novel genetic screen for snRNP assembly factors in yeast identifies a conserved protein, Sad1p, also required for pre-mRNA splicing. Mol. Cell. Biol. **19:** 2008–2020.

Makarov, E. M., O. V. Makarova, H. Urlaub, M. Gentzel, C. L. Will *et al.*, 2002 Small nuclear ribonucleoprotein remodeling during catalytic activation of the spliceosome. Science **298:** 2205–2208.

Makarova, O. V., E. M. Makarov and R. Luhrmann, 2001 The 65 and 110 kDa SR-related proteins of the U4/U6.U5 tri-snRNP are essential for the assembly of mature spliceosomes. EMBO J. **20:** 2553–2563.

Martemyanov, K. A., and A. T. Gudkov, 1999 Domain IV of elongation factor G from Thermus thermophilus is strictly required for translocation. FEBS Lett. **452:** 155–159.

Martin, A., S. Schneider and B. Schwer, 2002 Prp43 is an essential RNA-dependent ATPase required for release of lariat-intron from the spliceosome. J Biol. Chem. **277:** 17743–17750.

Maytal-Kivity, V., N. Reis, K. Hofmann and M. H. Glickman, 2002 MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. BMC Biochem. **3:** 28.

Mohr, D., W. Wintermeyer and M. V. Rodnina, 2002 GTPase activation of elongation factors Tu and G on the ribosome. Biochemistry **41:** 12520–12528.

Moore, M. J., C. C. Query and P. A. Sharp, 1993 Splicing of precursors to mRNA by the spliceosome, pp. 303–357 in *The RNA World*, edited by R. F. Gesteland and J. F. Atkins. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Muhlrad, D., R. Hunter and R. Parker, 1992 A rapid method for localized mutagenesis of yeast genes. Yeast **8:** 79–82.

Murzin, A. G., 1995 A ribosomal protein module in EF-G and DNA gyrase. Nat. Struct. Biol. **2:** 25–26.

Nissen, P., M. Kjeldgaard, S. Thirup, G. Polekhina, L. Reshetnikova *et al.*, 1995 Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. Science **270:** 1464–1472.

Noble, S. M., and C. Guthrie, 1996 Transcriptional pulse-chase analysis reveals a role for a novel snRNP-associated protein in the manufacture of spliceosomal snRNPs. EMBO J. **15:** 4368–4379.

Ogg, S. C., W. P. Barz and P. Walter, 1998 A functional GTPase domain, but not its transmembrane domain, is required for function of the SRP receptor beta-subunit. J. Cell. Biol. **142:** 341–354.

Ohi, M. D., C. W. Vander Kooi, J. A. Rosenberg, W. J. Chazin and K. L. Gould, 2003 Structural insights into the U-box, a domain associated with multi-ubiquitination. Nat. Struct. Biol. **10:** 250–255.

Peng, J., D. Schwartz, J. E. Elias, C. C. Thoreen, D. Cheng *et al.*, 2003 A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. **21:** 921–926.

Rader, S. D., and C. Guthrie, 2002 A conserved Lsm-interaction motif in Prp24 required for efficient U4/U6 di-snRNP formation. RNA **8:** 1378–1392.

Raghunathan, P. L., and C. Guthrie, 1998a RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. Curr. Biol. **8:** 847–855.

Raghunathan, P. L., and C. Guthrie, 1998b A spliceosomal recycling factor that reanneals U4 and U6 small nuclear ribonucleoprotein particles. Science **279:** 857–860.

Rodnina, M. V., A. Savelsbergh, V. I. Katunin and W. Wintermeyer, 1997 Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. Nature **385:** 37–41.

Rodnina, M. V., H. Stark, A. Savelsbergh, H. J. Wieden, D. Mohr *et al.*, 2000 GTPase mechanisms and functions of translation factors on the ribosome. Biol. Chem. **381:** 377–387.

Rodnina, M. V., K. B. Gromadski, U. Kothe and H. J. Wieden, 2005 Recognition and selection of tRNA in translation. FEBS Lett. **579:** 938–942.

Ruby, S. W., T. H. Chang and J. Abelson, 1993 Four yeast spliceosomal proteins (PRP5, PRP9, PRP11, and PRP21) interact to promote U2 snRNP binding to pre-mRNA. Genes Dev. **7:** 1909–1925.

Sali, A., and T. L. Blundell, 1993 Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. **234:** 779–815.

Savelsbergh, A., N. B. Matassova, M. V. Rodnina and W. Wintermeyer, 2000 Role of domains 4 and 5 in elongation factor G functions on the ribosome. J. Mol. Biol. **300:** 951–961.

Schmitt, M. E., T. A. Brown and B. L. Trumpower, 1990 A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res. **18:** 3091–3092.

Schwer, B., and C. Guthrie, 1991 PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. Nature **349:** 494–499.

Schwer, B., and T. Meszaros, 2000 RNA helicase dynamics in pre-mRNA splicing. EMBO J. **19:** 6582–6591.

Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, G. L. Temeles, B. S. Wolanski *et al.*, 1986 Mutant ras-encoded proteins with altered nucleotide binding exert dominant biological effects. Proc. Natl. Acad. Sci. USA **83:** 952–956.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Sprang, S. R., 1997 G protein mechanisms: insights from structural analysis. Annu. Rev. Biochem. **66:** 639–678.

Staley, J. P., and C. Guthrie, 1998 Mechanical devices of the spliceosome: motors, clocks, springs, and things. Cell **92:** 315–326.

Staley, J. P., and C. Guthrie, 1999 An RNA switch at the 5′ splice site requires ATP and the DEAD box protein Prp28p. Mol. Cell **3:** 55–64.

Stark, H., M. V. Rodnina, H. J. Wieden, M. van Heel and W. Wintermeyer, 2000 Large-scale movement of elongation factor G and extensive conformational change of the ribosome during translocation. Cell **100:** 301–309.

Stevens, S. W., I. Barta, H. Y. Ge, R. E. Moore, M. K. Young *et al.*, 2001 Biochemical and genetic analyses of the U5, U6, and U4/U6 x U5 small nuclear ribonucleoproteins from Saccharomyces cerevisiae. RNA **7:** 1543–1553.

Stevens, S. W., D. E. Ryan, H. Y. Ge, R. E. Moore, M. K. Young *et al.*, 2002 Composition and functional characterization of the yeast spliceosomal penta-snRNP. Mol. Cell **9:** 31–44.

Strauss, E. J., and C. Guthrie, 1991 A cold-sensitive mRNA splicing mutant is a member of the RNA helicase gene family. Genes Dev. **5:** 629–641.

Tarn, W. Y., K. R. Lee and S. C. Cheng, 1993 Yeast precursor mRNA processing protein PRP19 associates with the spliceosome concomitant with or just after dissociation of U4 small nuclear RNA. Proc. Natl. Acad. Sci. USA **90:** 10821–10825.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Umen, J. G., and C. Guthrie, 1995 A novel role for a U5 snRNP protein in 3′ splice site selection. Genes Dev. **9:** 855–868.

Umen, J. G., and C. Guthrie, 1996 Mutagenesis of the yeast gene PRP8 reveals domains governing the specificity and fidelity of 3′ splice site selection. Genetics **143:** 723–739.

van Nues, R. W., and J. D. Beggs, 2001 Functional contacts with a range of splicing proteins suggest a central role for Brr2p in the dynamic control of the order of events in spliceosomes of *Saccharomyces cerevisiae*. Genetics **157:** 1451–1467.

Varani, G., and K. Nagai, 1998 RNA recognition by RNP proteins during RNA processing. Annu. Rev. Biophys. Biomol. Struct. **27:** 407–445.

Verma, R., L. Aravind, R. Oania, W. H. McDonald, J. R. Yates, 3rd *et al.*, 2002 Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science **298:** 611–615.

Vidaver, R. M., D. M. Fortner, L. S. Loos-Austin and D. A. Brow, 1999 Multiple functions of *Saccharomyces cerevisiae* splicing protein Prp24 in U6 RNA structural rearrangements. Genetics **153:** 1205–1218.

Vijayraghavan, U., M. Company and J. Abelson, 1989 Isolation and characterization of pre-mRNA splicing mutants of Saccharomyces cerevisiae. Genes Dev. **3:** 1206–1216.

Wang, Y., and C. Guthrie, 1998 PRP16, a DEAH-box RNA helicase, is recruited to the spliceosome primarily via its nonconserved N-terminal domain. RNA **4:** 1216–1229.

Zeidler, W., C. Egle, S. Ribeiro, A. Wagner, V. Katunin *et al.*, 1995 Site-directed mutagenesis of Thermus thermophilus elongation factor Tu. Replacement of His85, Asp81 and Arg300. Eur. J. Biochem. **229:** 596–604.

Zhang, M., and M. R. Green, 2001 Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56. Genes Dev. **15:** 30–35.

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