MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2787–2795 Vol. 16, No. 6
0270-7306/96/$04.00+0

Identification and Characterization of a Yeast Gene Encoding the U2 Small Nuclear Ribonucleoprotein Particle B″ Protein

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Received 20 February 1996/Returned for modification 19 March 1996/Accepted 20 March 1996

The inessential yeast gene *MUD2* encodes a protein factor that contributes to U1 small nuclear ribonucleoprotein particle (snRNP)–pre-mRNA complex (commitment complex) formation. To identify other genes that contribute to this early splicing step, we performed a synthetic lethal screen with a *MUD2* deletion strain. The first characterized gene from this screen, *MSL1* (*MUD* synthetic lethal 1), encodes the yeast homolog of the well studied mammalian snRNP protein U2B″. The yeast protein (YU2B″) is a component of yeast U2 snRNP, and it is related to other members of the U1A-U2B″ family, the human U2B″ protein, the human U1A protein, and the yeast U1A protein. It binds in vitro to its RNA target, U2 snRNA stem-loop IV, without a protein cofactor, and the target resembles more closely the U1 snRNA binding site of the human U1A protein than it does the U2 snRNA binding site of human U2B″. Surprisingly, the YU2B″ protein lacks a C-terminal RNA binding domain, which is conserved in all other family members. Possible functional and evolutionary relationships among these proteins are discussed.

The removal of introns from eukaryotic pre-mRNAs takes place in large complexes called spliceosomes. Spliceosomes are assembled on pre-mRNA in an ordered pathway that includes the addition of protein factors and four small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U5, and U4/U6 snRNPs. U1 snRNP recognizes the 5′ splice site, U2 binds to the branch point, and the U4/U5/U6 tri-snRNP is subsequently added to form a catalytic complex (9, 24, 26, 28, 29).

Extensive biochemical studies of metazoan splicing snRNPs have led to the characterization of their components. Each snRNP has one or two snRNAs, several specific proteins, and eight core Sm proteins common to all four snRNPs (26, 37). These biochemical studies as well as molecular cloning have shown that U2 snRNP contains two specific proteins, U2B″ and U2A′ (10, 42). U2B″ belongs to a large family of RNA-binding proteins which share a conserved motif, the RNA binding domain (RBD) (5, 16). In association with U2A′, U2B″ binds to U2 snRNA stem-loop IV with high affinity (6, 36).

In the yeast *Saccharomyces cerevisiae*, the general mechanism and machinery of splicing are quite similar to those in mammals. Although yeast U2 snRNA is six times larger than mammalian U2 snRNA, the sequences and structures of important regions of the molecule are conserved. These include the branch point recognition site, the Sm binding site, and stem-loop IV (3). Much of the "extra" sequence can be removed from the yeast U2 snRNA with little or no consequence, e.g., the molecule functions well with an internal deletion of about 900 nucleotides (27, 40). Moreover, it has been shown that human U2 snRNA can substitute for yeast U2 snRNA (41). All of these data indicate that the overall features of U2 snRNP are conserved from yeasts to humans, and yeast counterparts of mammalian U2A′ and U2B″ proteins are likely to exist.

Genetic tools have been successfully employed with *S. cerevisiae* to identify and study splicing factors (14, 15). Through the characterization of temperature-sensitive mutants and their suppressors, several U2 snRNP-associated factors (e.g., Prp9p, Prp11p, and Prp21p) have been cloned and characterized (2, 18, 35, 44). Another genetic approach, synthetic lethal screening, has been used to isolate mutations that enhance the phenotype of mild mutations in splicing genes (8, 21). From one such screen, the genes encoding two inessential U1 snRNP-associated proteins, *MUD1* and *MUD2*, were cloned (1, 21).

Mud1p is the yeast counterpart of the well studied metazoan U1 snRNP protein U1A, which has two RBDs. The N- and C-terminal RBDs are not very closely related, suggesting that they carry out distinct functions. U1A is closely related to the metazoan U2 snRNP protein U2B″, which also has two RBDs. The two N-terminal RBDs are extremely well studied from the RNA binding point of view; they are very similar in primary sequence and bind to similar stem-loop sequences on U1 and U2 snRNAs, respectively (36). However, the U2B″ N-terminal RBD does not bind in vitro to its target without the aid of another U2 snRNP protein, U2A′, whereas the U1A N-terminal RBD alone binds to its stem-loop target sequence with high affinity. The C-terminal RBDs of these two proteins (also well conserved between U1A and U2B″) do not contribute to this binding. Even their contribution to splicing is uncertain; there is evidence only that the C-terminal RBD of U1A participates in polyadenylation (23).

Mud2p has homology to the human U2AF65 protein, and it also participates in yeast commitment complex formation, the earliest stable splicing complex that includes U1 snRNP and pre-mRNA. It has been proposed that Mud2p functions as a bridge between U1 snRNP and U2 snRNP during prespliceosome formation, in part by interacting with the U2 snRNP-associated splicing factor Prp11p (1).

To identify additional protein factors that function in U2 snRNP addition and interact (directly or indirectly) with Mud2p, we conducted a synthetic lethal screening to isolate mutants that are inviable in the absence of *MUD2* (*MSL* genes as *MUD* synthetic lethals). From this screen, we identified a mutant strain that showed U2 snRNP defects, namely, lower levels of U2 snRNA and the inability of splicing extracts prepared from this strain to form prespliceosomes. The cloning of

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the gene and the characterization of its product are the subjects of this communication.

MATERIALS AND METHODS

Strains and plasmids. The yeast strain used in mutagenesis was constructed by deleting the *MUD2* coding sequence in the wild-type strain MGD 353-13D and replacing it with the *ADE2* gene. This strain also carries plasmid pXL79, containing the *MUD2* gene in the YCP50 vector (1).

UV mutagenesis was performed on individual cells spread on Ura$^-$ plates as previously described (21). Viable colonies (10% of mutagenized cells) were replicated to 5-fluoroorotic acid (5-FOA)-containing plates. Those that failed to grow on these selective plates were collected from a parallel replica on Ura$^-$ plates.

Cloning the *MSL1* gene. The strain, M2S6, obtained in this screening, characterized as containing low levels of U2 snRNA, was also temperature sensitive at 37°C. This mutant strain was crossed to an otherwise wild-type strain in which the *MUD2* gene was replaced with the *LEU2* marker. Tetrads were dissected, and the U2 levels were measured in all spores from four complete tetrads. The synthetic lethality and low U2 levels cosegregated at a 2:2 ratio, but the temperature-sensitive phenotype did not. A spore from this cross (2C2) was chosen for cloning the wild-type gene and for further experiments. The haploid strain 2C2 was transformed with a yeast genomic library constructed by P. Hieter in the *LEU2-CEN* vector p366 as previously described (21). Transformants were selected on Leu$^-$ plates and replica plated to 5-FOA plates. Total RNA was prepared from 20 selected transformants and used as a template for PCR amplification with *MUD2*-specific primers and for primer extension with a U2 snRNA-specific oligonucleotide and reverse transcriptase. Samples from only 5 of the 20 colonies were positive for amplification with *MUD2* primers. Like the 2C2 strain, all five had low levels of U2 snRNA when examined by primer extension. The other 15 samples had wild-type levels of U2. Plasmids were rescued from these 15 colonies by transformation into *Escherichia coli*. These represented four different plasmids with overlapping restriction fragments. The smallest common 1.8-kb *Bam*HI-*Hin*dIII fragment was subcloned, used to rescue the synthetic lethality of 2C2, and sequenced. A search of the database indicated that this sequence is contained within the previously deposited sequence of cosmid 9167 from *S. cerevisiae* chromosome IX (accession no. Z37996) and encodes a hypothetical protein of 12.8 kDa (43). PCR primers specific for this putative open reading frame (ORF) were designed and used with the femtomole sequencing kit (Promega) to sequence the PCR product obtained from the mutant strain 2C2.

Deletion of the 12.8-kDa ORF. A plasmid (pU2B″KO) in which the *LEU2* gene is flanked by the 770 nucleotides immediately upstream of the *MSL1*-initiating ATG and the 660 nucleotides immediately downstream of the termination codon, effectively deleting the entire 12.8-kDa ORF, was constructed. Convenient unique *Bam*HI and *Sph*I sites were created by PCR to generate a linear fragment for yeast transformation and one-step gene disruption (33). The wild-type haploid strain MGD 353-13D was transformed to leucine prototrophy, and successful deletion of the 12.8-kDa ORF was determined by PCR with a battery of specific primers. Analysis of U1 and U2 snRNA levels was also performed by cDNA synthesis with reverse transcriptase and specific primers. The haploid strain U2B″KO-LEU was thus generated.

Plasmid Gal-U2B″HA was constructed by inserting the hemagglutinin (HA) epitope after the last amino acid of the 12.8-kDa ORF. The tagged coding sequence was inserted in place of the *MUD2* coding sequence in plasmid Gal-MUD2HA (1). Expression of the tagged protein is driven by the Gal regulatory sequences. A mutant plasmid, Gal-U2B″ mutant, was constructed as described above, except that the coding region was amplified from the genomic DNA of mutant strain 2C2 in order to recreate precisely the amino acid sequence of the truncated protein.

Plasmid GEX-U2B″ was constructed by introducing *Bam*HI and *Sal*I sites at the 5′ and 3′ ends of the coding sequence of *MSL1* and cloning it into pGEX 4-T for the production and purification of glutathione *S*-transferase (GST)–U2B″ protein from *E. coli.*

In vitro biochemical assays. The preparation of splicing extracts, analysis of splicing complexes, and reverse transcription were done as described previously (1). The absolute level of spliceosome and commitment complex formation is always somewhat variable between different extract preparations, in part because of differences in cell density and growth media. This is best controlled by preparing extracts from all strains at the same time.

Primers. U snRNA primers complementary to U1 (nucleotides 113 to 135), U2 (nucleotides 100 to 120), U4 (nucleotides 70 to 91), and U6 (nucleotides 27 to 54) were labeled at their 5′ ends and used for primer extensions with reverse transcriptase as previously described (1).

Immunoprecipitations. Immunoprecipitations were performed with Gamma-Bind Plus Sepharose beads and the 12CA5 monoclonal antibody as previously described (4), except that both the binding and washes were carried out in NET-150 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Nonidet P-40).

For the determination of salt stability, incubations were done in NET-100 and washes were done in NET-100, -200, -300, or -400. (Each number indicates the NaCl concentration.)

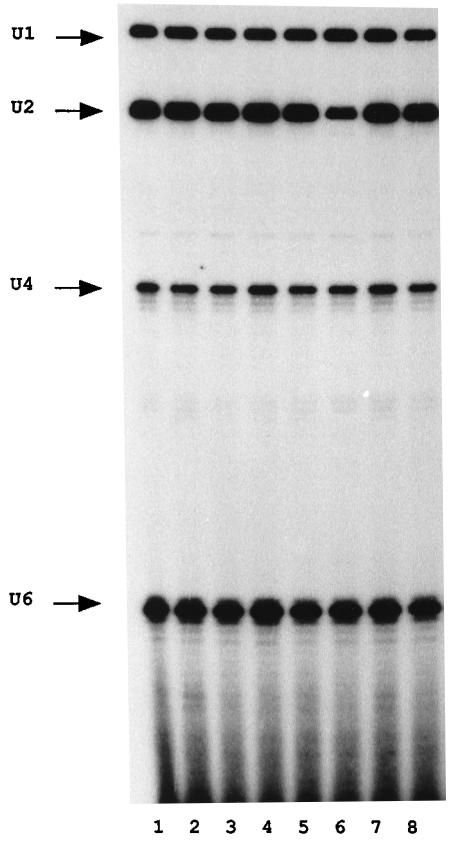


FIG. 1. snRNA analysis of the candidate mutants synthetic lethal with *MUD2*. Total RNA was extracted from the different strains, and equal amounts were analyzed by primer extension with reverse transcriptase and oligonucleotides complementary to U1, U2, U4, and U6 snRNAs. Lanes 1 through 6, mutant candidates; lane 7, M2-A, the *MUD2* deletion parental strain used for mutagenesis; lane 8, wild-type strain MGD 353-13D from which the *MUD2* gene was deleted.

Native gel mobility shift assay. The GST-U2B″ fusion protein was overexpressed in *E. coli* BL21 and purified through a reduced glutathione column. When stained with Coomassie brilliant blue, the purified protein appeared to be at least 90% pure on a sodium dodecyl sulfate-polyacrylamide gel (data not shown). RNA oligonucleotides, the sequences of which are shown in Fig. 6, were chemically synthesized and labeled with [γ-^{32}P]ATP. A control unrelated duplex with the sequence CUC GUC GAC GUG was also synthesized and ^{32}P labeled. There was no detectable degradation of labeled RNA oligonucleotides, as assayed on a denaturing polyacrylamide gel. Different amounts of purified GST-U2B″ fusion protein were incubated with 0.5 ng of RNA oligonucleotides in 10-μl reaction mixtures, containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, and 0.1 μg of tRNA per μl. After a 30-min incubation at room temperature, samples were electrophoresed at 150 V for 5 h on 7.5% polyacrylamide native gels with 0.5× Tris-borate-EDTA buffer. The affinity-purified GST protein was used as a negative control; it did not gel shift any of the RNA oligonucleotides used in the above experiments (data not shown).

Nitrocellulose filter binding assay. ^{32}P-labeled RNA oligonucleotides (10^5 cpm [about 0.05 ng]) were incubated with increasing amounts of GST-U2B″ fusion protein at room temperature for 30 min in 50 μl of buffer A (10 mM Tris-HCl [pH 6.8], 2 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 0.1 μg of tRNA per μl, 0.1 μg of bovine serum albumin per μl). The binding reaction mixtures were filtered through nitrocellulose under vacuum, and the membrane was washed once with buffer A prior to exposure on a PhosphorImager screen. The signals were quantitated in a Bio-Rad molecular imager system.

The competition assay was done as described above, except that GST-U2B″ protein (10 ng/μl) was mixed with 10^5 cpm (0.05 ng) of ^{32}P-labeled YU2 RNA oligonucleotide in the presence of increasing amounts of different cold RNA oligonucleotides (YU2, M1, or M2).

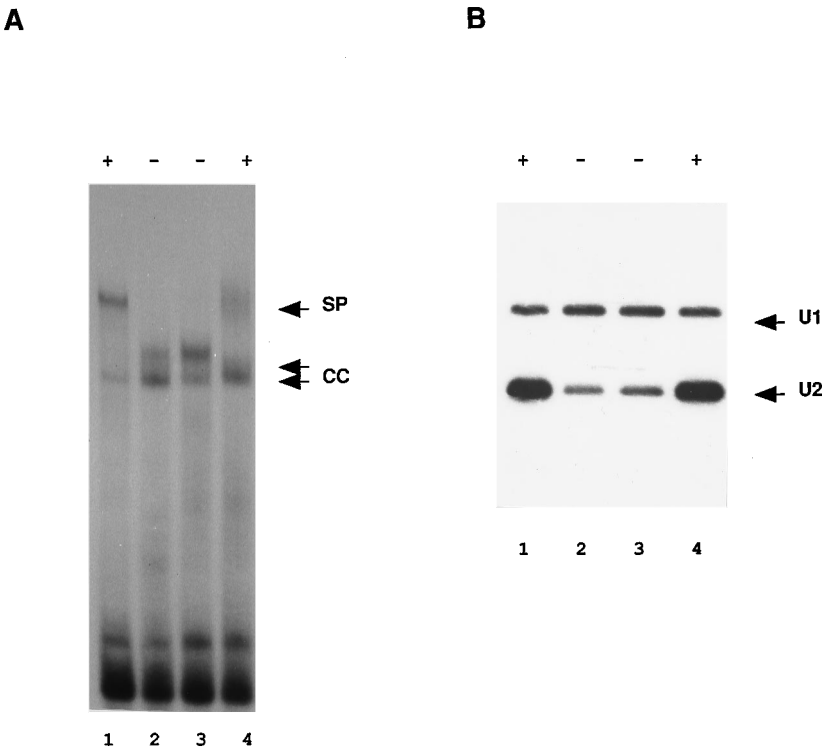


FIG. 2. Rescue of the biochemical phenotypes of the M2S6 mutant strain. (A) Restoration of spliceosome assembly in vitro. Splicing extracts were prepared from the strains indicated below. Standard splicing reactions were performed with radioactively labeled pre-mRNA substrate, and the complexes were analyzed by native gel electrophoresis. The positions of commitment complexes (CC) and spliceosomes (SP) are indicated on the right. Lane 1, the parental strain for mutagenesis; lane 2, mutant strain M2S6; lane 3, strain 2C2 rescued for viability on 5-FOA plates with a *LEU2* plasmid containing the *MUD2* gene; lane 4, strain 2C2 rescued for viability on 5-FOA plates with a *LEU2* plasmid containing the YIB9w sequence (*MSL1*). +, wild-type YU2B″ is present in the strain; −, the absence of wild-type YU2B″. (B) Restoration of U2 snRNA levels. Analysis of the cDNA products synthesized from total RNA with oligonucleotides complementary to U1 and U2 snRNAs. The lanes are the same as in panel A. The positions of the U1 and U2 snRNAs are indicated on the right.

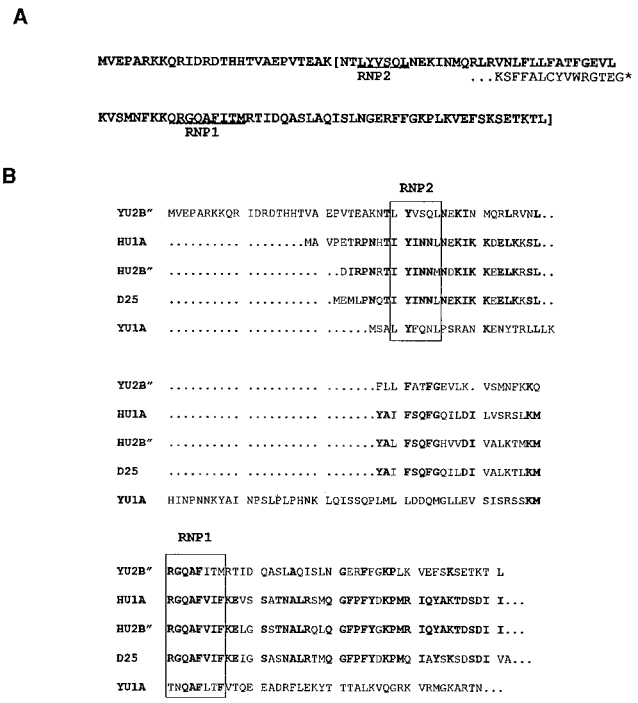
RESULTS

Synthetic lethal screen and biochemical phenotype of the mutant. A strain in which the *MUD2* gene was deleted and replaced with the *ADE2* selectable marker was constructed. A wild-type copy of the *MUD2* gene was introduced on a *URA3*-marked centromeric plasmid. Cells were UV irradiated to 10% viability, and 34,000 colonies were examined by replica plating on 5-FOA plates. Twelve colonies failed to grow on these selective plates, suggesting that they were dependent on *MUD2* function. We analyzed the U1, U2, U4, and U6 snRNA levels in total RNA prepared from six candidate strains and compared them with the levels in the parental strain. Strain M2S6 showed a profile with a low U2 snRNA level (Fig. 1, lane 6), suggesting that U2 snRNP assembly or turnover is aberrant in this mutant strain.

We then prepared in vitro splicing extracts from M2S6 and examined complex formation with a wild-type pre-mRNA substrate. After depleting the extracts of U2 snRNP activity by oligonucleotide-directed RNase H digestion (19, 38), commitment complex formation was assayed; the mutant extract was indistinguishable from a wild-type extract (data not shown). However, in a complete reaction, no spliceosome formation was observed in the mutant extract, suggesting that U2 snRNP addition was not normal (Fig. 2A; compare lane 2 with lane 1).

Cloning and phylogenetic comparisons. A yeast genomic library in a *LEU2*-marked centromeric vector was transformed into the mutant strain, 2C2, derived from a cross of M2S6 to a nonmutagenized strain as described in Materials and Methods. Transformants were selected for the suppression of synthetic lethality. Of 20 viable colonies selected on 5-FOA plates, 5 carried plasmids containing the *MUD2* gene. The remaining 15 contained four overlapping DNA fragments. DNA sequencing of the smallest common fragment revealed a single ORF identical to that encoding a putative 12.8-kDa ORF, YIB9w (43) (Fig. 3A). A 1.8-kb DNA subclone containing this gene (*MSL1*) rescued the biochemical phenotypes of 2C2 (Fig. 2, lanes 4); the *MUD2* gene did not (Fig. 2, lanes 3).

Sequencing of the endogenous copy of the *MSL1* (Y1B9w) ORF in the 2C2 mutant strain indicated an early frameshift in the coding region, presumably resulting in a null allele (*msl1*) (Fig. 3A), as discussed below. On the basis of sequence comparisons, Voss et al. suggested that this ORF is the yeast U2B″ homolog (43). Our own more recent search indicated that the mammalian and plant U1A-U2B″ family members are indeed the closest relatives in the database. In addition, the search identified the *Drosophila* D25 protein (13) (which is itself closely related to the U1A and U2B″ proteins [see Discussion]). However, the search did not identify the yeast U1A protein. This is due in part to the extensive additional loop that this protein contains between the alpha 1 and beta 2 regions of its N-terminal RBD (21). When this loop is deleted and replaced with the four amino acids present at this location in human U1A, the modified yeast U1A sequence identifies all of the family members, including (eventually) the YIB9w ORF. Taken together with the data shown below, this search indicates that the YIB9w ORF encodes yeast U2B″ protein (YU2B″).



C

	HU1A	HU2B	D25	YU2B	YU1A
HU1A	1.0000	0.9022	0.8495	0.5171	0.3478
HU2B		1.0000	0.8901	0.4776	0.3153
D25			1.0000	0.5025	0.3122
YU2B				1.0000	0.2589
YU1A					1.0000

FIG. 3. Sequence analysis of the hypothetical 12.8-kDa protein. (A) The hypothetical 12.8-kDa protein coded by the YIB9w gene ends immediately after the single RBD (shown in brackets). The highly conserved motifs RNP2 and RNP1 are underlined. The frameshift mutation in strain 2C2 leads to a truncated protein (indicated by an asterisk). The sequence changed by the frameshift is shown below the first line of the wild-type sequence. (B) The amino acid sequence coded by YIB9w (YU2B″) was aligned with the sequences of human U1 snRNP protein A (HU1A), human U2 snRNP protein B″ (HU2B″), *Drosophila* D25 protein (D25), and yeast U1 snRNP protein A (YU1A) by using the GCG70 PileUp program. The homologous region is limited to the N-terminal RBD of HU2B″, HU1A, D25, and YU1A. The C-terminal regions of these four proteins are not shown. The amino acid residues that are highly conserved among these five proteins are shown in bold. (C) On the basis of the multiple sequence alignment, the genetic distance between each pair of proteins was measured by the Genetics Computer Group DISTANCES program. The distance value is the number of matches between each sequence pair divided by the sequence length. The highest and lowest scores for YU2B″ protein are underlined.

YU2B″ is much smaller than the other family members, and a multiple sequence alignment shows that it contains only the N-terminal RBD with a modest N-terminal extension (Fig. 3B). All other family members, including yeast U1A, contain a second (C-terminal) RBD. Not surprisingly, human U1A, human U2B″, and D25 are much more similar to each other than to the two yeast proteins (Fig. 3B and C). The latter are also quite distant from each other; indeed, the Genetics Computer Group DISTANCES program suggests that YU2B″ is more closely related to the metazoan proteins than to yeast U1A (Fig. 3C). Although this may reflect weaknesses of the program (see Discussion), YU2B″ is certainly more diverged from yeast U1A than human U2B is from human U1A″ (Fig. 3C).

We proceeded to generate a true null allele by deleting the complete 12.8-kDa ORF and replacing it with the *LEU2* gene (see Materials and Methods). A haploid strain (U2B″KO-LEU or Δ*MSL1*) was obtained, and it had no obvious growth disadvantage compared with a wild-type strain. U2 snRNA levels are lower in the knockout strain than in the wild-type parent strain (data not shown) (i.e., similar to what was originally observed in the M2S6 mutant strain [Fig. 1]).

We prepared splicing extracts from the knockout strain and examined spliceosome formation (Fig. 4). There is almost no detectable spliceosome formation in the knockout extract compared with that in a wild-type extract; only commitment complex formation is detectable (Fig. 4; compare lanes 1 and 2). This is identical to what was observed in the original mutant strain (Fig. 2A). Spliceosome formation was restored in extracts from a rescued strain, i.e., the disrupted strain transformed with plasmid Gal-U2B″HA and grown in galactose (Fig. 4, lane 4) (see Materials and Methods); growth in glucose gave rise to much lower levels of spliceosome formation (lane 3), indicating that the effects on spliceosome formation are due to the presence or absence of the *MSL1* gene product. To verify that the phenotype of the 2C2 mutant strain was also due to the absence of the wild-type *MSL1* product, we cloned the *msl1* mutant gene from this strain and expressed it under Gal control (see Materials and Methods). There was little or no

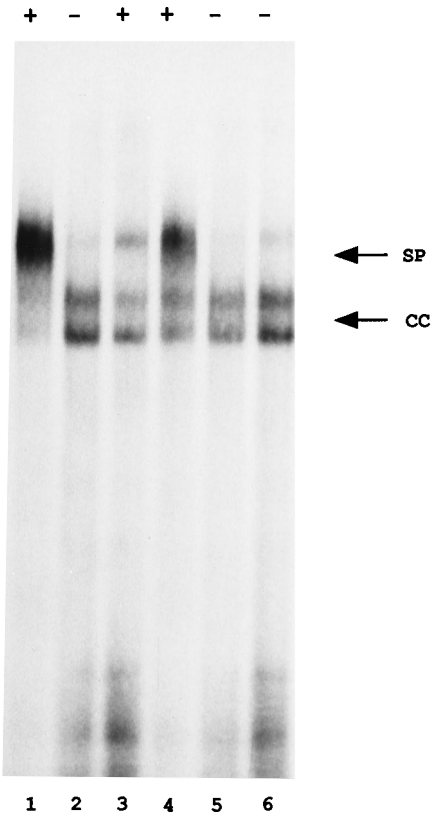


FIG. 4. Spliceosome assembly in the YU2B″ deletion strain. Lane 1, wild-type strain MGD 353-13D; lane 2, the YU2B″ knockout strain; lanes 3 and 4, the same strain as in lane 2 transformed with plasmid Gal-U2B″HA grown in glucose and galactose, respectively; lanes 5 and 6, the YU2B″ knockout strain transformed with plasmid Gal-U2B″ mutant grown in glucose and galactose, respectively. The positions of commitment complexes (CC) and spliceosomes (SP) are indicated on the right.

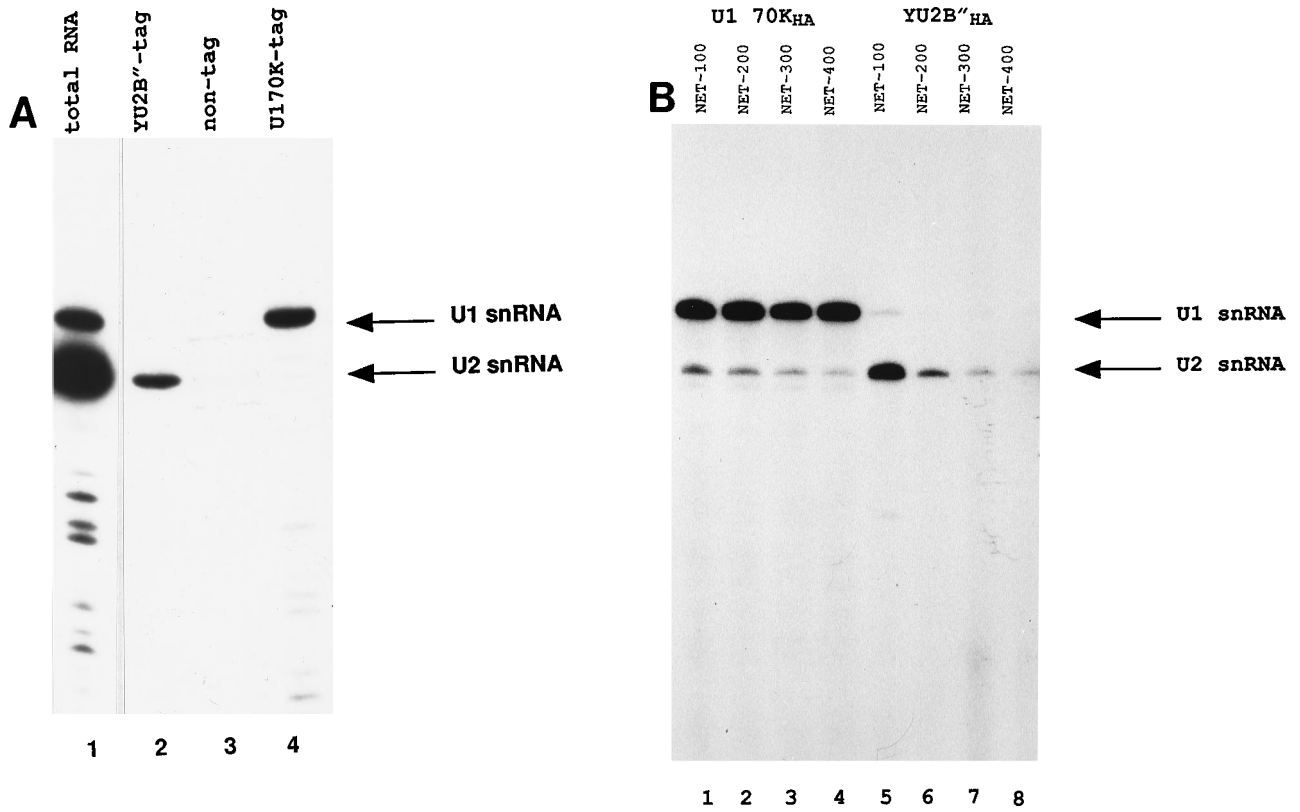


FIG. 5. Immunoprecipitation of snRNAs. (A) Splicing extracts from the indicated strains were incubated with the 12CA5 monoclonal antibody, and the immune complexes were isolated on GammaBind Plus Sepharose beads as described in Materials and Methods. The RNA was extracted and used as a template for cDNA synthesis with oligonucleotides complementary to U1 and U2 snRNAs. Lane 1, 20% of total extract RNA; lanes 2 through 4, immunoprecipitated RNAs from Gal-U2B″HA strain (YU2B″-tag), a non-HA-tagged control (non-tag), and a U1 70K$_{HA}$-tagged strain (U170K-tag), respectively. (B) Antibody and splicing extract incubation was done in NET-100 buffer, and the complex isolated on GammaBind beads was washed three times with NET-100, -200, -300, or -400, as indicated above each lane. Lanes 1 through 4, U1 70K$_{HA}$ extract; lanes 5 through 8, Gal-U2B″HA (YU2B″$_{HA}$) extract.

spliceosome formation in extracts from this putative mutant rescued strain (Fig. 4, lanes 5 and 6), indicating that the truncated protein is not functional.

We conclude that the *MSL1* gene encodes an inessential protein. In its absence, in vivo U2 snRNA levels are decreased. There is also less robust U2 snRNP addition during in vitro spliceosome assembly. The deletion strain phenotypes are similar if not identical to those of the original mutant strain 2C2, which has a very truncated version of this protein. The phenotypes and database searches make it very likely that the gene encodes the *S. cerevisiae* version of the U2 snRNP protein U2B″ (YU2B″).

Immunoprecipitations. To verify that there is direct interaction between the YU2B″ protein and U2 snRNP, immunoprecipitation experiments were performed with the HA epitope-tagged version of this protein expressed under galactose control. The tagged protein also rescues the synthetic lethality of the *msl1*-Δ*MUD2* combination (data not shown). As predicted, anti-HA antibodies specifically immunoprecipitated U2 snRNA from this YU2B″$_{HA}$ extract (Fig. 5A, lane 2). The immunoprecipitation is considerably less efficient than is that of the positive control, in which approximately 20% of U1 snRNA is immunoprecipitated by an HA-tagged version of the yeast U1 70K protein (U1 70K$_{HA}$) (Fig. 5A, lane 4). The difference in immunoprecipitation efficiency is due in part to the qualitative features of the interactions between the proteins and RNAs; the association of YU2B″$_{HA}$ to U2 snRNA is more salt sensitive than is that of U1 70K$_{HA}$ to U1 snRNA (Fig. 5B).

In vitro binding assays. To further characterize YU2B″, we examined its in vitro binding properties. U2 snRNA stem-loop IV is the binding site of the protein's N-terminal RBD in vertebrate systems, and a putative yeast stem-loop IV is present at approximately the correct location in yeast U2 snRNA (40). It resembles human U2 snRNA stem-loop IV and human U1 snRNA stem-loop II, the binding site of the closely related human U1A protein (Fig. 6A). We overexpressed a GST-YU2B″ fusion protein in *E. coli*, and the purified protein was assayed for its ability to bind to (i.e., to gel shift) several RNA oligonucleotides (Fig. 7A). Consistent with the notion that yeast U2 stem-loop IV is its natural binding site, YU2B″ bound well to this oligonucleotide (Fig. 7A, lane 3). It also bound to human U1 stem-loop II, which resembles yeast U2 stem-loop IV and is the natural binding site for the human U1A protein (Fig. 7A, lane 6). YU2B″ bound much more poorly to human U2 stem-loop IV and to a random RNA duplex that served as a negative control (Fig. 7A, lanes 9 and 12, respectively). Apparently, in vitro RNA binding by the YU2B″ protein does not require an A′ protein.

As the human U2 stem-loop IV sequence differs from that of the comparable yeast sequence at several critical residues, we designed two additional oligonucleotides with relevant single or double mutations in the yeast U2 stem-loop IV sequence (see below) (Fig. 6B). YU2B″ bound both mutant RNA sequences poorly, i.e., the mutants displayed at least 10-fold decreases in binding affinity compared with that of the wild-type U2 stem-loop IV sequence (Fig. 7B; compare lanes 4, 8, and 10).

To verify these conclusions, a nitrocellulose filter binding

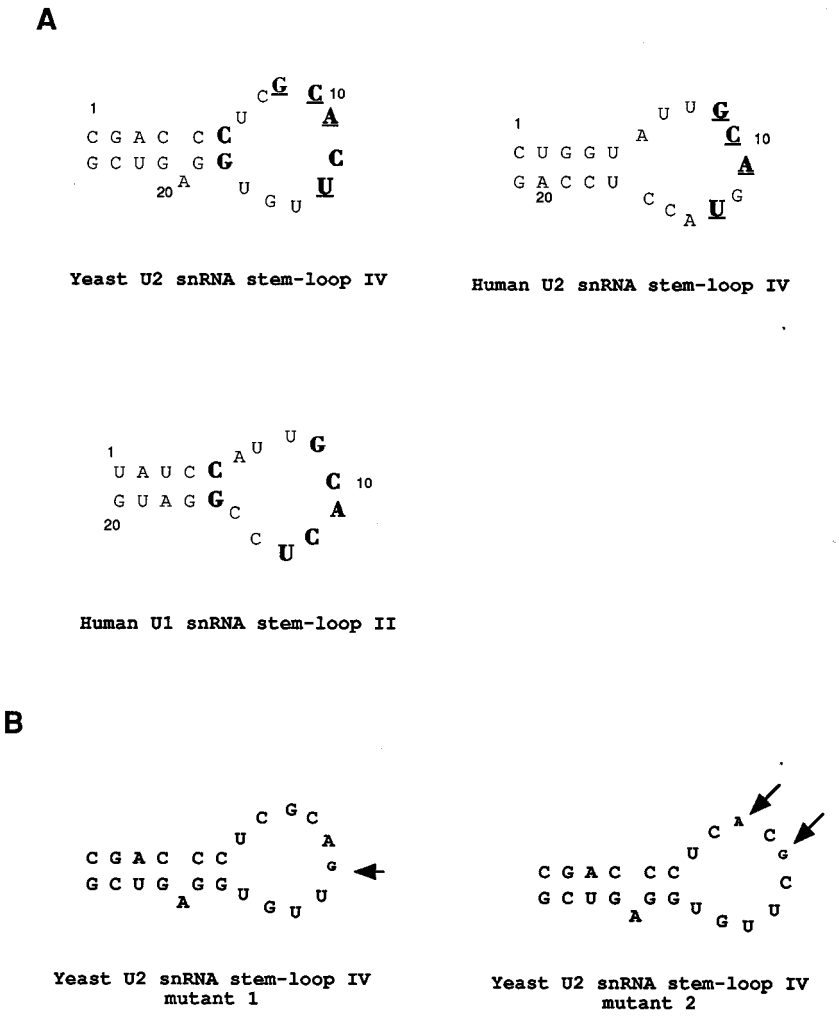


FIG. 6. Sequence of yeast U2 snRNA stem-loop IV. (A) The sequence and structure of yeast U2 snRNA stem-loop IV are compared with those of its close relatives, human U2 snRNA stem-loop IV and human U1 snRNA stem-loop II. The nucleotides conserved between yeast U2 snRNA stem-loop IV and human U1 snRNA stem-loop II are shown in bold. The nucleotides conserved between yeast and human forms of U2 snRNA stem-loop IV are underlined. (B) Two yeast U2 snRNA stem-loop IV mutants are shown; the altered nucleotides, in smaller letters, are indicated by arrows.

assay with the same protein and RNA oligonucleotides was also utilized. As expected, point mutations in yeast U2 snRNA stem-loop IV (M1 and M2) had strong negative effects on protein-RNA interaction (Fig. 8A). The oligonucleotides were also compared by competition. Cold M1 and M2 competed poorly with ^{32}P-labeled wild-type yeast U2 stem-loop IV for YU2B″ binding (Fig. 8B). The results indicate that the YU2B″ protein binds to its target stem-loop IV sequence in vitro and that loop residue C-12, as well as G-9 or A-11, is important for YU2B″ binding.

DISCUSSION

The inessential yeast factor Mud2p bears some resemblance, in sequence as well as function, to the essential mammalian splicing factor U2AF65. Both proteins contribute to early steps in splicing complex formation by associating with U1 snRNP complexes and by contributing to subsequent U2 snRNP addition (1, 45, 46). To identify other factors that affect early splicing complex formation, we searched for mutations in other genes that were lethal in a *MUD2* knockout strain. This strategy led to the identification of an inessential yeast gene, *MSL1*, that encodes the U2 snRNP-specific protein YU2B″.

We had previously demonstrated a synthetic lethal interaction between a deletion of *MUD2* and a temperature-sensitive allele of *PRP11*, which also encodes a U2 snRNP-associated protein. Moreover, we had described a physical interaction between these two proteins in the yeast two-hybrid system, suggesting that a direct interaction between them constitutes a bridge between U1 and U2 snRNPs during prespliceosome formation (1). It is attractive to imagine that the synthetic lethality between *MUD2* and *MSL1* might also reflect a physical interaction between the two proteins during spliceosome formation. We have, however, failed to detect an interaction between them in the two-hybrid system (data not shown), making it more likely that their interaction is indirect.

Since U2 snRNA is essential (40), the *MSL1* gene is inessential, and the Δ*MSL1* strain is without a marked growth phenotype, U2 snRNP must be functional without the U2B″ protein. However, U2 snRNP addition was severely compromised in extracts derived from the mutant and the deletion strains (Fig. 2 and 4). The in vitro phenotype could be due to

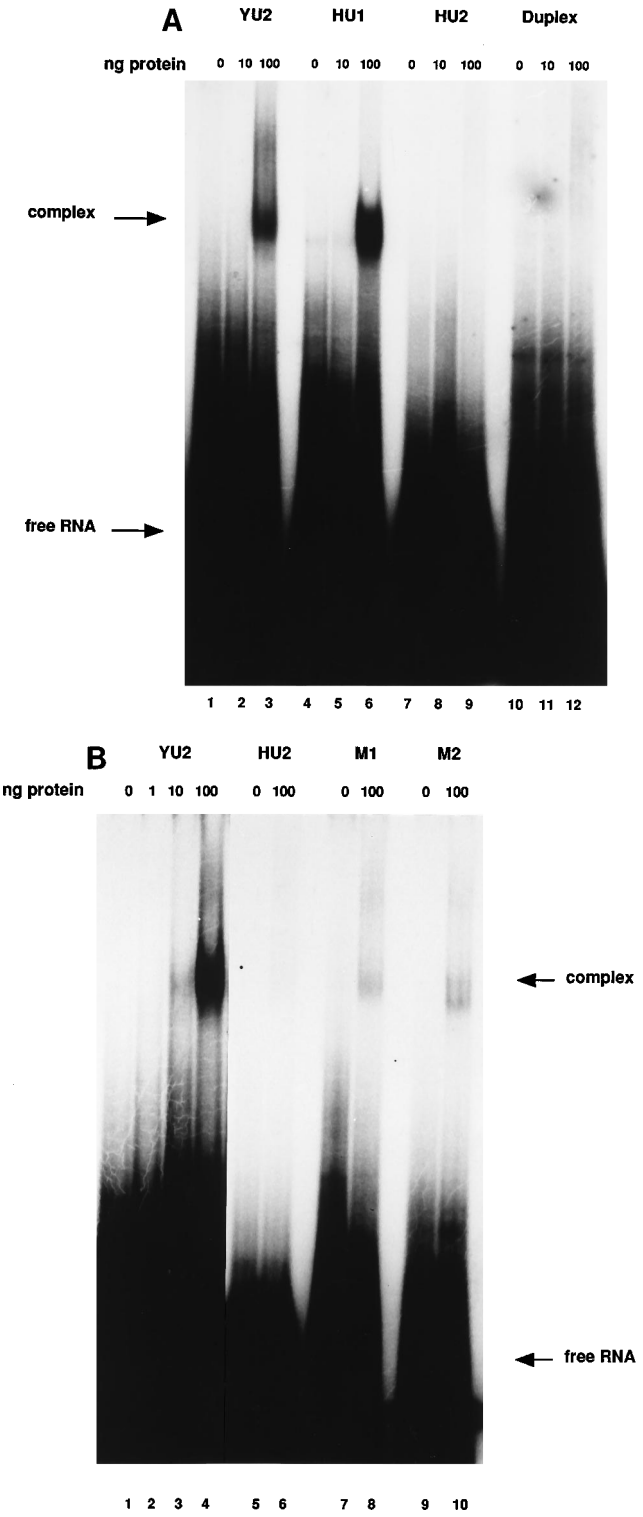


FIG. 7. YU2B″ protein binds to yeast U2 stem-loop IV in a sequence-specific manner. (A) The binding of GST-YU2B″ fusion protein to different RNAs was assayed by native gel mobility shift. Samples (10 μl) contained the amounts of protein (in nanograms) indicated. YU2B″ binds to yeast U2 (YU2) snRNP stem-loop IV (lanes 1 through 3) and human U1 (HU1) snRNA stem-loop II (lanes 4 through 6), but not human U2 (HU2) snRNA stem-loop IV (lanes 7 through 9) or a nonrelated RNA duplex (lanes 10 through 12). (B) The binding of YU2B″ to two YU2 snRNA stem-loop IV mutants (M1 and M2 [Fig. 6B]) was assayed by native gel mobility shift. Wild-type YU2 snRNA stem-loop IV was used as a positive control (lanes 1 through 4), and HU2 snRNA stem-loop IV was used as a negative control (lanes 5 and 6). Samples (10 μl) contained the amounts of protein (in nanograms) indicated. At the same concentration, YU2B″ binds much more weakly to M1 (lane 8) and M2 (lane 10) than to the wild-type sequence (lane 4). The positions of the complex and of free RNA are indicated beside each gel.

the approximately threefold decrease in U2 snRNA levels (estimated by eye) in the mutant or the deletion strain (Fig. 2B). Genetic depletions of U2 snRNA have been carried to even lower levels with little or no influence on growth rates (38), and it would not be surprising if in vitro splicing is more sensitive to a decrease in U2 snRNP levels than is in vivo splicing. Alternatively, the ΔB″-U2 snRNP could function less well without the B″ protein. in vivo snRNP function could be less markedly affected, or the growth rate could be a rather insensitive indicator of the splicing efficiency decrease (20). In either case, the reduced quantity or quality of the ΔB″-U2 snRNP is probably responsible for the synthetic lethal interaction with the *MUD2* deletion.

The results of immunoprecipitation experiments confirm that the protein is associated with U2 snRNP. However, immunoprecipitation of U2 snRNA is not complete. We have tagged several U1 and U2 snRNP proteins with the HA epitope. Approximately 20% of U1 snRNA is immunoprecipitated with the tagged U1 70K protein; this value is the highest we have achieved. It is not known why these values are not higher. The reason may be technical in nature, or only a fraction of U1 snRNPs may contain the relevant snRNP protein. Immunoprecipitation of U2 snRNA by the tagged U2B″ is also compromised by the salt sensitivity of the protein-RNA interaction. However, this might be affected by the tag; i.e., we have not used different epitopes, we have not placed them in different locations, and we do not know the salt sensitivity of the interaction between wild-type U2B″ and U2 snRNA. Despite these uncertainties, the sequence conservation, as well as the rest of the biochemical and genetic characterization, indicates that YU2B″ is a U2 snRNP component.

The target sequence of vertebrate U2B″ is U2 snRNA stem-loop IV, and it is quite conserved in sequence and position between yeasts and metazoans. Previous work has shown that this region of the yeast molecule is dispensable for splicing and for growth (27, 41). Similar conclusions have been drawn for metazoan U2 snRNA (12, 31), and these observations are consistent with the lack of a dramatic growth phenotype for the *MSL1* null mutant strain. On the other hand, deletion or mutagenesis of stem-loop IV caused defects in U2 snRNA 3′ end processing (40, 41). We examined the U2 snRNA 3′ ends in the 2C2 strain and found a U2 snRNA RNase protection pattern identical to that of the wild-type strain (data not shown). This suggests that the previously reported defect in 3′ end processing is not uniquely due to the absence of the YU2B″ protein.

Consistent with the prediction that the target sequence of the yeast protein is also stem-loop IV, YU2B″ binds in vitro to an oligonucleotide designed to mimic this target. It also binds to the human U1A target, human U1 snRNA stem-loop II, but not to the human U2B″ target, human U2 stem-loop IV (Fig. 7A). When comparing these three target sequences, we noticed that yeast U2 stem-loop IV resembles more closely the human U1A target than it does the human U2B″ target (Fig. 6A). For example, position 12 of yeast U2 stem-loop IV is occupied by a C, as in human U1 stem-loop II, rather than the G of human U2 stem-loop IV. Moreover, it is known that C-12 of human U1 stem-loop II makes a major contribution to the binding affinity of human U1A (36). On the basis of these studies and the mutagenesis data for human U1 stem-loop II

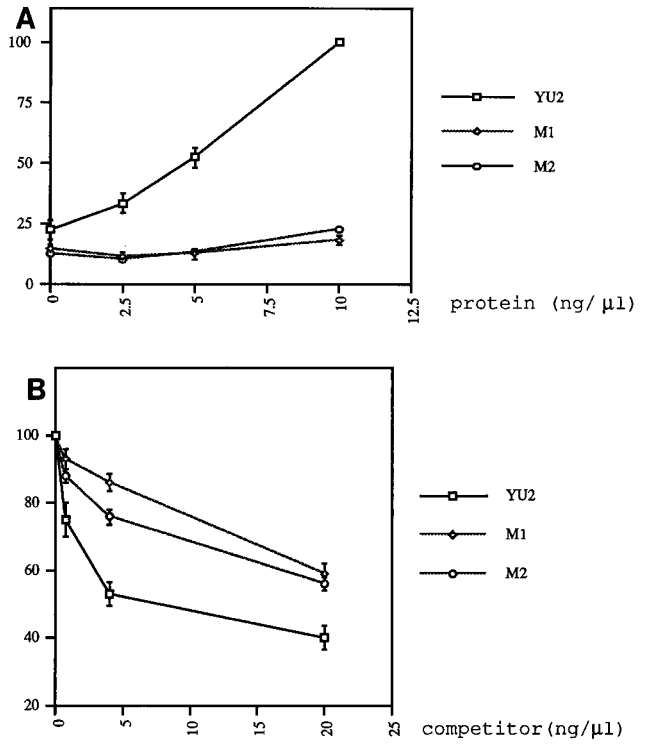


FIG. 8. Nitrocellulose filter binding. (A) The binding of GST-YU2B″ fusion protein to ^{32}P-labeled RNA oligonucleotides was assayed by nitrocellulose filter binding. The relative counts were measured in a PhosphorImager and plotted against the protein concentrations. The highest binding point was arbitrarily set at 100, and the other points were determined accordingly. The wild-type yeast U2 snRNA stem-loop IV (YU2) had a higher affinity for the YU2B″ protein than did the mutagenized loops (M1 and M2). The results from two independent experiments were averaged, with bars showing the actual values from these two experiments. (B) The binding of GST-YU2B″ to ^{32}P-labeled YU2 was analyzed by competition with cold YU2, M1, and M2. The largest molar excess of cold RNA to hot RNA was about 20,000-fold. The relative counts on the filter were plotted against the competitor concentrations. The results from two independent experiments were averaged and plotted as described above for panel A.

(11), we changed three nucleotides that should be important for binding affinity (Fig. 6B). Indeed, YU2B″ bound these mutant RNAs much more weakly (Fig. 7B). This indicates that binding is sequence specific and supports the biological significance of the results. The importance of C-12 also suggests that certain features of the YU2B″–stem-loop IV interaction do indeed more closely resemble the human U1A-RNA interaction than they do the human U2B″-RNA interaction. This provides a reasonable explanation for the relative binding affinities of the two human stem-loops (Fig. 7A). A similar conclusion has been independently reached by Polycarpou-Schwartz et al. (32).

The fact that in vitro binding of YU2B″ to RNA is autonomous also indicates that it resembles more closely the human U1A-RNA interaction than it does the human U2B″-RNA interaction. Human U2B″ does not bind to its target in vitro without the addition of another U2 snRNP protein, U2A′ (6, 36). An unidentified yeast homolog of U2A′ may exist, but we have been unable to potentiate or alter the U2B″ interaction with added yeast extract or added micrococcal-nuclease-treated yeast extract (data not shown). Almost identical in vitro binding results have been independently obtained by Polycarpou-Schwarz et al., who also concluded that the YU2B″ protein binds autonomously to the yeast U2 snRNA stem-loop IV sequence (32).

Consistent with the target sequence and binding results, the yeast protein also shows somewhat more primary sequence conservation to human U1A protein than to human U2B″ (Fig. 3). An evolutionary tree of this family suggests that this may be incorrect, but the yeast protein is certainly not much more closely related to human U2B″ than to human U1A (32); it is also much more distantly related to both human proteins than the human proteins are to each other (Fig. 3) (32). A conservative conclusion is that the YU2B″ protein is related to the human U1A protein as well as to the human U2B″ protein.

There are three additional noteworthy features of the YU2B″ protein sequence and the newly expanded U1A-U2B″ family. First, the relationship of YU2B″ to yeast U1A is also distant, much more distant than the relationship between the human U2B″ and U1A proteins or between any of the metazoan family members (Fig. 3 and data not shown). This reflects at least in part the unusual nature of the yeast U1A protein; for example, its N-terminal RBD contains an unusual loop that is not present in the other family members, including YU2B″ (21). This is consistent with the fact that the binding site of yeast U1A's N-terminal RBD on yeast U1 RNA must be quite different from the canonical U1 stem-loop II and U2 stem-loop IV sequences; yeast U1 snRNA does not contain a comparable stem-loop sequence (17). Recent identification of the binding site supports this interpretation (42b).

Second, the database search recognizes the *Drosophila* D25 protein as a relative of YU2B″. This protein has been previously considered the *Drosophila* U1A protein (13), but all of the data did not fully support this interpretation. It has now been shown that D25 serves as *Drosophila*'s U1A protein as well as its U2B″ protein (32). This nicely explains the observed sequence relationship between D25 and YU2B″.

Third, as noted above, YU2B″ has only one RBD. The C-terminal RBD conserved among all other members of this family (including yeast U1A) is absent. Although the function of this domain in yeast cells is uncertain, its sequence conservation between yeast U1A and the metazoan family members is impressive (21). Indeed, it is more conserved between *S. cerevisiae* and humans than is the N-terminal RBD, suggesting that functional conservation is likely. In the absence of definitive data, there are two ways to interpret the presence of two RNA-binding modules in a protein. They might collaborate to strengthen specificity or affinity to a single target, or they might bind to separate targets (7, 39). In the latter case, two targets may contribute to the same function (e.g., splicing) or may indicate two separate functions.

For human U1A, in which the two RBDs have been well studied, the target of the N-terminal RBD is clearly U1 snRNA stem-loop II. Moreover, it appears that all of the specificity and affinity of full-length U1A for this target resides within the N-terminal RBD (30, 36). Thus, the C-terminal RBD has one or more other targets (not even certain to be RNA [22]). Among these targets may be a region of pre-mRNA, and the human U1A C-terminal RBD–pre-mRNA interaction has been shown to contribute to polyadenylation (23).

The C-terminal RBD of yeast U1A might similarly contribute to polyadenylation. However, a pre-mRNA–C-terminal RBD interaction might also contribute to splicing, because mutations in this domain of yeast U1A have negative effects on in vivo splicing efficiency (42a). If the mammalian U1A and U2B″ C-terminal RBDs also contribute to splicing, one can imagine that the absence of the domain from YU2B″ might be related to an apparent difference between yeast and mammalian in vitro splicing complex formation. In mammalian extracts, U2 snRNP can form a complex with pre-mRNA in the

absence of U1 snRNP, even on a substrate missing a proper 5′ splice site (28). In yeast extracts, no comparable U1 snRNP independence has been demonstrated (25, 34). We speculate that this apparent difference, the U1-independent binding of mammalian U2 snRNP to pre-mRNA, may be related to the presence of the U2B″ C-terminal RBD in mammalian U2 snRNP and its absence from yeast U2 snRNP.

ACKNOWLEDGMENTS

We thank I. W. Mattaj and B. Séraphin for sharing results prior to publication. We thank B. Séraphin, I. W. Mattaj, M. J. Moore, and H. V. Colot for constructive comments on the manuscript.

This work was supported in part by grant GM33205 from the NIH to M. Rosbash.

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