A comprehensive biochemical and genetic analysis of the yeast U1 snRNP reveals five novel proteins

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ABSTRACT

The U1 snRNP is essential for recognition of the pre-mRNA 5′-splice site and the subsequent assembly of the spliceosome. Yeast U1 snRNP is considerably more complex than its metazoan counterpart, which suggests possible differences between yeast and metazoa in early splicing events. We have comprehensively analyzed the composition of yeast U1 snRNPs using a combination of biochemical, mass spectrometric, and genetic methods. We demonstrate the specific association of four novel U1 snRNP proteins, Snu71p, Snu65p, Nam8p, and Snu56p, that have no known metazoan homologues. A fifth protein, Npl3p, is an abundant cellular component that reproducibly co-purifies with the U1 snRNP, but its association is salt-sensitive. Therefore, we are unable to establish conclusively whether it binds specifically to the U1 snRNP. Interestingly, Nam8p and Npl3p were previously assigned functions in (pre-m)RNA-metabolism; however, so far, no association with U1 snRNP has been demonstrated or proposed. We also show that the yeast SmB protein is a U1 snRNP component. Yeast U1 snRNP therefore contains 16 different proteins, including seven snRNP core proteins, three homologues of the metazoan U1 snRNP-specific proteins, and six yeast-specific U1 snRNP proteins. We have simultaneously continued the characterization of additional mutants isolated in a synthetic lethal (MUD) screen for genes that functionally cooperate with U1 snRNA. Consistent with the biochemical results, *mud10*, *mud15*, and *mud16* are alleles of *SNU56*, *NAM8*, and *SNU65*, respectively. *mud10* and *mud15* affect the in vivo splicing efficiency of noncanonical introns. Moreover, mud10p strongly affects the in vitro formation of splicing complexes, and extracts from the *mud15* strain contain a U1 snRNP that migrates aberrantly on native gels. Finally, we show that Nam8p/Mud15p contributes to the stability of U1 snRNP.

Keywords: mass spectrometry; MUD screen; Nam8p; Npl3p; RNA-binding proteins; splicing regulation; yeast SmB

INTRODUCTION

Pre-mRNA splicing occurs via a two-step trans-esterification reaction that is catalyzed by a dynamic ribonucleoprotein complex called the spliceosome. Spliceosome formation involves the ordered assembly of the snRNPs U1, U2, and the [U4/U6.U5] tri-snRNP complex, together with an as yet undefined number of non-snRNP splicing factors on the pre-mRNA substrate (for reviews see Green, 1991; Rymond & Rosbash, 1992; Moore et al., 1993). The U1 snRNP binds to the pre-mRNA first, followed by U2 and finally U4/U6 and U5, which are pre-assembled into a trimeric complex prior to their incorporation into the spliceosome.

The first detectable splicing complex, the commitment complex in yeast or E-complex in metazoa, contains the U1 snRNP and an unknown number of non-snRNP splicing factors (Legrain et al., 1988; Séraphin & Rosbash, 1989; Michaud & Reed, 1991, 1993; Rosbash & Séraphin, 1991). The U1 snRNA component of U1 snRNP base pairs with the 5′-splice site in an ATP-independent manner (Séraphin et al., 1988; Séraphin & Rosbash, 1989; Liao et al., 1992). Several

other yeast commitment complex components are recruited to the pre-mRNA. Mud2p has sequence similarity to mammalian U2AF[65], which binds to the polypyrimidine tract upstream of the 3′-splice site of metazoan introns (Abovich et al., 1994). The pre-mRNA-cap binding complex consists of two subunits, Mud13p and Sto1p (homologues of the mammalian CBP20 and CBP80 proteins), and influences the stability of the commitment complex, although it is not essential for cell viability (Colot et al., 1996; Lewis et al., 1996). Recently, a protein was identified that binds specifically to the branchpoint sequence upstream of the 3′-splice site (BBP; Abovich & Rosbash, 1997; Berglund et al., 1997). The current model suggests that BBP is a mediator of a bridging event that brings the U1 snRNP also into contact with the branchpoint, possibly by interacting with the intrinsic U1 snRNP protein Prp40p. BBP was also shown to interact with Mud2p, although the functional significance of BBP's interactions is not yet clear (Abovich & Rosbash, 1997; Fromont-Racine et al., 1997). The identification of yeast U1 snRNP proteins is an essential prerequisite for obtaining an accurate picture of U1 snRNP and commitment complex formation.

The human U1 snRNP has been extensively characterized. It contains eight core proteins denoted SmB/B′, D1, D2, D3, E, F, and G, which are also shared by the other snRNPs, and three U1 snRNP-specific proteins, U1-70K, U1-A, and U1-C (Will et al., 1995). The analysis of isolated and fractionated yeast snRNPs indicated that yeast U1 snRNP is biochemically more complex (Fabrizio et al., 1994). Yeast U1 snRNP sediments as a 17–18 S particle (metazoan U1 snRNP: 12 S), and its snRNA is almost four times longer than its metazoan counterpart (Kretzner et al., 1987; Siliciano et al., 1987). Although yeast U1 snRNP apparently has an equivalent set of common snRNP proteins, it seemed to contain up to nine specific proteins in contrast to only three for metazoan U1 snRNP. The difference in size and composition of the yeast and metazoan U1 snRNPs suggested that the early splicing events may also differ between yeast and metazoa.

Initially, yeast U1 snRNP proteins were identified by homology to their metazoan counterparts and by genetic approaches. Sequence analysis identified the yeast U1-70K protein, Snp1p (Smith & Barrell, 1991). The yeast SmD1, D3, E, F, and G proteins were identified by sequence homology (Rymond, 1993; Roy et al., 1995; Séraphin, 1995; Bordonné & Tarassov, 1996). Synthetic lethal approaches were designed to identify genes that functionally cooperate with the U1 snRNA. The MUD screen (for mutant-U1-die) detected mutants that cause synthetic lethality with otherwise viable U1 snRNA mutations and identified the yeast U1-A homologue, Mud1p (Liao, 1992; Liao et al., 1993). Remarkably, additional genetic screens identified two yeast-specific U1 snRNP proteins without metazoan counterparts (Prp39p

and Prp40p: Lockhart & Rymond, 1994; Kao & Siliciano, 1996), confirming that yeast U1 snRNP is more complex than metazoan U1 snRNP (Fabrizio et al., 1994).

We have recently further developed the biochemical approach allowing us to specifically isolate the yeast U1 snRNP. We co-purified 15 proteins tightly associated with the U1 snRNP, which we identified by peptide sequencing, using nanoelectrospray mass spectrometry (Wilm & Mann, 1996; Wilm et al., 1996) and subsequent database screening. We have already described 11 of these proteins, including six common proteins (SmD1, D2, D3, E, F, and G), homologues of the metazoan U1 snRNP-specific proteins U1-70K (Snp1p), U1-A (Mud1p), U1-C, and the yeast-specific U1 snRNP proteins Prp39p and Prp40p (Neubauer et al., 1997; Tang et al., 1997).

Here we present a comprehensive biochemical and genetic analysis of the remaining four U1 snRNP proteins. Three of them, Snu71p, Nam8p, and Snu56p, are specifically and stably associated with the U1 snRNP. Surprisingly, although Nam8p was previously shown to be involved in (pre-m)RNA metabolism, it was not recognized as a U1 snRNP protein (Ekwall et al., 1992; Ogawa et al., 1995; Nakagawa & Ogawa, 1997). The fourth protein, Npl3p, associates weakly and in a salt-sensitive manner, not only with U1 but, to a lesser extent, also with the other snRNPs. Therefore, we are unable to place it unambiguously in the U1 snRNP. We also have characterized additional mutants from the MUD screen (Liao et al., 1993; Stutz et al., 1997). Remarkably, *mud10* and *mud15* are alleles of *SNU56* and *NAM8*. *mud16* is an allele of an additional, weakly associated U1 snRNP protein that we refer to as Snu65p. Snu65p was simultaneously studied in the laboratory of Brian Rymond, where it was named Prp42p (McLean & Rymond, 1998). We have characterized the mutants functionally. The *mud10* mutant strain forms fewer commitment complexes and spliceosomes than a wild-type strain in vitro. *mud15* contains a U1 snRNP that migrates aberrantly on native gels. Interestingly, both mutations decrease the in vivo splicing efficiency of noncanonical introns.

In summary, this work establishes a comprehensive picture of yeast U1 snRNP. It contains seven common proteins, including the SmB protein, and nine particle-specific proteins, of which only three have counterparts in the metazoan U1 snRNP.

RESULTS AND DISCUSSION

Identification of the protein components of purified yeast U1 snRNP by mass-spectrometric peptide sequencing

We isolated the yeast U1 snRNP by two affinity-chromatographic steps from total cell extracts as de-

scribed previously (Neubauer et al., 1997). The RNA and protein composition of the preparation is shown in Figure 1. The U1 snRNP always co-purifies with low amounts of U5 snRNP (see Fig. 1A), probably due to interactions between U1 and U5 snRNP proteins. An interaction might exist between Prp40p that contains two WW-domains and Prp8p (Abovich & Rosbash, 1997). The N terminus of Prp8p contains a proline-rich sequence of the type known to interact with WW-domains. In fact, the N-terminal 88 amino acids of Prp8p, fused to GST, specifically precipitate U1 snRNP from extracts (data not shown). Our purified U1 snRNP fraction was therefore expected to be contaminated by U5 snRNP components. Indeed, the 216- and 114-kDa proteins are identical to the U5 snRNP-specific proteins Prp8p and Snu114p (Jackson et al., 1988; Fabrizio et al., 1997). In addition, the 42-kDa protein belongs to U5 snRNP, as indicated by immunoprecipitation (data not shown). Separation of the U1 and U5 snRNPs by glycerol gradient centrifugation demonstrated further the co-sedimentation of these three proteins with U5 snRNA (Neubauer et al., 1997). The 36- and 38-kDa proteins are nonspecific contaminants (namely glyceraldehyde-3-phosphate dehydrogenases 1 and 2, as identified by nanoelectrospray mass spectrometry peptide sequencing, data not shown). The remaining 15 proteins are all associated with U1 snRNP.

We have already characterized 11 of these proteins (Neubauer et al., 1997). The remaining four proteins, with apparent molecular weights of 77, 57, 55, and 52 kDa (indicated by asterisks in Fig. 1B), were identified by mass spectrometry as previously described (Neubauer et al., 1997). The sequences of the four proteins are shown in Figure 2. The proteins of 77 and 52 kDa are novel proteins of unknown function. They are named Snu71p and Snu56p because they are "*snur*p"-associated and their predicted molecular weights are 71 and 56 kDa, respectively. The 57- and 55-kDa proteins are identical to Nam8p and Npl3p, respectively. Although they were studied previously and have been assigned functions in (pre-m)RNA metabolism, no association with snRNPs has been described (Ekwall et al., 1992; Russell & Tollervey, 1992; Ogawa et al., 1995; Siebel & Guthrie, 1996, and references therein; Nakagawa & Ogawa, 1997). These proteins are described below.

Snu71p

SNU71 encodes a protein of 620 amino acid residues (Fig. 2A). The sequence contains 19 RS, RD-, or RE-dipeptides, which are characteristic of the SR-family of metazoan splicing factors (for reviews, see Fu, 1995; Manley & Tacke, 1996). Unlike in metazoan SR-proteins that contain a single domain with many SR-dipeptides, the eight SR- or RS-dipeptides found in Snu71p are spread over the entire sequence. The protein is rich in
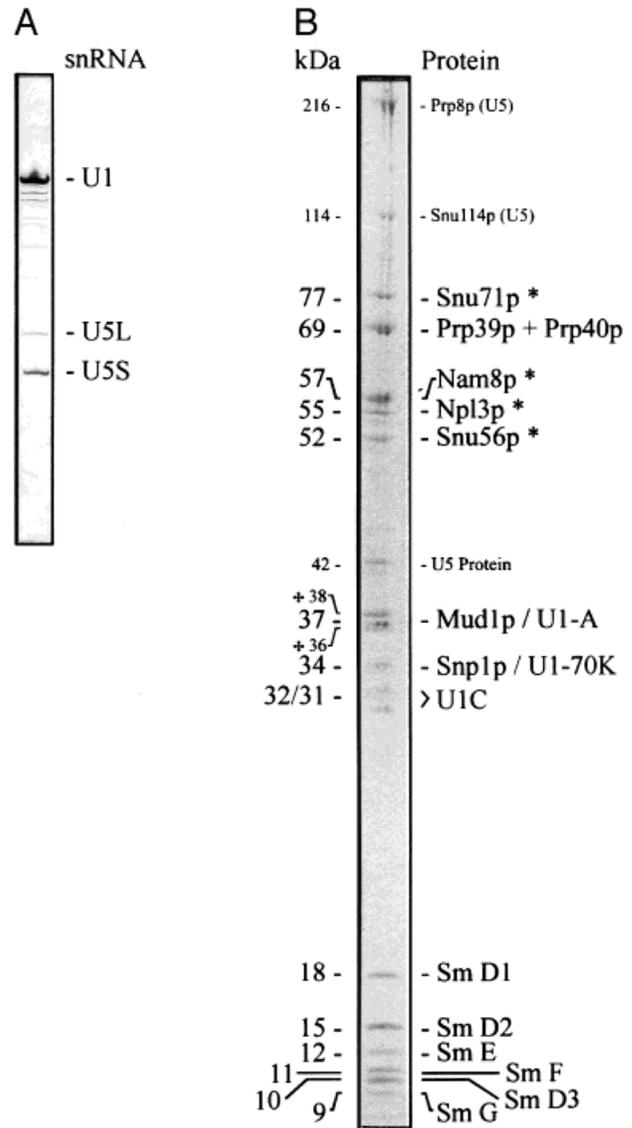

FIGURE 1. Purified yeast U1 snRNP is associated with 15 proteins. The U1 snRNP was isolated at either 250 or 300 mM KCl by two chromatographic steps with identical results. First, all snRNPs were isolated from splicing extracts by anti-m₃G-cap immunoaffinity chromatography. The U1 snRNP, containing a 6xHis-tagged specific protein (Snp1p), was purified by subsequent Ni-NTA chromatography (together with low amounts of U5 snRNP; Neubauer et al., 1997). **A:** Silver stain of the RNAs found in the Ni-NTA eluate of the U1 snRNP isolation. RNAs were extracted by phenol/chloroform/isoamylalcohol and run on an 8 M urea, 8% polyacrylamide gel. The identity of the RNA bands is indicated on the right. Minor bands migrating below the U1 snRNA are degradation products of U1, as demonstrated by northern analysis (data not shown). **B:** Coomassie-stained proteins of the Ni-NTA eluate of the U1 snRNP isolation. Proteins were acetone-precipitated from the organic phase after extraction of the RNAs and separated on a high-TEMED, 12% polyacrylamide SDS-gel. The proteins were identified by nanoelectrospray mass spectrometry and are indicated on the right; apparent molecular weights are indicated on the left. Printed in small letters are proteins that belong to the U5 snRNP. Novel U1 snRNP-associated proteins described in this work are indicated by asterisks. The two contaminating proteins (glyceraldehyde-3-phosphate dehydrogenases 1 and 2) are indicated by crosses.

A

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MRDIVFVSPQ LYLSSQEGWK SDSAKSGFIP ILKNDLQRFQ DSLKHIVDAR    50
NSLSETLLNS NDDGSIHNSD QNTGLNKDKE ASIADNNSAN KCATSSSRYQ   100
ELKQFLPISL DQQIHTVSLQ GVSSSFSRGQ IESLLDHCLN LALTETQSNS   150
ALKVEAWSSF SSFLDTQDIF IRFSKVDEDE AFVNTLNYCK ALFAFIRKLH   200
EDFKIELHLD LNTKEYVEDR TGTIPSVKPE KASEFYSVFK NIEDQTDERN   250
SKKEQLDDSS TQYKVDTNTL SDLPSDALDQ LCKDIIEFRT KVVSIEKEKK   300
MKSTYEESRR QRHQMQKVFD QIRKNHSGAK GSANTEEEDT NMEDEDEEDD   350
TEDDLALEKR KEERDLEESN RRYEDMLHQL HSNTEPKIKS IRADIMSAEN   400
YEEHLEKNRS LYLKELLHLA NDVHYDHHRS FKEQEERRDE EDRAKNGNAK   450
ELAPIQLSDG KAISAGKAAA ITLPEGTVKS ENYNADKNVS ESSEHVKIKF   500
DFKKAIDHSV ESSSEDEGYR ESELPPTKPS ERSAAEDRLP FTADELNIRL   550
TNLKESRYVD ELVREFLGVY EDELVEYILE NIRVNQSKQA LLNELRETFD   600
EDGETIADRL WSRKEFRLGT                                    620
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B

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MRPRRRGLAY HHTKPKGQLS QGHYPTTSND GQRRKVGNSE AFQSFDIWKN    50
LDRIRSTKKN AGQFIKGSLL ILPMRTEDKQ QFDECMDELH KYISKDILRC   100
YPQKEQKDEG MLFYIVLKDF NILDSCFVLS VLLAFQKRLW MAPSEKSYFR   150
                              (*)
VPKNINLTGS FYLPKNIETG RGHIITSYRR EQPSSSIVEV GFNVVPDFQQ   200
FQVKACHVSK FMNELSNFFS QVEFGKCEAN VINYFKREYN RTYSQISLAL   250
YELPLIGDGL FDIKSYISKT RPIIETSKAQ MIKHISEMKA YNEISGLQGD   300
QFPRQQRPLS NSPSSNSISS SQTIEAGATS YQTQPQRHAV NKPSNVLNSS   350
NRHSGPKTFE DGRYSEGNKP GFMTQDEIKQ HCIGTIKASM DAVKKKSSYQ   400
ILKTYVRCPR QNYIDIVYQN LNDLRSKTNC NIVVLNLNNL HESQMWLESL   450
NTTNYTIFAQ APHPSTIRVI SIGGVGEYIV KALELILNIL EH           492
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C

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                                              RBD 1
MSYKQTTYYP SRGNLVRNDS SPYTNTISSE TNNSSTSVLS LQGASNVSLG    50
TTGNQLYMGD LDPTWDKNTV RQIWASLGEA NINVRMMWNN TLNNGSRSSM   100
     RNP-2
GPKNNQGYCF VDFPSSTHAA NALLKNGMLI PNFPNKKLKL NWATSSYSNS   150
  RBD 2  RNP-1                                (*)
NNSLNNVKSG NNCSIFVGDL APNVTESQLF ELFINRYAST SHAKIVHDQV   200
                RNP-2
TGMSKGYGFV KFTNSDEQQL ALSEMQGVFL NGRAIKVGPT SGQQQHVSGN   250
    RNP-1
NDYNRSSSSL NNENVDSRFL SKGQSFLSNG NNNMGFKRNH MSQFIYPVQQ   300
    RBD 3
QPSLNHFTDP NNTTVFIGGL SSLVTEDELR AYFQPFGTIV YVKIPVGKCC   350
             RNP-2
GFVQYVDRLS AEAAIAGMQG FPIANSRVRL SWGRSAKQTA LLQQAMLSNS   400
RNP-1                                ↑
LQVQQQQPGL QQPNYGYIPS STCEAPVLPD NNVSSTMLPG CQILNYSNPY   450
ANANGLGSNN FSFYSNNNAT NTQATSLLAD TSSMDLSGTG GQQVIMQGSE   500
AVVNSTNAML NRLEQGSNGF MFA                                523
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D

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MSEAQETHVE QLPESVVDAP VEEQHQEPPQ APDAPQEPQV PQESAPQESA    50
PQEPPAPQEQ NDVPPPSNAP IYEGEESHSV QDYQEAHQHH QPPEPQPYYP   100
                         RBD 1
PPPPGEHMHG RPPMHHRQEG ELSNTRLFVR PFPLDVQESE LNEIFGPFGP   150
                              RNP-2              RBD 2
MKEVKILNGF AFVEFEEAES AAKAIEEVHG KSFANQPLEV VYSKLPAKRY   200
        RNP-1
RITMKNLPEG CSWQDLKDLA RENSLETTFS SVNTRDFDGT GALEFPSEEI   250
  RNP-2                              RNP-1
LVEALERLNN IEFRGSVITV ERDDNPPPIR RSNRGGFRGR GGFRGGFRGG   300
FRGGFSRGGF GGPRGGFGGP RGGYGGYSRG GYGGYSRGGY GGSRGGYDSP   350
RGGYDSPRGG YSRGGYGGPR NDYGPPRGSY GGSRGGYDGP RGDYGPPRDA   400
YRTRDAPRER SPTR                                         414
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FIGURE 2. Sequences of the novel yeast U1 snRNP-specific proteins. In all sequences, the peptides identified by mass spectrometry are underlined. **A:** Sequence of the yeast U1 snRNP-specific protein Snu71p (EMBL accession number, Z72798; PIR accession number, S64304; ORF, YGR013w). A putative bipartite nuclear localization signal is found between residues 298 and 312. A highly acidic stretch is found between residues 336 and 375. SR/RS-, RD/DR-, and RE/ER-dipeptides are printed in bold. **B:** Sequence of the yeast U1 snRNP-specific protein Snu56p (EMBL accession number, Z49701; PIR accession number, S54536; ORF, YDR240c). The point mutation S125F found in the *mud10* mutant is indicated below the sequence by a star. **C:** Sequence of the yeast U1 snRNP-specific protein Nam8p (Swissprot accession number, Q00539). The three RNA-binding domains are indicated by arrows above the sequence. RNP-2 and RNP-1 motifs are printed in bold italics and are indicated below the sequence. A premature stop codon is found in the *mud15* mutant at position 140, marked by a star. An arrow marks the C terminus of the GST-fusion protein used for the immunization of rabbits. **D:** Sequence of the (putatively associated) yeast U1 snRNP protein Npl3p (Swissprot accession number, Q01560). The two RNA-binding domains are indicated by arrows above the sequence. RNP-2 and RNP-1 motifs are printed in bold italics and indicated below the sequence. The C-terminal RGG-domain is boxed with a thin line. SR/RS-, RD/DR-, and RE/ER-dipeptides are printed in bold letters.

serines (10.65%), glutamic acid (11.77%), and aspartic acid (8.87%), and is therefore negatively charged and acidic (calculated pI = 4.78). In addition, a putative bipartite nuclear localization signal (NLS) is located between residues 298 and 312. A highly acidic stretch is found between residues 336 and 375. Despite these sequence elements, Snu71p has no known motifs of possible functional significance.

Snu56p

SNU56 encodes a protein of 492 amino acids (Fig. 2B) that is relatively rich in serines (9.76%) and asparagines (6.71%). Eight serine residues cluster between positions 310 and 321. Snu56p is positively charged and basic (calculated pI = 9.35). Recombinant Snu56p exhibits some RNA-binding affinity in vitro (data not shown), although the sequence does not contain any consensus RNA-binding motifs. No other known motifs that would suggest a function for this protein are detected in the sequence.

Nam8p

The 57-kDa protein is identical to Nam8p, an RNA-binding protein of 523 amino acids that contains three RNA-binding domains (RBDs, see Fig. 2C). Nam8p was previously identified as a suppressor of mitochondrial splicing defects, when overexpressed, and shown to be inessential for vegetative cell growth (Ekwall et al., 1992). Other studies proved that Nam8p is essential during meiosis and for splicing of a meiosis-specific pre-mRNA, the MER2-transcript (Ogawa et al., 1995; Nakagawa & Ogawa, 1997). The 5'-splice site of MER2

is not canonical and probably does not interact stably with the U1 snRNA-5′-arm (Nandabalan et al., 1993). Splicing of MER2 is dependent on a meiosis-specific factor, Mer1p (Engebrecht et al., 1991), which binds through its KH motif to the 5′-exon and in the intron of the MER2-transcript, and stabilizes the interaction of MER2 with the U1 snRNA-5′-arm (Nandabalan & Roeder, 1995). Consistent with this model, splicing of the MER2 pre-mRNA becomes Mer1p-independent when its 5′-splice site is changed to the consensus. Splicing of MER2 is also affected by a mutation in the second RBD of Nam8p (Nakagawa & Ogawa, 1997). This suggests that Nam8p, like Mer1p, stabilizes the interaction of the noncanonical MER2-intron with the U1 snRNP. However, when the *MER2* gene was replaced by an intron-less version, some but not all of the *NAM8* deletion phenotypes were suppressed, indicating that Nam8p might also affect the splicing of other meiosis-related transcripts (Nakagawa & Ogawa, 1997).

Npl3p

The 55-kDa protein (calculated M_r: 45 kDa) is identical to the RNA-binding protein Npl3p (Fig. 2D), which has been studied extensively and assigned a number of possible functions (Russell & Tollervey, 1992; Siebel & Guthrie, 1996, and references therein). Concerning its structural organization, Npl3p is one of the few yeast proteins with similarity to the vertebrate hnRNP proteins (Lee et al., 1996). It has two RBDs in the middle of the sequence and a C-terminal RGG-domain. Npl3p was shown to shuttle between the nucleus and the cytoplasm, another characteristic of hnRNP proteins (Flach et al., 1994). It is also implicated in various aspects of RNA metabolism, such as mRNA export from the nucleus and ribosomal precursor–RNA processing, but an influence on pre-mRNA splicing was not reported (Russell & Tollervey, 1992; Flach et al., 1994). Depending on genetic background, Npl3p is essential (Loo et al., 1995; Russell & Tollervey, 1995). In addition, Npl3p contains several SR- or RS-dipeptides as part of the C-terminal RGG-domain. Its RBDs exhibit similarity to the SR-protein ASF/SF2 (Birney et al., 1993; Russell & Tollervey, 1995).

Snu71p, Nam8p, and Snu56p, but not Npl3p, are bound to the U1 snRNP in a salt-resistant manner

The identification of four novel proteins without known homologues in the metazoan spliceosome was striking. Although Nam8p and Npl3p were shown previously to be involved in (pre-m)RNA metabolism, there was no known association with U1 snRNP. Therefore, we verified the association of these new U1 snRNP proteins with an independent method, namely co-immunoprecipitation.

We raised antisera against all of the four U1 snRNP protein candidates and tested the antisera for specificity in western blots, using purified U1 snRNP proteins (Fig. 3A). All antisera recognize their antigen specifically, whereas the pre-immune sera show no reactivity (see Fig. 3A legend). Immunoprecipitations from yeast splicing extracts were performed at salt concentrations of 150, 400, and 700 mM NaCl and the precipitated snRNAs were identified by northern blotting, using ^{32}P-labeled DNA probes specific for the U1, U2, U4, U5, and U6 snRNAs (Fig. 3B). The antisera against Snu71p, Nam8p, and Snu56p specifically precipitate U1 snRNP (Fig. 3B, lanes 2–4, 6–8, 10–12, respectively), even at 700 mM salt, whereas the non-immune sera do not precipitate any of the snRNPs. The antisera against Nam8p and, to a lesser extent, Snu71p, co-precipitate some U2 snRNP at low salt (150 mM NaCl; Fig. 3B, lanes 2, 6); this is probably nonspecific background. Identical results (data not shown) were obtained with extracts from two strains, namely BJ2168 and BSY283, the latter being the one expressing the polyhistidine-tagged Snp1p (from which we isolated the U1 snRNP). We conclude that Snu71p, Nam8p, and Snu56p are stably associated U1 snRNP-specific proteins.

The anti-Npl3p antiserum precipitates considerably less U1 snRNP from the BSY283 extract compared with the other sera and only at 150 mM salt (Fig. 3B, lane 18). To a lesser extent, it also precipitates all other snRNPs. In addition, the non-immune serum precipitates detectable amounts of all snRNPs at 150 mM salt, in contrast to the other non-immune sera (Fig. 3B, lanes 17, 21). When the extract from BJ2168 was used (Fig. 3B, lanes 14–17), the amount of precipitated U1 snRNP was even less (compared with strain BSY283 in Fig. 3B, lanes 18–21), whereas the precipitation of the other snRNPs was identical. This experiment shows only a weak, salt-sensitive, and moderately specific association of Npl3p with the U1 snRNP.

To investigate the fraction of the soluble cellular Npl3p actually associated with snRNPs, we performed a glycerol-gradient centrifugation of whole-cell extracts and analyzed the snRNAs and proteins of each fraction separately. Sedimentation of the snRNAs was followed by northern blot analysis, whereas Npl3p was assayed by western blot analysis (Fig. 4). Npl3p is abundant and the majority of it sediments as free protein in the top fractions of the gradient (2–5 S, Fig. 4, fractions 1–6). The rest is more or less equally distributed over all fractions, without a detectable peak in the U1 snRNP region (around 17 S, Fig. 4, fractions 11–19). This implies that no more than a small fraction of the cellular Npl3p is associated with U1 or the other snRNPs.

The results presented above do not allow a definitive conclusion about the association of Npl3p with the U1 snRNP. Negative results were also obtained when assaying for the presence of Npl3 in the commitment complexes using specific antibodies (B. Rutz, pers.

comm.), whereas Nam8p tested positive in the same assays. Npl3p is either not present or not accessible in the commitment complexes. Although we reproducibly co-isolate Npl3p with the U1 snRNP, we are unable to demonstrate convincingly that it is stably associated only with the U1 snRNP. We cannot exclude at this time that Npl3p binds unspecifically to snRNAs through its RNA-binding domains. However, it may act transiently in concert with the U1 snRNP in the spliceosome.

Yeast U1 snRNP also contains the core snRNP protein SmB

In metazoa, the SmB/B′ proteins are part of the Sm-core of proteins that are common to all snRNPs. SmB and SmB′ originate from one gene through alternative splicing and differ only slightly at their C-termini. We had never co-isolated a homologue of the SmB/B′ proteins as a yeast U1 snRNP component and concluded that we might have lost this protein during snRNP isolation. Because there is a general conservation of the splicing apparatus between yeast and metazoa, it would be quite unlikely if this protein is not present in yeast. Indeed, when we analyzed purified yeast [U4/U6.U5]-tri-snRNPs, we could clearly identify a SmB/B′ homologue with an apparent molecular weight of 28 kDa by mass spectrometry (our unpubl. results). We investigated the association of this protein with the U1 snRNP by immunoprecipitation, using a strain expressing both a genomic copy of *SMB* and a protein-A-tagged SmB protein from a multicopy plasmid. As a comparison, we used the same strain harboring a multicopy plasmid encoding protein-A-tagged SmF (Séraphin, 1995). Primer extension of the precipitated U1 snRNA (Fig. 5A) showed that the U1 snRNP could be precipitated in an indistinguishable manner by SmF and SmB even at 300 mM NaCl (as were all other snRNPs, not shown). This demonstrates that the yeast SmB protein is a U1 snRNP component and is probably lost upon chromatographic isolation of the U1 snRNP.

An alignment of the yeast SmB and human SmB′ proteins is shown in Figure 5B. Yeast SmB is shorter than its human counterpart (196 versus 240 amino acids). However, the two proteins are well conserved in the N-terminal half (49% identity, 73% similarity), which contains the Sm-motifs 1 and 2 (Hermann et al., 1995). In addition, the C-terminal 85 amino acids of the yeast SmB protein align to the C terminus of the human SmB′ protein. The C terminus of yeast SmB contains a proline-rich stretch, but, unlike human SmB/B′, it has only one repeat of this motif. Overall, the proteins share 31% identity and 49% similarity.

Identification of U1 snRNP protein candidates by a synthetic lethal (MUD) screen

Because loosely associated U1 snRNP proteins might be lost during biochemical isolation, we also used an independent, genetic strategy for the identification of U1 snRNP proteins. This MUD (for mutant-U1-die) synthetic lethal screen was designed to identify mutations that cause lethality with otherwise viable mutations in U1 snRNA. Many of the mutant genes encode proteins that either cooperate with, or are integral components of U1 snRNP (Liao, 1992; Liao et al., 1993; Abovich et al., 1994; Colot et al., 1996; for review, see also Stutz et al., 1997).

We have characterized several novel *mud* mutants, and we show here that three of them, *mud10*, *mud15*, and *mud16*, encode mutant U1 snRNP proteins. A wild-type genomic library was transformed into the *mud* mutant strains to rescue the synthetic lethal phenotype (Liao et al., 1993). The rescuing plasmids were isolated and sequenced to identify the genes whose mutation caused the synthetic lethality.

Strikingly, the *MUD10* and *MUD15* genes were identical to *SNU56* and *NAM8*, respectively, clearly demonstrating the convergence of the biochemical and genetic approaches. We isolated the viable mutant alleles from the *mud10* and *mud15* strains and compared their sequences to the wild type. *mud10* contains the point mutation S125F and *mud15* has a premature stop codon before the second RBD (see also Fig. 2B,C). The *mud10* mutation was synthetically lethal with a U1 snRNA mutant, which has a point mutation in loop II and a large deletion of the U1 snRNA-core region (termed 18–84; for a description of the mutated regions of the yeast U1 snRNA, see Liao et al., 1992). The *mud15* mutation was lethal in combination with the U1-4U mutation in the 5′-arm of the U1 snRNA (changing nt 4 from C to U and thereby destabilizing the base pairing interaction between the U1 snRNA and the 5′-splice site).

MUD16 codes for a 65-kDa protein that we refer to as Snu65p. We did not detect Snu65p in the wild-type U1 snRNP by mass spectrometry. It is likely that this results from its low abundance in the U1 snRNP rather than from a complete absence. Although Nam8p and Snu65p migrate in the same band (compare Figs. 1B and 10), peptides from both proteins would have been distinguished by mass spectrometry if one of the proteins was not present in more than fivefold molar excess (as demonstrated for Prp39p and Prp40p, Neubauer et al., 1997). Snu65p was simultaneously identified in the laboratory of Brian Rymond and was termed Prp42p (McLean & Rymond, 1998). *mud16* was lethal in combination with the U1 snRNA double mutation 18–84 (see above). We studied the association of Snu65p with the U1 snRNP by immunoprecipitation from an extract containing protein-A-tagged Snu65p. Primer extension of the precipitated snRNAs demonstrated the specific association of Snu65p with the U1 snRNP at 150 mM salt (Fig. 6A, lane 14). In comparison, considerably more U1 snRNA was precipitated from an extract containing a protein-A-tagged Nam8p, demonstrating
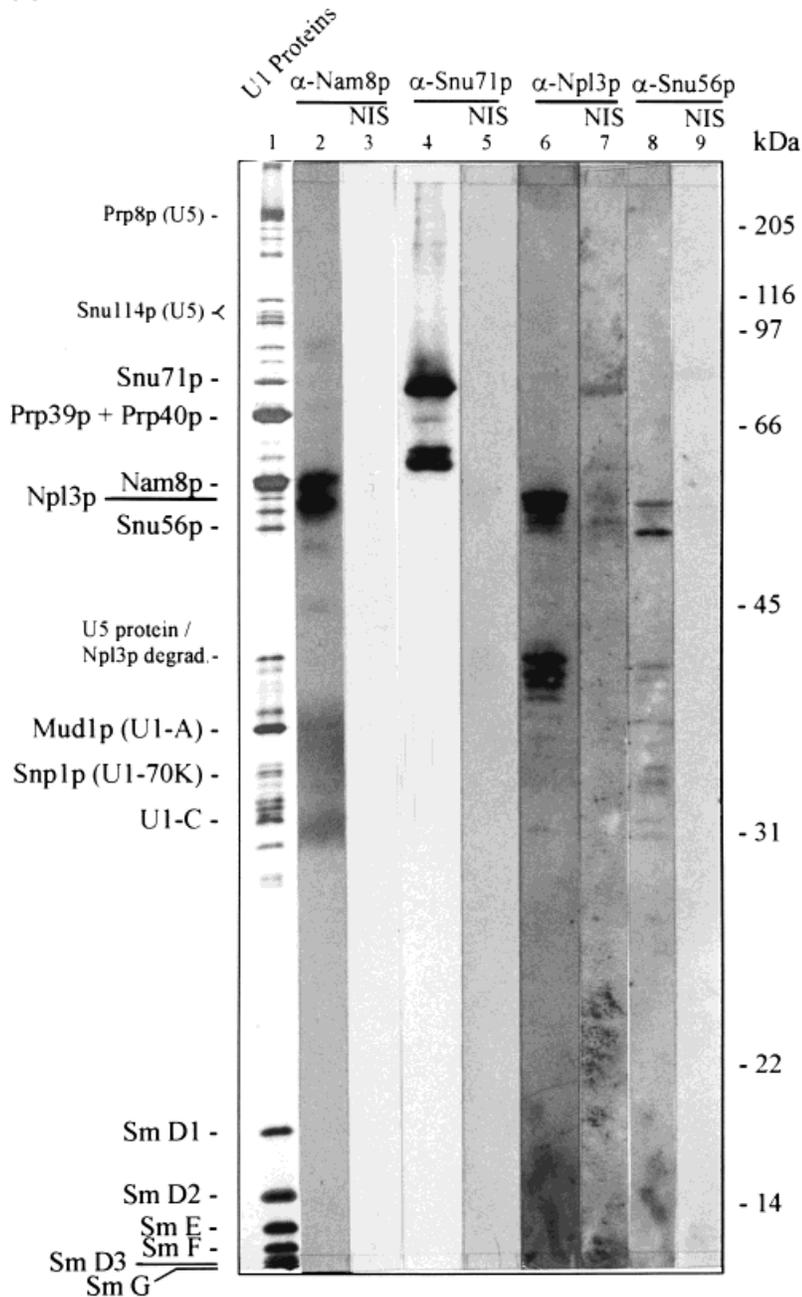
A

FIGURE 3. Snu71p, Nam8p, and Snu56p are tightly associated yeast U1 snRNP-specific proteins, whereas Npl3p is weakly associated. **A:** Western blot of purified yeast U1 snRNP proteins probed with antisera against Nam8p (lane 2), Snu71p (lane 4), Npl3p (lane 6), and Snu56p (lane 8), using the enhanced luminescence (ECL) detection system (Amersham). The following antisera dilutions were used: α-Nam8p, 1:500; α-Snu71p, 1:500; α-Npl3p, 1:250; α-Snu56p, 1:500. Non-immune sera (NIS) were used as controls in lanes 3, 5, 7, and 9, diluted as the corresponding antisera. Silver-stained yeast U1 (and U5) snRNP proteins are shown in lane 1, proteins are indicated on the left, molecular weights on the right. All antisera detected several bands below the main bands. These are most likely degradation products. For Npl3p, the band(s) around 42 kDa and also the three bands seen below Snu114p in the silver-stained protein gel (lane 1) were identified as degradation products of these proteins by mass spectrometry. Western blot analysis of U1 snRNP proteins from a protease-deficient strain revealed no degradation bands (see Fig. 4, lower panel for Npl3p). Most of Snu56p (lane 8) is degraded, because the band of higher molecular weight is represented to a lesser extent when compared with the degradation product. **B:** Immunoprecipitation of yeast splicing extracts using the antisera against the novel U1 snRNP-specific proteins. Precipitated snRNAs were probed with ^{32}P-labeled DNA probes specific for the U1, U2, U4, U5, and U6 snRNAs, respectively. The antisera against Nam8p (lanes 2–4), Snu71p (lanes 6–8), and Snu56p (lanes 10–12) all precipitate the U1 snRNP specifically from extract BJ2168 at salt concentrations of 150, 400, and 700 mM NaCl. Immunoprecipitations with the anti-Npl3p antiserum are shown in lanes 14–16 (using extract BJ2168) and in lanes 18–20 (using extract BSY283 that contains the 6x-histidine tagged Snp1p). The α-Npl3p antiserum precipitates considerably less U1 snRNP compared with the other antisera and only at 150 mM NaCl, and this precipitation depends also on the extract used (compare lanes 14 and 18). Low amounts of U2, U4, U5, and U6 snRNPs are also precipitated by the α-Npl3p antiserum. The corresponding non-immune sera were used as controls at 150 mM NaCl (shown in lanes 5, 9, 13, 17, and 21). Lane 1, immunoprecipitation of all snRNPs using the monoclonal α-m$_3$G-cap antibody H20. Positions of the snRNAs are indicated on the left. Some U1 snRNA in lane 10 was lost during gel loading. (*Figure continues on facing page.*)

that Nam8p associates more tightly with the U1 snRNP (Fig. 6A, lane 11). The sequence of Snu65p (Fig. 6B) contains eight repeats of the tetratrico peptide repeat motif (TPR, Ordway et al., 1994; Lamb et al., 1995; for an alignment, see Fig. 6C and also McLean & Rymond, 1998). TPRs are thought to form amphipathic α-helices that are involved in protein–protein interactions. Although the TPR motif has been defined as a 34-amino acid repeat, not all TPRs found in Snu65p strictly obey

this rule. Introduction of gaps reveals some more homology between the TPRs of Snu65p than an alignment based strictly on 34 amino acid blocks. The TPRs from Snu65p do not correspond very well to the TPR consensus that can loosely be defined for TPR proteins involved in the cell cycle (Lamb et al., 1995). Rather, Snu65p falls into a (putative) class of TPR proteins that are involved in mRNA metabolism. These include the yeast U1 snRNP-specific protein Prp39p
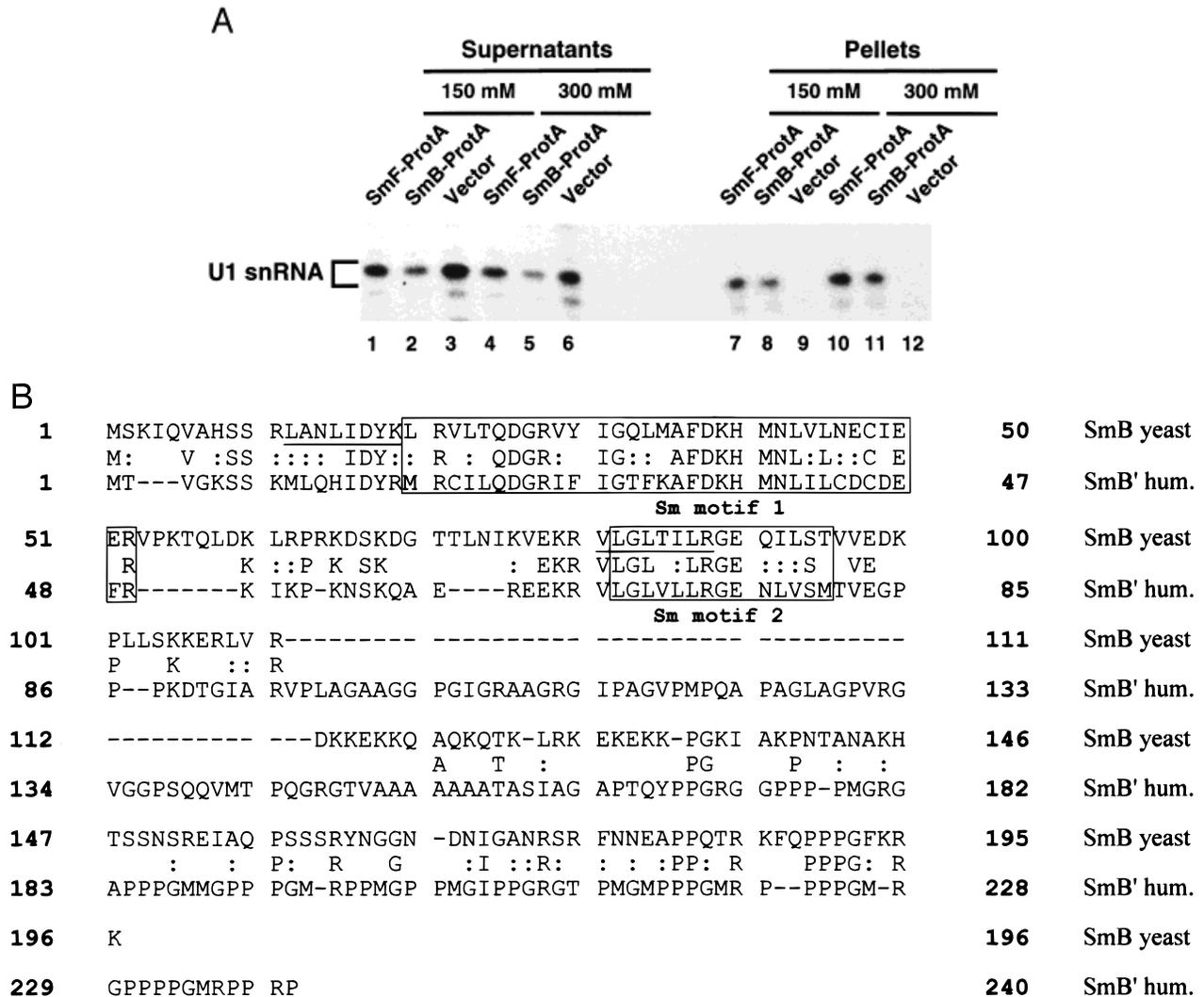
FIGURE 3. (*Continued.*)

FIGURE 4. Minor amounts of the cellular Npl3p are associated with U1 snRNPs. A 300-μL aliquot of splicing extract prepared form strain BJ2168 was separated by (10–30%) glycerol gradient centrifugation at 200 mM KCl. Fractions of 500 μL each were taken from the top and proteins and RNAs contained in the fractions were separated by phenol/chloroform/isoamylalcohol extraction. Upper panel: RNAs were probed with ^{32}P-labeled DNA probes specific for U1, U2, U4, U5, and U6 snRNAs, respectively. The U1 snRNP sedimented between fractions 11 and 19, with a peak in fraction 15, corresponding to a sedimentation coefficient of 17 S. Positions of the snRNAs are indicated on the right. Lower panel: Proteins isolated from the glycerol gradient fractions were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti-Npl3p antiserum (diluted 1:5,000) and bound primary antibodies were detected with the ECL system. Most of the cellular Npl3p sediments in fractions 1–6 as free protein (2–5 S). Lower amounts of Npl3p are found in all fractions, but Npl3p does not peak with U1 snRNP.

A

B

```
  1  MSKIQVAHSS RLANLIDYK|L RVLTQDGRVY IGQLMAFDKH MNLVLNECIE|   50   SmB yeast
     M:      V :SS  :::: IDY:|: R : QDGR: IG:: AFDKH MNL:L::C E|
  1  MT---VGKSS KMLQHIDYR|M RCILQDGRIF IGTFKAFDKH MNLILCDCDE|   47   SmB' hum.
                                            Sm motif 1

 51  |ER|VPKTQLDK LRPRKDSKDG TTLNIKVEKR V|LGLTILRGE QILST|VVEDK  100   SmB yeast
     |R |      K ::P K SK          : EKR V|LGL :LRGE :::S| VE
 48  |FR|-------K IKP-KNSKQA E----REEKR V|LGLVLLRGE NLVSM|TVEGP   85   SmB' hum.
                                            Sm motif 2

101  PLLSKKERLV R--------- ---------- ---------- ----------   111   SmB yeast
     P   K   :: R
 86  P--PKDTGIA RVPLAGAAGG PGIGRAAGRG IPAGVPMPQA PAGLAGPVRG   133   SmB' hum.

112  ---------- ---DKKEKKQ AQKQTK-LRK EKEKK-PGKI AKPNTANAKH   146   SmB yeast
                          A    T   :           PG       P  :  :
134  VGGPSQQVMT PQGRGTVAAA AAAATASIAG APTQYPPGRG GPPP-PMGRG   182   SmB' hum.

147  TSSNSREIAQ PSSSRYNGGN -DNIGANRSR FNNEAPPQTR KFQPPPGFKR   195   SmB yeast
           :    :  P: R   G      :I ::R:  : : :PP: R    PPPG: R
183  APPPGMMGPP PGM-RPPMGP PMGIPPGRGT PMGMPPPGMR P--PPPGM-R   228   SmB' hum.

196  K                                                        196   SmB yeast

229  GPPPPGMRPP RP                                            240   SmB' hum.
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FIGURE 5. Yeast U1 snRNP contains a stably associated SmB protein. **A:** Immunoprecipitation demonstrating the stable association of (protein-A-tagged) SmB with the U1 snRNP. Extracts containing protein-A-tagged SmB (lanes 2, 5, 8, 11) and SmF (lanes 1, 4, 7, 10) and no tagged proteins (lanes 3, 6, 9, 12) were used for immunoprecipitation using rabbit IgG-agarose at 150 (lanes 1–3, 7–9) and 300 mM NaCl (lanes 4–6, 10–12). The U1 snRNA content of the precipitates (lanes 7–12) and supernatants (lanes 1–6) was assessed by primer extension using a ^{32}P-labeled U1-specific primer. Extension products were separated by denaturing gel electrophoresis and the gel was autoradiographed. **B:** Alignment of yeast SmB (Swissprot accession number, P40018) and human SmB′ proteins. The proteins share an overall identity of 31% (49% similarity). Both proteins contain the conserved motifs Sm 1 and Sm 2 (boxed and indicated under the sequence) and the N-termini from both proteins (containing the Sm 1 and Sm 2 motifs) exhibit 49% identity and 73% similarity. Conserved residues are indicated between the sequences; identical residues by letters, conservative exchanges by a colon. Conservative exchanges were defined by these groups of amino acids: acidic, D, E; basic, H, K, R; hydrophobic, A, F, I, L, M, P, V, W; polar, C, G, N, Q, S, T, Y. Peptides identified by mass spectrometric analysis of yeast SmB that was co-purified with [U4/U6.U5] tri-snRNPs are underlined.

(Lockhart & Rymond, 1994), the splicing factor, Prp6p, and finally, Rna14p, which is involved in poly-adenylation as part of cleavage factor (CF) IA (Minvielle-Sebastia et al., 1991, 1994). Homologues of Rna14p are CStF 77K in mammals and suppressor of forked (su[f]) in *Drosophila* (Mitchelson et al., 1993; Takagaki & Manley, 1994). TPRs that obey the latter sequence consensus are also found in the *Drosophila* crooked neck (crn) protein and its likely yeast homologue, although these proteins might be involved in the cell cycle (McLean & Rymond, 1998). We do not have any information about

the role of TPRs in the U1 snRNP. The presence of two TPR-proteins in the U1 snRNP (Snu65p and Prp39p) suggests that they might either bind to each other, to other (U1 snRNP) proteins, or even to RNA.

The presence of two TPR proteins in yeast U1 snRNP might also explain the co-isolation of Npl3p (see above). A temperature-sensitive mutant of Npl3p was suppressed by mutant alleles of Hrp1p and Rna15p (Henry et al., 1996). Interestingly, Rna15p is also part of CF IA, in a tight complex with the TPR-protein Rna14p. Because Npl3p and Rna15p both belong to the few yeast
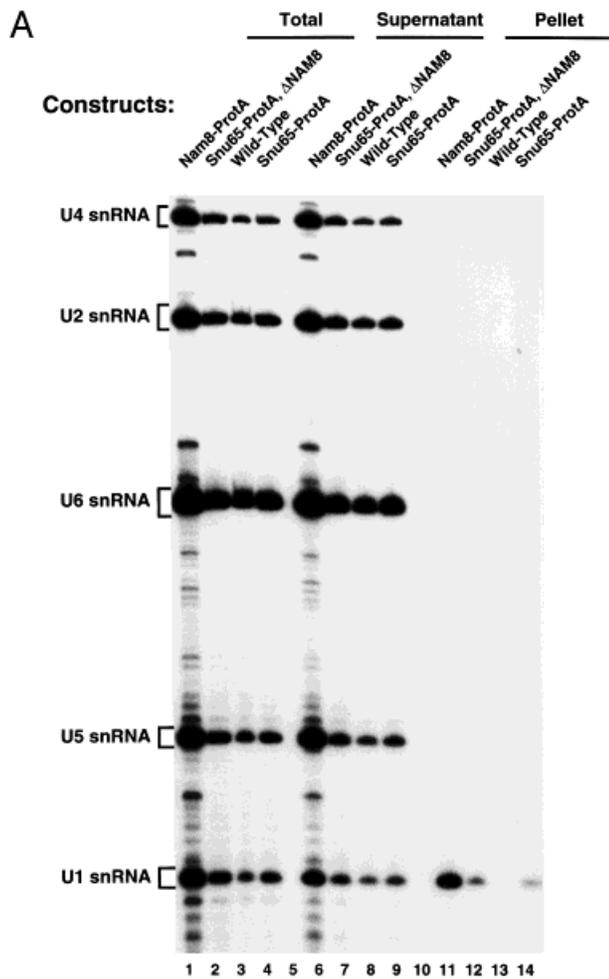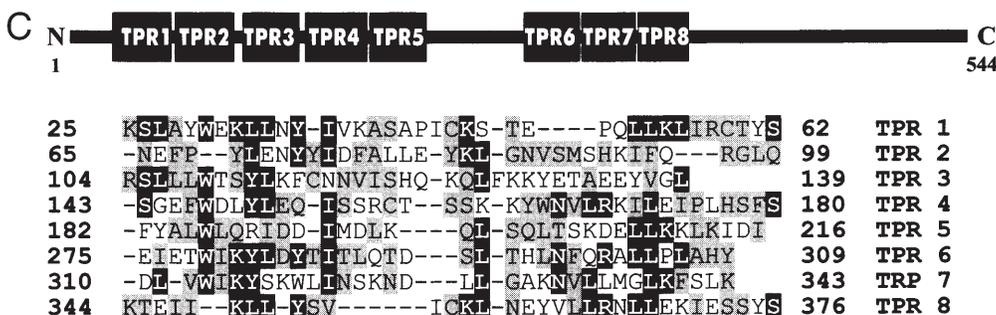
A

FIGURE 6. Yeast U1 snRNP contains weakly associated Snu65p/Mud16p/Prp42p. **A:** Immunoprecipitation of U1 snRNPs from extracts containing protein-A-tagged Snu65p at 150 mM NaCl. Precipitations from wild-type and Δ*nam8* extracts that contain protein-A-tagged Snu65p are shown in lanes 14 and 12, respectively. As a control, extracts containing no tagged protein (lane 13) or protein-A-tagged Nam8p (lane 11) were also used. Further, primer extension products from snRNAs isolated from the supernatants and from the input are shown in lanes 1–4 and 6–9, respectively, as indicated. Lanes 5 and 10 are empty. Snu65p is specifically associated with the U1 snRNP (compare in lane 14 the U1 signal with the other snRNA signals and with all signals in the control lane 13). Note that a reduced amount of U1 snRNA is precipitated by Snu65p–ProteinA compared with Nam8p–ProteinA (compare lanes 11 with 14), even though the Nam8p–ProteinA-containing extract is slightly more concentrated. Note also that more than 95% of the tagged proteins were precipitated as indicated by western blotting. In the absence of Nam8p (lane 12), about twice the amount of U1 snRNP is precipitated by a tagged Snu65p (compared with lane 14), indicating a hyperstabilized association of Snu65p with the Δnam8p U1 snRNP. **B:** Sequence of the novel U1 snRNP-specific protein Snu65p/Mud16p/Prp42p (EMBL accession number, Z49701; PIR accession number, S54531; ORF, YDR235w). The sequences of the peptides that were identified by mass spectrometry are underlined. **C:** Snu65p contains eight degenerate tetratrico peptide repeats (TPRs) that cluster as a pentamer and as a trimer in the N-terminal two thirds of the protein. An alignment of the TPRs is shown in the lower panel. Printed white on black are identical amino acids that are found in at least three sequences. Highlighted in grey are functionally conserved amino acids that are found in at least three sequences. Equivalent amino acids were grouped as in Figure 5B.

B

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MDKYTALIHD ENFSTLTLNV SRYPKSLAYW EKLLNYIVKA SAPICKSTEP    50
QLLKLIRCTY SSMLNEFPYL ENYYIDFALL EYKLGNVSMS HKIFQRGLQA   100
FNQRSLLLWT SYLKFCNNVI SHQKQLFKKY ETAEEYVGLH FFSGEFWDLY   150
LEQISSRCTS SKKYWNVLRK ILEIPLHSFS KFYALWLQRI DDIMDLKQLS   200
QLTSKDELLK KLKIDINYSG RKGPYLQDAK KKLKKITKEM YMVVQYQVLE   250
IYSIFESKIY INYYTSPETL VSSDEIETWI KYLDYTITLQ TDSLTHLNFQ   300
RALLPLAHYD LVWIKYSKWL INSKNDLLGA KNVLLMGLKF SLKKTEIIKL   350
LYSVICKLNE YVLLRNLLEK IESSYSDNVE NVDDFEIFWD YLQFKTFCQN   400
SLYSSRYSDS QSNGLLNKEL FDKVWKRLSC KEKKSGQEIL LNNLVQFYSK   450
DTVEFVEKNI FQKIIEFGWE YYLQNGMFWN CYCRLIYFDT SRSYLDKRQY   500
IVRKIWPQID KKFAQSVLPS LTEFCESYFP EEMDTLEEMF TEEP         544
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C


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 25  KSLAYWEKLLNY-IVKASAPICKS-TE----PQLLKLIRCTYS   62  TPR 1
 65  -NEFP--YLENYYIDFALLE-YKL-GNVSMSHKIFQ---RGLQ   99  TPR 2
104  RSLLLWTSYLKFCNNVISHQ-KQLFKKYETAEEYVGL        139  TPR 3
143  -SGEFWDLYLEQ-ISSRCT--SSK-KYWNVLRKILEIPLHSFS  180  TPR 4
182  -FYALWLQRIDD-IMDLK----QL-SQLTSKDELLKKLKIDI   216  TPR 5
275  -EIETWIKYLDYTITLQTD---SL-THLNFQRALLPLAHY     309  TPR 6
310  -DL-VWIKYSKWLINSKND---LL-GAKNVLLMGLKFSLK     343  TRP 7
344  KTEII--KLL-YSV------ICKL-NEYVLLRNLLEKIESSYS  376  TPR 8
```

proteins with similarity to metazoan hnRNP proteins (Lee et al., 1996), an analogous interaction could exist in U1 snRNP between Npl3p and Snu65p or Prp39p.

Snu71p, Snu56p, and Snu65p are essential for cell viability

Because no information was available about whether *SNU71*, *SNU65*, and *SNU56* are essential for cell viability, we have independently disrupted one chromosomal copy of *SNU71*, *SNU65*, or *SNU56* with marker genes by homologous recombination in diploid strains. Transformants were selected on the corresponding media and sporulated subsequently. After tetrad dissection, only two of the four spores were viable for each of the genes and these spores were auxotrophic for the disruption marker, indicating that *SNU71*, *SNU65*, and *SNU56* are all essential for cell viability (data not shown). For *SNU71* and *SNU56*, the heterozygous parental strains were transformed with plasmids containing the wild-type genes, sporulated, and dissected. In at least 2 of 12 tetrads, four spores were viable and cosegregation of the markers used for disruption and the markers carried by the corresponding plasmids was found, demonstrating that lethality was due to the loss of *SNU71* and *SNU56* (data not shown).

Functional analysis of *mud10/snu56* and *mud15/nam8* in vivo and in vitro

We have performed in vivo and in vitro experiments to test whether *mud10*, *mud15*, and *mud16* result in any splicing or commitment complex formation defects. The influence of *mud10*, *mud15*, and *mud16* on splicing was assayed in vivo. First, the copper-resistance gene, *CUP1*, was knocked out in the mutant strains. We reintroduced a recombinant *cup1* that was interrupted by different introns into the *mud/Δcup1* strains as a reporter for splicing activity. Only when splicing was efficient could the strains grow on copper-containing medium (Lesser & Guthrie, 1993). We used the following introns to interrupt *CUP1* (Fig. 7A): (1) an efficiently spliced intron (HZ18; Pikielny & Rosbash, 1985); (2) an intron with a mutation in the 5′-splice site (HZ12; Jacquier et al., 1985); and (3) an inefficiently spliced artificial intron (ACC; Legrain & Rosbash, 1989). Moreover, we assayed for the in vitro formation of commitment complexes and spliceosome. Splicing extracts were prepared from the *mud10* and *mud15* mutant strains, as well as from mutant strains that were transformed with rescuing plasmids containing the wild-type *MUD10* and *MUD15* genes.

In the *mud10* background, splicing of the efficient HZ18-intron was not compromised (Fig. 7B). Splicing of the HZ12- and ACC-introns was mildly affected compared with a wild-type background. Moreover, the *mud10* extract formed less commitment complexes and splice-

osome compared with a wild-type extract or the extract from *mud10* that was rescued with *MUD10* (Fig. 8). These results indicate that Mud10p/Snu56p contributes to the stability of the commitment complex and/or efficiency of complex formation, possibly by stabilizing the interaction of the U1 snRNP with the intron. The mutation S125F mildly impairs the function of Mud10p/Snu56p so that only the splicing efficiency of noncanonical introns is affected. We expect that a deletion of Snu56p (complete loss of its function) would affect splicing of all introns, because *MUD10/SNU56* is essential for cell viability.

In the *mud15* background, only splicing of the (mutated) HZ12-intron was strongly affected (Fig. 7C). Splicing of consensus-introns was normal, and the *mud15* extract formed normal levels of complexes with a consensus pre-mRNA substrate (Fig. 8, compare lanes 4 and 5 and see legend). The situation concerning splicing of the HZ12-intron and of the MER2-transcript (see above) is similar. Both contain a noncanonical 5′-splice site and splicing of both is strongly affected in the absence of (wild type) Nam8p/Mud15p. We propose that Nam8p/Mud15p is involved in the stabilization of the U1 snRNA-5′-splice site interaction. Nam8p could do this either directly through binding and/or induction of a certain conformation of the pre-mRNA, or indirectly by fixing the U1 snRNP in a conformation that favors this interaction. This model is supported by the fact that the *mud15* mutation (which is likely to be a null allele) leads to cell death when the U1 snRNA carries the U1-4U mutation in the 5′-arm. This mutation could have an equivalent, destabilizing effect on the U1 snRNA-5′-splice site interaction as have the noncanonical 5′-splice sites in MER2 or the HZ12-intron, only that it now affects all wild-type splice sites. In the presence of Nam8p, the U1-4U mutation is viable, possibly because Nam8p stabilizes the mutant base pairing interaction.

mud16 had a minor effect on splicing of the ACC- and HZ12-introns (data not shown). However, *MUD16/ SNU65/PRP42* was indeed shown to be essential for in vivo splicing and in vitro commitment complex formation (McLean & Rymond, 1998).

Deletion of Nam8p destabilizes the overall structure of yeast U1 snRNP yet stabilizes the association of Snu65p

The phenotypes of the *mud* mutations could either be due directly to the particular mutation itself, or they could be caused by an indirect effect that they impose on the U1 snRNP. Mutations or deletions of other U1 snRNP proteins have been shown previously to affect the structural integrity and mobility of the U1 snRNP on native gels (e.g., Mud1p and Prp39p; see Liao et al., 1993; Lockhart & Rymond, 1994). We tested the influence of the novel *mud* mutants on the structure of the
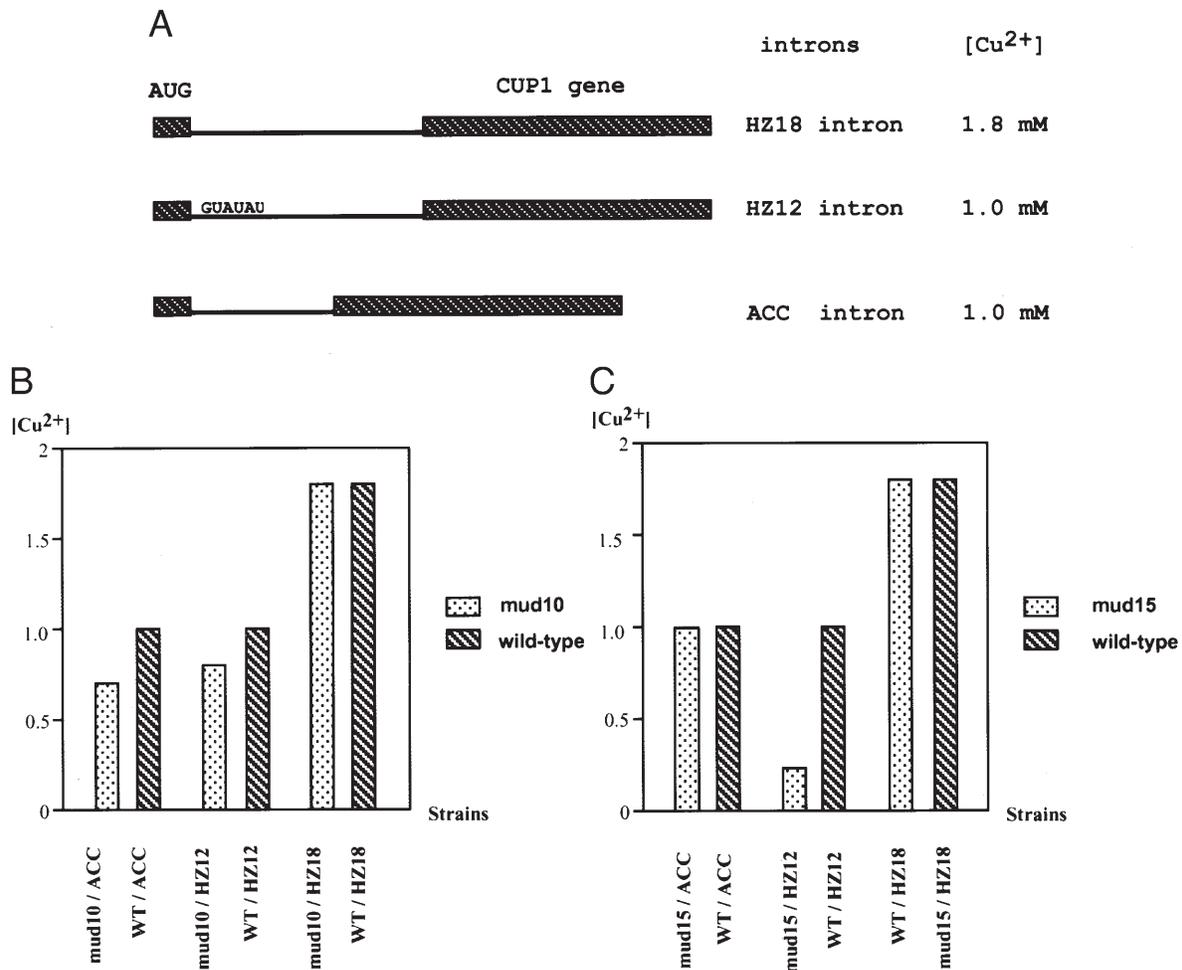
FIGURE 7. In vivo splicing assay using the *CUP1* reporter. **A:** The endogenous *CUP1* gene was knocked out in the *mud10* and *mud15* mutant strains. A reporter *CUP1* gene that was interrupted by a wild-type intron (HZ18), an intron with a 5′-splice site mutation (HZ12), or an inefficiently spliced artificial intron (ACC) was transformed into the Δ*cup1/mud* strains. Splicing efficiency was measured by the ability of the resulting strain to grow on copper-containing medium. A Δ*cup1* strain that did not contain any *mud* mutations was simultaneously transformed with the *CUP1*-reporter constructs. The highest copper concentrations that the resulting strains could grow on are indicated. **B:** Splicing efficiency of *mud10* and *MUD10* strains. **C:** Splicing efficiency of *mud15* and *MUD15* strains that were transformed with the *CUP1*-reporter constructs was measured as the highest copper concentrations that the strains were able to grow on.

U1 snRNP by native gel electrophoresis. Extracts from the *mud15* strain and from *mud15* rescued with wild-type *MUD15* on a plasmid were fractionated and the position of the U1 snRNA was identified by northern analysis (Fig. 9, lanes 3, 4). As a comparison, a wild-type extract and an extract that was genetically depleted of Mud1p (U1-A) were also assayed (Fig. 9, lanes 1, 2). The extract from the *mud15* strain showed a strong phenotype, because its U1 snRNP migrated even faster than the U1 snRNP in the ΔU1-A control. This result indicates that the *mud15* mutation (likely equivalent to a knockout of Nam8p/Mud15p) has a dramatic effect on the structure of the U1 snRNP. It might even lead to an overall destabilization of the complex and, as a consequence, to the loss of other U1 snRNP proteins. In contrast, commitment complex formation was normal in the *mud15* mutant, as assayed using the

identical gel system (Fig. 8, lanes 4, 5). This suggests that additional factors present in the commitment complexes (e.g., Snu65p) or just the pre-mRNA help to stabilize the *mud15*-U1 snRNP. The *mud10* mutation had no effect in the U1 snRNP stability assay (data not shown).

To analyze the contribution of Nam8p/Mud15p to U1 snRNP in more detail, we deleted the *NAM8* gene in strain BSY283, which carries the polyhistidine-tagged Snp1p. First, we studied the association of other U1 snRNP proteins in the Δ*nam8* strain by immunoprecipitation (data not shown). We used antisera against Snu71p, Snu56p, Npl3p, and, as a control, Nam8p. As expected, the α-Nam8p antiserum did not precipitate any snRNP. The association of Snu71p and Snu56p with the U1 snRNP was greatly reduced already at 150 or 400 mM salt, respectively. In contrast, binding of Npl3p
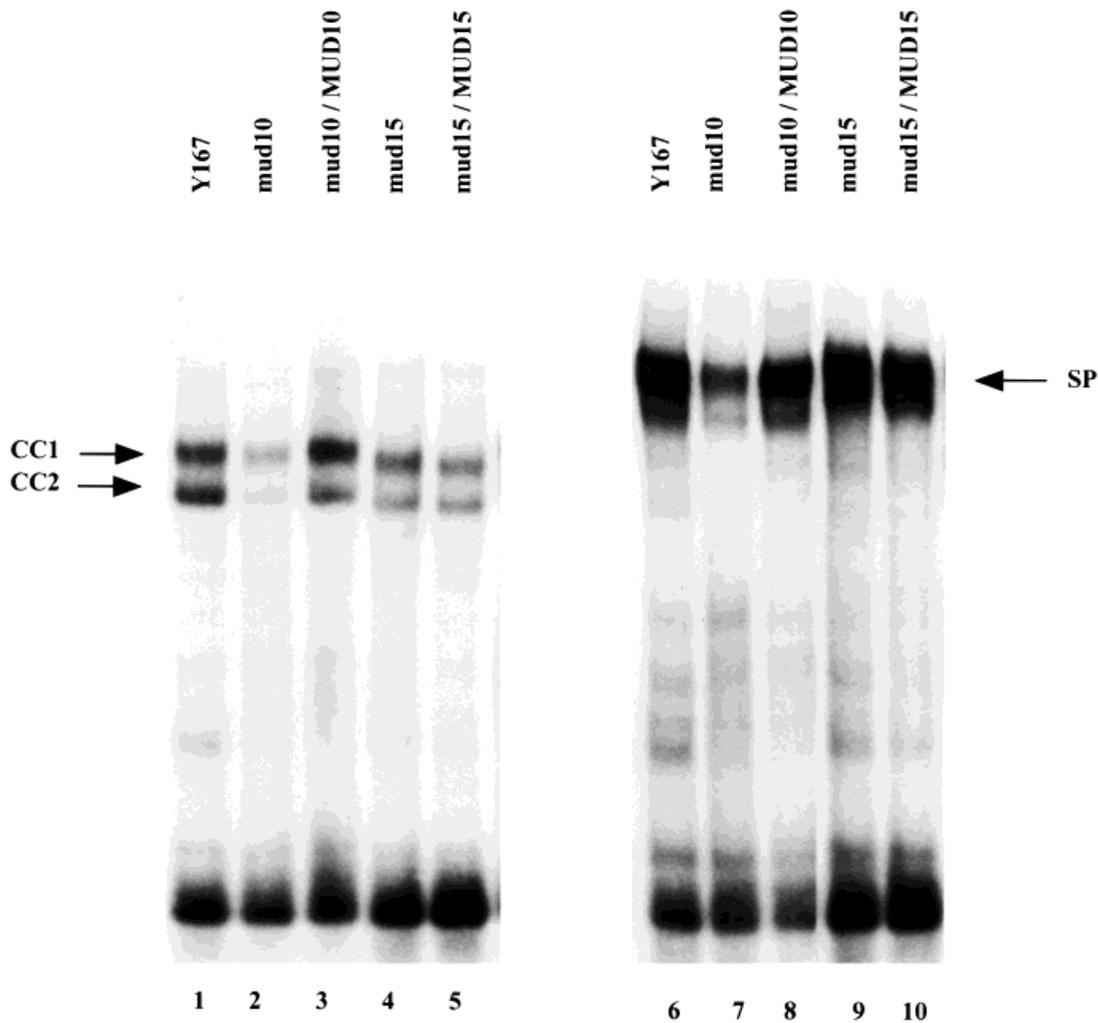
FIGURE 8. Formation of splicing complexes in the *mud* mutant strains. Splicing extracts were prepared from different strains and incubated with ³²P-labeled pre-mRNA under splicing conditions. The complexes associated with pre-mRNA were assayed by native gel electrophoresis. For commitment complex formation (lanes 1–5), U2 snRNA was selectively inactivated by adding U2-specific oligonucleotides (to direct RNAseH digestion) to the extracts. Lanes 1, 6, wild-type strain; lanes 2, 7, *mud10* mutant; lanes 3, 8, *mud10* mutant transformed with *MUD10*/p366; lanes 4, 9, *mud15* mutant; lanes 5, 10, *mud15* mutant transformed with *MUD15*/p366. Positions of commitment complexes and spliceosome are indicated. The small mobility change in lanes 4 and 5 is due to a gel artifact.

was not affected by the loss of Nam8p. These results suggest that Nam8p stabilizes the association of Snu71p and Snu56p with the U1 snRNP at least in vitro.

We purified the Δnam8p U1 snRNP as described for the wild type (Neubauer et al., 1997). Δnam8p U1 snRNP contained all of the proteins that were co-isolated with wild-type U1 snRNP, although it contained less Snu71p and Snu56p, as judged from Coomassie-staining of the proteins (Fig. 10, compare with Fig. 1B). Surprisingly, a novel protein that had an apparent molecular weight of 57 kDa and a mobility on gels identical to that of Nam8p was present in Δnam8p U1 snRNP. However, as expected, this protein was not detected by the anti-Nam8p antiserum in a western blot (data not shown) and was not co-isolated with wild-type U1 snRNP under identical conditions. ES-MS sequencing of this 57-kDa protein revealed it to be Snu65p/Mud16p/Prp42p.

The association of Snu65p with the Δnam8p U1 snRNP was again studied by immunoprecipitation from an *SNU65–protein-A/Δnam8* strain. About twice as much U1 snRNA was co-precipitated from the Δ*nam8* extract as from the wild-type extract (Fig. 6A, compare lane 12 and 14). This confirmed the biochemical result; namely, Snu65p binds only weakly to wild-type U1 snRNP, but is associated more strongly in the absence of Nam8p. The significance, if any, of this observation on U1 snRNP function will require further studies. The deletion of Nam8p might just leave more space for Snu65p to bind to the U1 snRNP or it could induce a conformation that favors the association of Snu65p. Nam8p and Snu65p might also compete for the same or overlapping binding sites on the U1 snRNP.

When we subjected isolated Δnam8p U1 snRNP to glycerol gradient centrifugation at higher salt concen-

FIGURE 9. The *mud15* mutation affects U1 snRNP mobility on native gels. Splicing extracts were incubated with *Xenopus* total RNA before native gel electrophoresis (identical gel system as in Fig. 8). Separated RNAs were blotted and probed with a ^{32}P-labeled DNA probe specific for U1 snRNA. Lane 1, wild-type strain; lane 2, U1-A knockout strain; lane 3, *mud15* mutant strain transformed with *MUD15*/p366; lane 4, *mud15* mutant strain. Position of wild-type U1 snRNP is indicated. Bands of higher mobility are most likely free U1 snRNA or immature U1 snRNP complexes.

FIGURE 10. The knockout of *NAM8* hyperstabilizes the association of Snu65p with the U1 snRNP. Δnam8p U1 snRNP was isolated from strain AGY001; co-isolated proteins are indicated on the right. These particles contained stably associated Snu65p. Note that Snu56p (and Snu71p) are barely visible on this Coomassie-stained gel, indicating an impaired association of these two proteins with Δnam8p U1 snRNPs (compare with Fig. 1B). However, Snu56p is present and was clearly stained with silver (not shown). Marked by an asterisk is a contaminating protein that is also found in isolated wild-type U1 snRNP (see Fig. 1B).

trations, we observed a more general effect of the Nam8p deletion on other U1 snRNP proteins. The Δnam8p U1 snRNP sedimented as a 17 S complex at 200 mM salt and included all of the U1 snRNP proteins in addition to Snu65p. In contrast, at 450 mM salt, Δnam8p U1 snRNP sedimented as a 13–14 S complex and not only Snu71p and Snu56p, but also Prp39p and Prp40p dissociated from the complex and remained at the top of the gradient. Snu65p behaved remarkably different, because it remained stably associated with the Δnam8p U1 snRNP even at 700 mM salt (data not shown). In comparison, wild-type U1 snRNP co-sedimented with all of the U1 snRNP proteins (except Npl3p and Snu65p) at 700 mM salt as the intact 1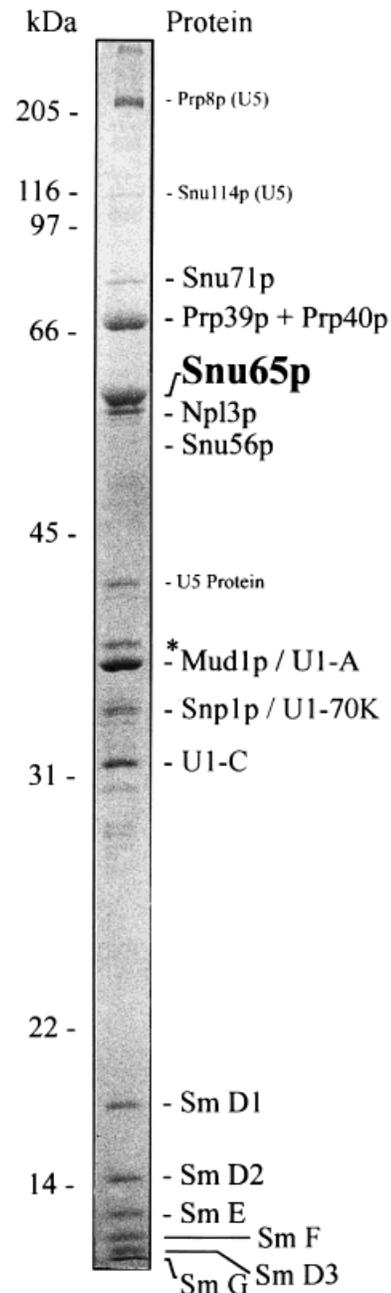7 S complex (data not shown). We therefore conclude that, at least in vitro, Nam8p has a stabilizing effect on the association of the yeast-specific U1 snRNP proteins Snu71p, Snu56p, Prp39p, and Prp40p. Surprisingly, the association of Snu65p is hyperstabilized in the absence of Nam8p.

CONCLUSIONS

Using a combination of biochemical, mass spectrometric, and genetic approaches, we have comprehensively analyzed the protein composition of yeast U1 snRNP. It is substantially more complex than its metazoan counterpart. Assuming a stoichiometric contribution of every protein and considering the unclear association of Npl3p, yeast U1 snRNP contains 16–17 proteins, of which 9 are particle-specific, and its predicted molecular weight is 809 or 764 kDa, respectively. In contrast, the metazoan counterpart consists of 11 proteins, of which only 3 are U1 snRNP-specific (Table 1). In addition, yeast U1 snRNA is almost four times larger than its metazoan counterpart and contains yeast-specific domains (Kretzner et al., 1987, 1990). During the biochemical two-step isolation, the U1 snRNP is tightly associated with 15 proteins either at 250 or 300 mM KCl (Neubauer et al., 1997). By mass-spectrometric peptide sequencing, we identified six common proteins, SmD1, D2, D3, E, F, and G, and nine specific proteins. These included the homologues of the metazoan U1-70K (Snp1p), U1-A (Mud1p), and U1-C, in addition to six proteins that are not found in metazoan U1 snRNP: Snu71p, Prp39p, Prp40p, Nam8p, Snu56p, and Npl3p (which is not exclusively associated with yeast U1 snRNP). Finally, by immunoprecipitation, we have shown that yeast U1 snRNP is also associated with an SmB protein.

The genetic approach revealed four yeast U1 snRNP-associated proteins, Mud1p, Mud15p/Nam8p, Mud10p/Snu56p, and Mud16p/Snu65p. The latter is a loosely associated U1 snRNP-specific protein that is not co-isolated with wild-type U1 snRNP at 250 mM

KCl, but remains stably associated in the absence of Nam8p.

Npl3p is associated weakly with snRNPs (but preferentially with U1). At this stage, it does not appear to act like the other bona fide U1 snRNP proteins and its presence in purified U1 snRNP is not unequivocal. However, it is also possible that the weak interaction between U1 snRNP and Npl3p is important at some specific stage of U1 snRNP function. Further studies will be required to solve this dilemma.

The biochemical and genetic approaches have led to congruent results and complement each other in a convincing manner. Emphasizing the potential inherent in our approach, even an extensive two-hybrid strategy using the U1 snRNP-specific Snp1p and Mud1p failed to reveal any of the novel U1 snRNP components (Fromont-Racine et al., 1997). The picture we can now draw of yeast U1 snRNP might well be definitive, although there might still be U1 snRNP proteins that have escaped both identification approaches. In particular, the genetics is far from complete, because many complementation groups contain only a single allele (Stutz et al., 1997). The biochemical results suggest the possibility of more bona fide U1 snRNP proteins that, like Snu65p/Mud16p, are only weakly associated with wild-type U1 snRNP in vitro.

Seven of nine of the yeast U1 snRNP-specific proteins are essential (only Nam8p and Mud1p are inessential for cell growth). This suggests an important role for these proteins in splice site definition. In contrast, it was shown that, in metazoa, high concentrations of certain SR-proteins can lead to U1 snRNP-independent splicing in vitro (Crispino et al., 1994; Tarn & Steitz, 1994). These proteins may functionally substitute for the additional yeast-specific U1 proteins. Alternatively, metazoan homologues of these proteins may exist and be more loosely associated with U1 snRNP than their yeast counterparts, i.e., in vitro biochemistry may identify them as non-snRNP-associated splicing factors. Consistent with this second possibility, two human proteins that share about 32% sequence identity with Nam8p and also contain three RBDs can be identified in the database, namely the nucleolysins, TIA-1 and TIAR. These proteins were found to be involved in apoptosis induced by cytolytic T-lymphocytes and were shown to trigger DNA fragmentation in the target cell nuclei (Tian et al., 1991; Kawakami et al., 1992). Interestingly, the *NAM8* deletion leads to the loss of DNA-doublestrand breaks during meiotic recombination (Nakagawa & Ogawa, 1997). Analogously, TIA-1 and TIAR could be splicing factors (both proteins interact with poly-A RNA, when overexpressed; Dember et al., 1996) that are involved in the expression of genes that function in DNA metabolism. A metazoan homologue of Prp40p might also exist, because BBP probably interacts with Prp40p in the commitment complex, and BBP has a human homologue, SF1 (Abovich &

TABLE 1. Protein components of human and yeast U1 snRNPs.

Protein class	*Homo sapiens*	App. M_r (kDa)	*Saccharomyces cerevisiae*	App. M_r (kDa)
Core proteins	SmG	9	SmG	9
	SmF	11	SmF	11
	SmE	12	SmE	12
	SmD1	16	SmD1	18
	SmD2	16.5	SmD2	15
	SmD3	18	SmD3	10
	SmB/B′	28/29	SmB	28
Specific proteins	U1-70K	70	Snp1p	34
	U1-A	34	Mud1p	37
	U1-C	22	yU1-C	32
			Snu56p/Mud10p	52
			(Npl3p)[a]	55
			Nam8p/Mud15p	57
			Snu65p/Mud16p/Prp42p	57
			Prp39p	69
			Prp40p	69
			Snu71p	77

[a]The nature of the association of Npl3p with the U1 snRNP remains unclear. For references, see Introduction.

Rosbash, 1997). Indeed, human and mouse ESTs that share significant sequence homology with Prp40p are found in the database (not shown; see also Abovich & Rosbash, 1997).

Why is the yeast U1 snRNP so much more complex than its metazoan counterpart? The metazoan spliceosome regulates splicing of an enormous variety of introns that are only moderately conserved in their consensus sequences. In addition, alternative and regulated splicing are of major importance, whereas these processes are almost nonexistent in yeast. Therefore, the metazoan U1 snRNP might be considered as a "core-activity" that needs to be differentially fine-tuned for splicing of particular introns by recruiting additional factors, more or less specific for these introns. The SR-family of metazoan splicing factors plays a crucial role in the definition of the splice sites, especially in regulated splicing (Fu, 1995; Manley & Tacke, 1996). On the contrary, the yeast U1 snRNP controls splicing of only a small number of introns (just about 255 are detected in the whole yeast genome). Therefore, rather few additional factors might be needed to help the yeast U1 snRNP, and these could well be integral components that need not be differentially recruited. Indeed, proteins that contain SR-dipeptides are associated with the yeast U1 snRNP (Snu71p and eventually Npl3p). However, the dipeptides found in these proteins are not part of extensive repeats as they are in metazoan SR-proteins. Therefore, although the yeast-specific U1 snRNP proteins and SR-proteins might have related functions, we expect that they act in a different manner.

Our work establishes, as demonstrated for the yeast U1 snRNP, a new approach for the characterization of complex biochemical particles. The remaining yeast snRNPs will be similarly analyzed and novel snRNP proteins will almost certainly be identified. Finally, this combination of biochemical and genetic analysis can be applied to splicing complexes as well as to snRNPs and should aid in understanding more dynamic features of snRNP structure and function.

MATERIALS AND METHODS

Isolation of yeast U1 snRNP and Δnam8p U1 snRNP, glycerol gradient centrifugation, and protein identification

Isolation of U1 snRNPs from strains BSY283 and AGY001 (lacking Nam8p, see below) was essentially as described (Neubauer et al., 1997), with minor changes. Isolations were performed in buffers containing 250 mM KCl (D250). U1 snRNPs bound to the Ni-NTA column were washed with 24 bed volumes D250 containing 10 mM imidazole and eluted with 8 bed volumes D250 containing 50 mM imidazole. Three 1-mL fractions containing U1 snRNP were obtained. Glycerol gradient centrifugation of wild-type and Δnam8p U1 snRNPs (performed at salt concentrations as indicated in the text) or total cell extracts (diluted with buffer G 200 to adjust the

glycerol content to 8%) and mass spectrometric analysis of U1 snRNP proteins were as described (Neubauer et al., 1997). To analyze silver-stained bands, we used a published protocol (Shevchenko et al., 1996).

Cloning and overexpression of Snu71p, Nam8p, Npl3p, and Snu56p; in vivo tagging of Snu65p, Nam8p, SmB, and SmF

The open reading frames *SNU71*, *NAM8*, *NPL3*, and *SNU56*, including the start and stop codons, were cloned by PCR from yeast genomic DNA using specific primers and Pfu DNA polymerase. The primers used were: for *SNU71* (upstream, 5′-GGCCGGGATCCATGAGGGATATTGTATTT-3′; downstream, 5′-GGCCGCTCGAGTCAGGTCCCCAAGCGAAA-3′); for *NAM8* (upstream, 5′-GGCCCGAATTCATGTCCTAT AAACAAACA-3′; downstream, 5′-GGCCCGTCGACCTCAA GCAAACATAAAACC-3′); for *NPL3* (upstream, 5′-GGCCGGG ATCCATGTCTGAAGCTCAAGAA-3′; downstream, 5′-GGCC GCTCGAGTTACCTGGTTGGTGATCT-3′); and for *SNU56* (upstream, 5′-GGCCGGGATCCATGCGCCCAAGAAGGAGA-3′; downstream, 5′-GGCCGCTCGAGTCAATGTTCTAATA TATT-3′). The upstream and downstream primers contained *Bam*H I and *Xho* I restriction sites (*Eco*R I and *Sal* I in the case of Nam8p), respectively, for subsequent fusion to *GST* by cloning into vector pGEX-4T1 (Pharmacia). DNA encoding the C-terminal 138 amino acids of Nam8p (between *Esp* I and *Sal* I) was removed from plasmid pGEX-4T1-*NAM8*, because truncated GST-Nam8p was expressed more easily in *Escherichia coli* (see also Fig. 2C). GST-fusion proteins were expressed in BL-21 cells after growing 2-L suspension cultures in LB-medium (containing 100 μg/mL ampicillin) at 37 °C to OD$_{600}$ = 0.8. Expression was induced using 1 mM IPTG at 30 °C for 4.5 h. GST-fusion proteins were purified according to the protocol supplied by Pharmacia.

The yeast SmB coding sequence and putative upstream promoter sequence was recovered by PCR from yeast genomic DNA using primers EM158, 5′-TTTGGATCCATGTATT GAATTTTACT-3′, and EM232, 5′-TCAGAGCTCTTTTTCTTTT AAAACCTGGTG-3′, digested with *Bam*H I and *Sac* I, and inserted together with a *Sac* I/*Hind* III fragment harboring the two IgG binding domains of protein A (Séraphin, 1995) into the pRS425 vector (Sikorski & Hieter, 1989), giving rise to plasmid pBS1361. The SmF-ProteinA fusion present in plasmid pBS806 (originally referred to as SmX3-ProtA) has been described previously (Séraphin, 1995). These plasmids were transformed in the yeast strain MGD453-13D (Séraphin et al., 1988) following the procedure of Ito et al. (1983).

To tag Nam8p and Snu65p, the sequence coding for two IgG-binding domains of the *Staphylococcus aureus* protein A protein together with the *Kluveromyces lactis URA3* marker (Langle-Rouault & Jacobs, 1995) was inserted in the genome downstream of, and in frame with, the *NAM8* and *SNU65* open reading frames by transformation with PCR fragments (Baudin et al., 1993; Puig et al., 1998). For *SNU65*, this fragment was generated by amplifying a *ProteinA–K. lactis URA3*-containing fragment from plasmid pBS1365 with oligonucleotides op31 (5′-CAGAGGAGATGGATACATTAGAG GAAATGTTTACTGAAGAACCTAAGCTGGAGCTCAAAAC-3′) and op32 (5′-CATTTACATATTTATACCTTTTAATAAATG ACAATGCCTTTTGGTACGACTCACTATAGGG-3′). This PCR

product was transformed into strains MGD453-13D (Séraphin et al., 1988) and BSY642 (see below). For *NAM8*, we followed a two-step PCR strategy (Puig et al., 1998) using oligonucleotides op4 (5′-CTTTTTCTTTCTTGGTATTT-3′) and op5 (5′-TGCTAGGATTTATTTGTGAG-3′) for the first amplification and oligonucleotides op5 and op6 (5′-GCAATGTT AAACCGTTTGGAACAAGGCAGTAATGGTTTTATGTTTAA GCTGGAGCTCAAAAC-3′) for the amplification of the first PCR ligated to Bsp120I-digested pBS1365. The resulting strains expressed the expected size proteins as revealed by western blot analysis. Correct integration of the PCR fragments was verified by genomic PCR using oligonucleotides OF-1 (5′-TATCAACGGTACCCTTAG-3′) and op34 (5′-AGTA TTTTCAAAGAGTGGC-3′) for *NAM8* tagging or OF-1 and op39 (5′-GCGGGAGTGATGAGG-3′) for *SNU65* tagging.

Preparation of antisera and western blot analysis

Rabbits were immunized every three weeks with 500 μg of purified GST-fusion proteins. Serum was taken from the rabbits 10 days after the third immunization and subsequently always 10 days after the injection of antigen. Western blot analysis was performed using the enhanced chemiluminescence (ECL) method, according to the protocol supplied by Amersham. The antisera were diluted as indicated in Figures 3A and 4.

Preparation of splicing extracts, immunoprecipitation, northern blot analysis, and primer extension

Yeast splicing extract was prepared from strains BSY283, AGY001, or BJ2168 (*MAT*a, *leu2*, *trp1*, *ura3*, *prb1-1122*, *pep4-3*, *prc1-407*, *gal2*) either by homogenizing cells in a Dounce homogenizer (Lin et al., 1985) or by grinding frozen cells under liquid nitrogen (Umen & Guthrie, 1995). Splicing extracts were dialyzed against buffer D50 containing 0.5 mM dithiothreitol (DTT) and 0.5 mM phenyl-methyl-sulfonyl-fluoride (PMSF). Immunoprecipitations were performed essentially as described (Lauber et al., 1996), only that 5 μL of antiserum or pre-immune serum or 10 μg of monoclonal antibody H20 (specific for the snRNA-m$_3$G-cap; Lührmann et al., 1982) were used to precipitate snRNPs from 50 μL splicing extract at salt concentrations as indicated in the text. For immunoprecipitation of protein A-tagged proteins, yeast mini-extracts were prepared as described (Séraphin & Rosbash, 1989). Incubation with rabbit IgG-agarose beads and RNA extractions from total extracts, supernatants, and pellets were done following a published protocol (Lygerou et al., 1994b). Buffers for immunoprecipitation and washes contained 150 mM NaCl except when otherwise stated (Fig. 5A). RNAs were detected by primer extension using primers specific for yeast U snRNAs (Séraphin & Rosbash, 1989; Lygerou et al., 1994a, 1994b). Primer extensions were separated on denaturing polyacrylamide gels, dried, and exposed to X-ray films with intensifying screen.

Synthetic lethal (MUD) screen and in vivo splicing phenotype of the *mud* mutants

The *mud* mutants were isolated through an enhancer screen (Liao et al. 1993; Stutz et al., 1997). The *MUD* genes were identified after transformation of the *mud* mutants with a wild-type genomic library, as described previously (Liao et al., 1993). Plasmid *MUD10*/p366 was constructed by inserting 5 kb of genomic DNA (*Aat* II/*Bgl* II, including the *MUD10* ORF) into vector p366 (*LEU*, *CEN*). *MUD15* coding sequence (2.3 kb) was amplified by PCR and cloned into vector p366 to give rise to *MUD15*/p366. The *CUP1* gene was knocked out from *mud10* and *mud15* strains as described previously (Lesser & Guthrie, 1993), giving rise to Δ*CUP/ mud10* and Δ*CUP/mud15*. The *CUP1* reporter gene was disrupted by HZ18, HZ12, or ACC introns (Jacquier et al., 1985; Pikielny & Rosbash, 1985; Legrain & Rosbash, 1989). The reporter genes were introduced into Δ*CUP/mud10* and Δ*CUP/ mud15* strains and the transformed strains were assayed on copper-containing plates.

Gene disruptions

SNU56/MUD10

The *KAN* gene was amplified by PCR with primers carrying sequences complementary to sequences upstream and downstream of the *MUD10* ORF. A wild-type diploid strain was obtained by crossing MGD353-13D (Séraphin et al., 1988) to MGD353-46D (*MAT*a, *trp1-289*, *ura3-52*, *leu2-3*, 112, *his3-Δ1*, *CYH^R*). This diploid strain was transformed with PCR fragments (Δ*mud10::KAN*) and selected for kanamycin resistance. Successful deletion of the *MUD10* gene was confirmed by Southern blotting. The heterozygous diploid was transformed with *MUD10*/p366 and sporulated again. When tetrads were dissected, some gave rise to four viable spores and co-segregation of *LEU* and *KAN* was observed, indicating that *MUD10* is essential.

Disruption of *SNU71* and *NAM8*

NAM8 and *SNU71* were cloned by PCR with primers specific for their complete 5′- and 3′-UTRs into vectors pRS306 (*URA3/NAM8*), pRS316 (*URA3/CEN/SNU71*), and pRS314 (*TRP1/CEN/SNU71*). Primers used for *NAM8*: upstream, 5′-GGGGGGGGTACCTGACGTTTCATAAGAATA-3′ (*Kpn* I); downstream, 5′-GGGGGGAGCTCGTTGATTTTATTTTGTGT-3′ (*Sac* I); for *SNU71*: upstream, 5′-GGCCCCTCGAGCAT AGATGTATAAATCAG-3′ (*Xho* I); downstream, 5′-GCCGC CCGCGGAGATGGGTATAATTAGGA-3′ (*Sac* II). *NAM8* was disrupted with the *URA3* gene between *Bam*H I and *Hpa* I restriction sites, removing 1,324 nt of the coding sequence. *SNU71* was disrupted between *Bgl* II and *Hin*d III sites with *URA3*, removing 821 nt of the coding sequence. The disrupted genes inclusive of their UTRs were cut from the vectors and used to transform the haploid yeast strain BSY283 (using Δ*nam8::URA3*; giving rise to strain AGY001) or diploid strain YPF1 (Fabrizio et al., 1997; using Δ*snu71::URA3*). Transformants that integrated the disrupted genes were selected on $-$URA plates and colonies were picked to prepare genomic DNA. Successful gene disruption was confirmed by Southern blot analysis and PCR. For *SNU71*, cells were streaked on sporulation plates, tetrads were dissected, and the spores were grown on YPD plates at 25 °C. Always only two spores were viable and *URA$^-$*, indicating that *SNU71* is essential for cell viability. The heterozygous parental strain (*SNU71/*

Δ*snu71::URA3*) was transformed with *SNU71* on plasmid pRS314 (*TRP1/CEN*). After sporulation and tetrad dissection, in 2 of 12 tetrads, all four spores were viable and the *URA3* and *TRP1* markers co-segregated. This demonstrated that *SNU71* is essential and that lethality of the cells was due to the disruption of *SNU71*.

SNU65 was disrupted in strain BSY320 (obtained by diploidization of MGD453-13D after transformation with an HO vector) following the strategy of Baudin et al. (1993), using the *K. lactis URA3* marker amplified with oligonucleotides op45 (5′-TTCAGAAAATACCCAGCTGACCATTAACTGAAG TAGCAAACATCTAAGCTGGAGCTCAAAAC-3′) and op32 (see above), resulting in strain BSY681. Correct integration was verified by PCR from genomic DNA with oligonucleotides op35 (5′-CTCCGCTTACATCAACAC-3′) and op34 (see above).

To construct the *SNU65-ProteinA/Δnam8* strain, *NAM8* was disrupted first in strain MGD453-13D (Séraphin et al., 1988) using the *TRP1* marker following a two-step PCR strategy (Puig et al., in prep.). Oligonucleotides op4 and op5 (see above) were used for the first amplification and oligonucleotides op5 and op27 (5′-ATATCCCGTCAAAAGTTCCTAATAC ATTGCGGGTACAAATGTCCAAGCTGGAGCTCAAAAC-3′) for the amplification of the first PCR ligated to *Xho* I–*Pvu* II-digested pBS1173 (Puig et al., 1998). The resulting strain, BSY642, was then used to insert the protein A tag downstream of *SNU65* as described above.

In vitro complex formation, native gel electrophoresis, and U1 snRNP blotting

The splicing extract preparation, ^{32}P-labeled substrate synthesis (RP51A-derived), spliceosome assembly with or without intact U2 snRNP, and native gel electrophoresis were performed as described previously (Séraphin & Rosbash, 1989). The gel was blotted as described previously and probed with ^{32}P-labeled U1-specific DNA probes (Legrain et al., 1988; Séraphin & Rosbash, 1989).

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