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Structurally Related but Functionally Distinct Yeast Sm D Core Small Nuclear Ribonucleoprotein Particle Proteins

JAGOREE ROY,[1] BINHAI ZHENG,[2] BRIAN C. RYMOND,[2] AND JOHN L. WOOLFORD, JR.[1]*

Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213,[1] and T. H. Morgan School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506-0225[2]

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Spliceosome assembly during pre-mRNA splicing requires the correct positioning of the U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) on the precursor mRNA. The structure and integrity of these snRNPs are maintained in part by the association of the snRNAs with core snRNP (Sm) proteins. The Sm proteins also play a pivotal role in metazoan snRNP biogenesis. We have characterized a *Saccharomyces cerevisiae* gene, *SMD3*, that encodes the core snRNP protein Smd3. The Smd3 protein is required for pre-mRNA splicing in vivo. Depletion of this protein from yeast cells affects the levels of U snRNAs and their cap modification, indicating that Smd3 is required for snRNP biogenesis. Smd3 is structurally and functionally distinct from the previously described yeast core polypeptide Smd1. Although Smd3 and Smd1 are both associated with the spliceosomal snRNPs, overexpression of one cannot compensate for the loss of the other. Thus, these two proteins have distinct functions. A pool of Smd3 exists in the yeast cytoplasm. This is consistent with the possibility that snRNP assembly in *S. cerevisiae*, as in metazoans, is initiated in the cytoplasm from a pool of RNA-free core snRNP protein complexes.

Elucidation of the order of U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particle (snRNP) addition in spliceosome assembly and the roles of the snRNPs in the first and second *trans*-esterification reactions of pre-mRNA processing has been greatly augmented by genetic analysis of the yeast *Saccharomyces cerevisiae* (for recent reviews, see references 19 and 42). However, studies of snRNP biogenesis have by and large been limited to biochemical assays of metazoan snRNPs. Little is known about the assembly of snRNPs in *S. cerevisiae.*

In metazoans, newly transcribed snRNAs are exported from the nucleus to the cytoplasm where they associate with core snRNP proteins (also known as Sm antigens) to form a pre-snRNP complex (see reference 2 and references therein). Assembly of these snRNP complexes is completed in the nucleus, in which the snRNPs then function in pre-mRNA splicing. The core snRNP proteins B, B′, D1, D2, D3, E, F, and G are instrumental in initiating snRNP assembly in the cytoplasm (28, 44). An ordered pathway for this process in which the D1, D2 and/or D3, E, F, and G proteins form an RNA-free 6S core cytoplasmic complex has been suggested (10, 45). This intermediate then assembles with the snRNA plus the B and B′ proteins to form an 11S-15S particle that is subsequently transported into the nucleus. Association of core snRNP proteins with snRNA is necessary for cytoplasmic modifications of the snRNA, such as hypermethylation of the 5′ cap of the snRNA (30). Both of these events, the association of the snRNAs with the core proteins and the hypermethylation of the 5′ cap, provide signals for reentry of the snRNA into the nucleus (11, 12). Assembly of the snRNPs is completed by the addition of snRNP-specific proteins, which occurs either during or after the transport of the pre-snRNPs to the nucleus.

Do core snRNP proteins have specific functions during spliceosome assembly and pre-mRNA processing or do they serve merely to maintain snRNP structure and integrity? The study of mammalian and *Xenopus* core proteins has left this question largely unanswered. A second intriguing question arises from the existence of three similar yet distinct snRNP D proteins: D1, D2, and D3 (1, 27). Do these three proteins represent a family of proteins with identical functions, or are there subtle differences in their roles in snRNP assembly and function? The genetic techniques available for *S. cerevisiae* make it an attractive system for addressing these questions.

The much lower abundance of snRNPs in *S. cerevisiae* compared with metazoans has made biochemical purification of core snRNP proteins from *S. cerevisiae* a difficult task. Furthermore, identification of core snRNP proteins has been largely refractile to the screens of temperature-sensitive mutants that have been employed for the isolation of the majority of defined yeast-splicing factors (reviewed in reference 18). However, several lines of evidence suggest that core snRNP proteins exist in *S. cerevisiae*. (i) Yeast U1, U2, U4, and U5 snRNAs contain the phylogenetically conserved Sm-binding site that has been demonstrated to directly associate with the core snRNP proteins in metazoans (17, 42). At least in the case of the U5 snRNA, this Sm site is essential for cell viability (23). (ii) The Sm sites in the yeast snRNAs are functional upon injection into *Xenopus* oocytes. Injected yeast snRNAs are able to assemble with the *Xenopus* core snRNP proteins (36). (iii) Antibodies directed against mammalian core snRNP proteins (Sm antisera) weakly coimmunoprecipitate the yeast U1, U2, U4, and U5 snRNAs (48, 50). (iv) The identification of the yeast gene *SMD1* encoding the functional homolog of the human D1 protein (40, 41) strengthens the possibility that other core snRNP proteins also exist in *S. cerevisiae*. (v) The recent isolation of the U1 and U4/U6.U5 snRNPs from *S. cerevisiae* (7) indicates that these snRNPs do indeed contain proteins that have sizes similar to those of the human core proteins.

We report here the characterization of a yeast gene *SMD3* that encodes a second D core snRNP protein. The Smd3 protein is similar in sequence to the human D3 protein and is

* Corresponding author. Phone: (412) 268-3193. Fax: (412) 268-7129. Electronic mail address: jw17@andrew.cmu.edu.

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
BJ2168	*MAT***a** *gal2 leu2 pep4-3 prc1-407 prb1-1122 trp1 ura3-52*	E. Jones
BJ3434	*MAT***a**/*MAT*α *ade6/+ his1/+ leu2-1/leu2-1 lys1/+ trp1/+ ura3-52/ura3-52 PEP3/ pep3*::*LEU2*	E. Jones
JWY1401	*MAT***a**/*MAT*α *ade2-101/+ can1/+ his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ101/trp1-Δ101 ura3-52/ura3-52*	31a
JWY2457	*MAT***a**/*MAT*α *ade2-101/+ can1/+ his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ101/trp1-Δ101 ura3-52/ura3-52 SMD3/smd3Δ2*::*HIS3*	This study
JWY2445	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + pYCp50-*SMD3*	This study
JWY2449	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + pRS314-*SMD3* (pIB6)	This study
JWY2450	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + p*GAL1*::*SMD3*	This study
JWY2451	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + pRS314-*SMD3HA-1*	This study
JWY2454	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + pRS314-*SMD3HA-2*	This study
JWY2455	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + pRS314-*SMD3HA-3*	This study
JWY2456	*MAT***a** *his1 leu2-1 trp1-Δ101 ura3-52 smd3Δ1*::*LEU2* + pRS424-*SMD3HA-3*	This study
BZY1-16D	*MAT*α *leu2-3,112 trp1-289 ura3-52 smd1*::*LEU2* + p*GAL*::*SMD1HA*	This study
BZY1-7D	*MAT*α *leu2-3,112 trp1-289 ura3-52*	This study
BRY100 (*SMD1* null)	*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52 smd1*::*LEU2* + p*GAL1*::*SMD1*	40, this study

structurally related to the yeast and human D1 proteins. This work demonstrates that Smd3 is functionally distinct from Smd1. We show that Smd3 is required for yeast pre-mRNA splicing and that it associates with the U snRNAs. Our results also indicate that snRNP biogenesis in *S. cerevisiae* may parallel its mammalian counterpart.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains used in this work are described in Table 1. The techniques used for growing yeast and bacteria are described elsewhere (32, 47). Yeast cells were transformed with DNA by the lithium acetate method (21).

Diploid yeast strain BJ3434 is heterozygous for the *pep3*::*LEU2* null allele of the nonessential *PEP3* gene. The 5′ 228 nucleotides (nt) of the adjacent *SMD3* coding sequence were also deleted in this disruption (34). A 0.95-kb *Eco*RI fragment containing *SMD3* cloned in plasmid YCp50 (pYCp50-*SMD3*) was transformed into BJ3434. The resulting strain was subjected to sporulation, and tetrads were dissected. Strain JWY2445 was identified among the haploid progeny by screening for the desired phenotypes.

A null allele of *SMD3* was created as follows. A 0.95-kb *Eco*RI fragment containing *SMD3* was cloned into the *Eco*RI site of plasmid pRS314 (18) to generate pIB6. This plasmid contains a unique *Bgl*II site in the coding region of *SMD3*. A 1.7-kb *Bam*HI fragment containing the entire *HIS3* gene was purified from plasmid pHIS3CEN3 (15) and was inserted within *SMD3* in the unique *Bgl*II site of pIB6, resulting in plasmid pIB14. A 2.6-kb *Eco*RI fragment from pIB14 containing *smd3Δ2*::*HIS3* was purified and used to replace one copy of *SMD3* in the diploid yeast strain JWY1401 by homologous recombination (39).

The *GAL1*::*SMD3* promoter fusion was constructed as follows. A 0.96-kb *Eco*RI-*Sal*I fragment from the plasmid pBM258T (45a), containing the *GAL1* promoter and transcription start sites, a multiple cloning site, and the *CYC1* transcription terminator, was cloned into plasmid pRS314. The 0.95-kb *Eco*RI fragment from pIB6 was purified and further digested with the enzyme *Hin*fI. A 460-bp fragment containing *SMD3* was purified from the resulting fragments, made blunt ended with the Klenow enzyme, and cloned into the *Sma*I site of pRS314-*GAL1*. In this construct, nucleotide −35 with respect to the ATG of *SMD3* is directly 3′ of the *GAL1* transcription start site, such that transcription of *SMD3* is under the control of the *GAL1* promoter. The plasmid containing the *GAL1*::*SMD3* allele was transformed into yeast strain JWY2445. Strain JWY2450 was derived from the transformants upon loss of the YCp50-*SMD3* plasmid on selective medium containing galactose and 5-fluoroorotic acid (5-FOA.)

The p*GAL1*::*HD1* plasmid containing the human D1 gene fused to the *GAL1* promoter was derived from the plasmid pHSMD1368 (38) (a gift from Sallie Hoch) as follows. A 1.2-kb *Eco*RI-*Sal*I fragment from pHSMD1368, containing the *GAL1*::*HD1* fusion, was cloned into the *URA3*-marked vector pUN75 (6).

Yeast strains expressing epitope-tagged alleles of *SMD3* were obtained by plasmid shuffling. Plasmids containing the epitope-tagged alleles of *SMD3* (see below) were transformed into JWY2445. Subsequent loss of the YCp50-*SMD3* plasmid was selected by using 5-FOA.

Growth curve and viability tests. Growth and viability of the JWY2450 yeast strain in galactose medium or after shifting to glucose medium were assayed as described previously (5). Briefly, cells were grown in liquid medium containing galactose to mid-log phase (optical density at 610 nm, ~0.5) and were then diluted into medium containing either galactose or glucose. The optical densities of the cultures at 610 nm and cell viabilities were monitored. The cultures were diluted to keep all A_{610} readings between 0.2 and 0.9 in order to maintain logarithmic growth.

Nucleic acid electrophoresis, transfer, and hybridization. The methods used for electrophoresis of DNA and RNA and nucleic acid transfer to Nytran membranes (Schleicher & Schuell, Keene, N.H.) were as described previously (32). Radiolabeling of DNA probes and their hybridization to Nytran membranes were done as described previously (9). A 370-bp DNA fragment internal to the *CRY1* gene was generated by PCR and used as a probe to detect *CRY1* pre-mRNA and mature mRNA. A 2.2-kb *Eco*RI-*Hin*dIII DNA fragment containing the *ACT1* gene was used as a probe for *ACT1* pre-mRNA and mature mRNA. Yeast poly(A)$^+$ RNA was isolated as described by Couto et al. (4).

For detection of snRNAs, Northern blot analysis was done as follows. Total RNA or RNA extracted from immunoprecipitates (see below) was boiled for 5 min, and an equal volume of urea dye (8 M urea, 20% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol in 2× Tris-borate-EDTA [pH 8.5]) was added. The sample was then subjected to electrophoresis on 5% acrylamide–7 M urea gels in 100 mM Tris–80 mM borate–1 mM EDTA (pH 8.5) (100 mM TBE) buffer for 5 h at 15 mA and electroblotted to Nytran membrane for 4 h in 50 mM TBE buffer at 35 V. The membranes were subsequently probed with ^{32}P-radiolabeled DNA complementary to snRNAs. These probes were generated by PCR of plasmids containing cloned snRNA genes. The intensity of the signal was quantified using the Ambis radioanalytic imaging system (Ambis, Inc., San Diego, Calif.). For detection of 5S rRNA, an oligonucleotide probe (5′-GGTAGATAT GGCCGCAACC-3′) was used. Hybridization of Nytran membranes to this oligonucleotide probe was done as described elsewhere (6).

Epitope tagging of *SMD3*. Oligonucleotide sequences encoding the 9-amino-acid influenza virus hemagglutinin (HA) epitope (20) were inserted at the 5′ and 3′ ends of *SMD3* by site-directed mutagenesis (25). The 61-nt oligonucleotide JR1 (5′-CCAGAGAAACGATATGTCCAGCGTAGTCTGGGACGTCGTAT GGGTACTGTGCCTCATTTAA-3′) containing the sequences encoding the HA epitope and flanking sequences from *SMD3* was used to insert the epitope sequences between codons 15 and 16 of *SMD3* to construct the amino-terminus-tagged allele of *SMD3* (*SMD3HA-1*). Similarly, the 60-nt oligonucleotide JR2 (5′-CGTTTCCTACCTAGCGTAGTCTGGGACGTCGTTGGGTATCTCT TAGGTCCTCT-3′) was used to insert the HA epitope between codons 100 and 101 in the wild-type *SMD3* gene as well as in the *SMD3HA-1* construct, resulting in the carboxy-terminus-tagged allele *SMD3HA-2* and the doubly tagged *SMD3HA-3* allele, respectively. For all of the constructs, the presence of the HA epitope was confirmed by restriction enzyme digestion and sequencing of the DNA. The tagged alleles were cloned into the yeast centromeric plasmid pRS314 (one to three copies per cell). The *SMD3HA-1* and *SMD3HA-3* alleles were also cloned into the yeast high-copy-number plasmid pRS424 (3).

Epitope tagging of *SMD1*. A *SMD1HA* construct was made by PCR with the 5′ oligonucleotide 5′-TTTTAGATCTATTATGAAGTTGGTTAACTTT-3′ introducing a *Bgl*II site (underlined) and the 3′ oligonucleotide 5′-ACCAGCAG CGAATCATAGAGCGTAGTCTGGAACGTCGTATGGGTA-3′ introducing the nine codons of the HA epitope after the penultimate codon of *SMD1*. This PCR fragment was cloned into the *Sma*I site of vector pTZ19u (U.S. Biochemicals, Cleveland, Ohio); the presence of the HA epitope codons was confirmed by sequence analysis. A *Bgl*II-*Bam*HI fragment containing the *SMD1HA* construct from this plasmid was inserted into the *Bam*HI site of the pBM150 vector (22). The correct orientation that places the *SMD1HA* gene under the control of the *GAL1* promoter was identified by restriction digestion analysis.

A diploid strain heterozygous for a null allele of *SMD1* (*SMD1*/*smd1*::*LEU2*) has been previously described (40). The *URA3* marked plasmid containing *GAL*::*SMD1HA* was transformed into this strain, the transformants were sporulated, and asci were dissected on galactose-containing medium. Strain BZY1-16D was identified among the meiotic progeny by screening for the desired phenotype.

Preparation of splicing extracts and immunoinhibition of splicing. Splicing extracts were prepared from yeast strains JWY2455 and BJ2168 by the method described by Lin et al. (29), with the modification that the spheroplasted cells were allowed to regenerate in rich medium containing 1.2 M sorbitol for 2 h at 30°C prior to homogenization. Extracts from yeast strains BZY1-7A, BZY1-16D, and JWY2456 were prepared as described elsewhere (29), with the following modifications: strain BZY1-16D was grown in galactose-based medium; yeast cells were spheroplasted using lyticase (Sigma Chemical Co.) at a concentration of 400 U/ml by gently shaking for 1 h at 30°C; buffers A and D contained 0.5 mM phenylmethylsulfonyl fluoride; and extracts were not centrifuged after dialysis but were directly frozen in aliquots at −80°C. The activity of the extracts was checked by standard splicing reactions using an *ACT1* pre-mRNA transcribed in vitro and radiolabeled with ^{32}P (29).

For immunoinhibition experiments, 15 μl of splicing extracts was incubated with 1 or 2 μg of purified anti-HA antibody, diluted in buffer D (20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.9) for 1 h on ice, and subsequently assayed for splicing.

Immunoblot and immunoprecipitation analyses. Total proteins were extracted from yeast cells as described elsewhere (32). For immunoblot analysis, protein samples were boiled in 1× Laemmli buffer (26) and subjected to electrophoresis in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. Proteins were then transferred by electroblotting to a nitrocellulose membrane. The immunoblots were probed with anti-HA epitope monoclonal antibodies (a kind gift of Peter Kolodziej) and then incubated with goat anti-mouse immunoglobulin G antibodies conjugated to alkaline phosphatase (Promega Corp., Madison, Wis.) and developed according to the manufacturer's instructions. For Fig. 5B and 6A, proteins were subjected to electrophoresis in SDS-4 to 15% gradient polyacrylamide gels and transferred to polyvinylidene difluoride membranes.

For immunoprecipitation of the Smd1HA and Smd3HA-3 proteins, 1 μg of purified anti-HA antibody 12CA5 (BAbCo, Berkeley, Calif.) was bound to 30 μl of protein A-agarose beads (Bio-Rad Laboratories, Hercules, Calif.) in 0.1 M borate buffer, pH 8.2, at room temperature for 1 h and subsequently cross-linked as described elsewhere (46). The protein A-antibody complexes were collected by brief centrifugation and washed extensively in buffer B (20 mM HEPES, pH 7.9, 50 mM NaCl, 0.05% Triton X-100). A 10-μl volume of splicing extracts (20 mg of total protein per ml) from strain BZY1-16D, JWY2455, JWY2456, or BZY1-7A was diluted in buffer B (to a final volume of 80 μl) and bound to the antibody beads for 1 h at room temperature. The immune complexes were collected by brief centrifugation and were washed six times with 1 ml of buffer B. Proteins were eluted in 1× Laemmli sample buffer and subjected to immunoblot analysis as described above.

Coimmunoprecipitation of snRNAs was assayed as follows. The immune complexes were collected as described above, and the supernatant fractions were saved. The pellet was washed with buffer B as described above. After the last wash, all buffer B was removed and 300 μl of proteinase K (PK) buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, 1% SDS) was added to the pellet fractions, the supernatant fractions, and 10 μl of total splicing extract. The proteins were digested with proteinase K (U.S. Biochemicals) at a concentration of 0.4 mg/ml for 10 min at 37°C, which was followed sequentially by two extractions with phenol-chloroform-isoamyl alcohol and one extraction with chloroform. The RNA was ethanol precipitated in the presence of 100 μl of 3 M sodium acetate, washed, suspended in 8 M urea dye, and subjected to Northern (RNA) analysis (see above). For experiments in which two consecutive immunoprecipitations were performed, the supernatants from the first precipitations were mixed with 30 μl of fresh antibody-bound agarose and processed as described above.

Immunoprecipitations of 5′ hypermethylated snRNAs. Cap modification of snRNAs was analyzed as follows. A 2-μg amount of an anti-trimethylguanosine (TMG) antibody (a generous gift from James Friesen and Reinhard Lührmann) was bound to 50 μl of immobilized protein A beads (Repligen Corporation, Cambridge, Mass.) in buffer B as described above. The protein A-antibody complexes were collected by brief centrifugation and washed in buffer B. A 10-μg amount of total RNA were diluted with 50 μl of buffer B, and the dilution was bound to the beads for 60 min at room temperature. For 15- or 20-h time points in glucose medium, approximately five times more RNA was coupled with 250 μl of antibody beads solution. The immune complexes were collected and washed with buffer B. The immune complexes or supernatant fractions were incubated in buffer K (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 1% SDS, 2 mg of proteinase K per ml) for 1 h at 37°C. Digested proteins were removed by phenol extraction, and the RNA was precipitated with ethanol in the presence of 20 mg of glycogen per ml at −20°C. The RNA was then washed and suspended in water. Half of the RNA sample was used for RNA blot analysis.

Nuclear and cytoplasmic fractionations. Nuclear and cytoplasmic fractions of yeast cells were obtained by the method described elsewhere (52). Proteins from equivalent amounts of nuclei or cytoplasm, i.e., nuclei and cytoplasm from equal numbers of cells, were subjected to immunoblot analysis as described above. Polyclonal antiserum to phosphoglucokinase was provided by Jeremy Thorner (University of California, Berkeley), and a monoclonal antibody to the 200-kDa subunit of RNA polymerase II was provided by Sha-Mei Liao and Richard Young (Massachusetts Institute of Technology, Cambridge). Quantification of bands on immunoblots was done using the optical image detection mode on the Ambis radioanalytic imaging system. Total RNA was extracted from equivalent amounts of nuclei or cytoplasm from approximately 2×10^{11} cells and suspended in 250 μl of water. A 15-μl volume of each sample was subjected to RNA blot analysis for detection of U snRNAs as above. These results were quantified as described above.

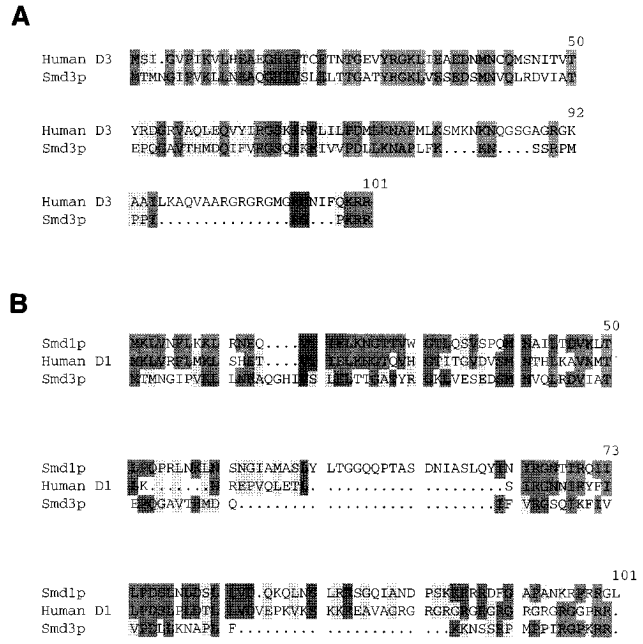

FIG. 1. The amino acid sequence of yeast Smd3 is similar to that of the human D3 protein as well as to those of yeast Smd1 and the human D1 protein. (A) An alignment of the predicted amino acid sequences of Smd3 and human D3 protein; (B) an alignment of the predicted amino acid sequences of Smd3, Smd1, and human D1 protein. Lightly and darkly shaded areas represent similar and identical amino acids, respectively. The numbering above each row of sequences corresponds to the amino acid sequence of Smd3.

Indirect immunofluorescence microscopy. Yeast cells were prepared for indirect immunofluorescence microscopy as described elsewhere (35). The primary antibody used was purified monoclonal anti-HA (see above) at a concentration of 50 μg/ml. Rhodamine-conjugated anti-mouse immunoglobulin G (Cappel, Organon-Teknika Corp., Durham, N.C.) was used as the secondary antibody. Slides were viewed with a Zeiss Axiophot microscope equipped with a 100× lens objective and Nomarski differential interference contrast (DIC) optics. Photographs were taken using a photometric cooled charge-coupled device (CCD) camera (Photometric Inc., Tucson, Ariz.) with 12-bit pixel depth. Subsequently, DIC images were flat-field divided to smooth out background and linearly stretched to 8-bit levels for display. Fluorescent images were likewise linearly contrast stretched.

DNA sequence analysis. DNA sequencing of HA-tagged *SMD3* alleles, which were subcloned in pBluescript II KS(+) plasmids (Stratagene, La Jolla, Calif.), was carried out by the dideoxy chain-termination method (43), using Sequenase version 2.0 (U.S. Biochemicals).

Analysis and comparisons of DNA sequences were performed by using computer programs developed by the Genetics Computer Group's Sequence Analysis software package at the University of Wisconsin Biotechnology Center, which was accessed through the Pittsburgh Supercomputing Center.

RESULTS

A yeast ORF encodes a protein similar to the human D3 core snRNP protein. An open reading frame (ORF) upstream of the previously characterized *S. cerevisiae PEP3* gene (34) is predicted to encode a protein similar in sequence to that of human core snRNP protein D3 (29b). A comparison between

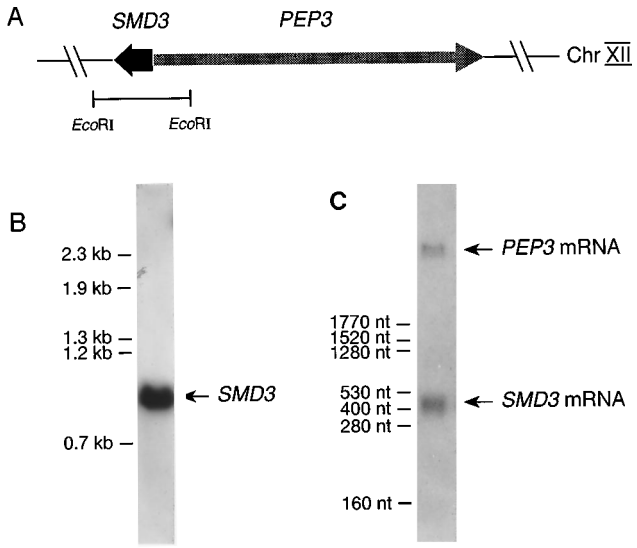

FIG. 2. (A) *SMD3* is a single-copy gene adjacent to *PEP3* on chromosome XII that expresses a 450-nt poly(A)$^+$ mRNA. A ^{32}P-radiolabeled 0.95-kb *Eco*RI DNA fragment containing *SMD3* was hybridized to a Southern blot of yeast genomic DNA digested with *Eco*RI (B) and a blot of yeast poly(A)$^+$ RNA (C).

the sequences (BestFit program, Genetics Computer Group) based on the Dayhoff algorithm (also depicted by an alignment in Fig. 1A) indicates that the putative protein encoded by this ORF is 78% similar and 51% identical to the human D3 core snRNP protein. This degree of similarity is significant; the human D1 protein is functionally equivalent to the yeast Smd1 (41), and its sequence is only 40% identical to that of Smd1 (40). Thus, we have named this gene *SMD3.*

The amino acid sequence of the predicted protein encoded by this ORF is also similar to that of the human core snRNP protein D1 (37) and its homolog in *S. cerevisiae*, Smd1 (40) (Fig. 1B). The sequence of Smd3 is 50% similar and 26% identical to that of human D1 protein and 49% similar and 25% identical to that of yeast Smd1. Three metazoan D snRNP proteins, D1, D2, and D3, are core components of spliceosomal snRNPs (1, 27). The mammalian D1, D2, and D3 proteins share considerable sequence similarity (29a). This structural similarity appears to be conserved in *S. cerevisiae.*

***SMD3* is an essential, single-copy gene.** A ^{32}P-radiolabeled 0.95-kb *Eco*RI DNA fragment containing the entire *SMD3* ORF was hybridized to a Southern blot of yeast genomic DNA that was digested with *Eco*RI or *Bam*HI. In each case, only one DNA fragment of the expected size was detected (Fig. 2B and data not shown), indicating that the *SMD3* gene is present in one copy in the haploid yeast genome.

Previously, a null allele of *PEP3* created by replacing sequences from *PEP3* and the upstream ORF (*SMD3*) with *LEU2* was found to be lethal (33a). However, the *PEP3* gene was determined to be nonessential for vegetative growth (34), from which it was inferred that the upstream ORF (*SMD3*) encodes an essential function. We confirmed this by two means. (i) We showed that a plasmid bearing only *SMD3* was capable of rescuing the lethality of the *pep3*::*LEU2* null allele. Therefore, in this paper, we refer to this allele as *smd3Δ1*::*LEU2*. (ii) We constructed a null allele of *SMD3*, *smd3Δ2*::*HIS3*, in which only sequences from the *SMD3* coding region are deleted and replaced with *HIS3*. One copy of the wild-type *SMD3* gene was replaced with this null allele in the diploid strain JWY1401 by homologous recombination. Genomic Southern blot analysis confirmed that the expected recombination events had occurred (data not shown). Upon sporulation of this heterozygous diploid, which was followed by tetrad dissection, only two viable spores were recovered from each of 20 tetrads. All of the meiotic offspring recovered were unable to grow on medium lacking histidine and therefore contained the wild-type allele of *SMD3*. This lethality can be complemented by a plasmid-borne 0.95-kb *Eco*RI fragment that contains all of the *SMD3* coding region. Thus, *SMD3* is essential for mitotic growth.

A blot of total yeast polyadenylated mRNAs fractionated by gel electrophoresis was probed with the ^{32}P-radiolabeled 0.95-kb *Eco*RI fragment containing all of the *SMD3* ORF and 91 codons of the *PEP3* ORF. Two mRNA species, approximately 3,100 and 440 nt long, were detected (Fig. 2C). The larger mRNA is identical in size to the previously characterized *PEP3* mRNA (34). The size of the smaller mRNA is in agreement with the 303-nt ORF in the *SMD3* sequence.

***SMD3* is required for pre-mRNA splicing.** To determine whether the *SMD3* gene is necessary for pre-mRNA splicing, we constructed yeast strain JWY2450 (*smd3Δ1*::*LEU2* plus p*GAL1*::*SMD3*), which is conditional for synthesis of Smd3, and assayed the effect of depletion of Smd3 on pre-mRNA splicing. The conditional null allele of *SMD3* was constructed by placing the *SMD3* coding region downstream of the yeast *GAL1* promoter such that transcription of the *GAL1*::*SMD3* allele is induced in galactose-containing medium and repressed in glucose-containing medium. *SMD3* mRNA expressed from *GAL1*::*SMD3* is present in the galactose-grown cultures at more than 10-fold higher levels than mRNA expressed from wild-type *SMD3* in glucose or galactose medium (Fig. 3B and data not shown). RNA blot analysis shows that *SMD3* mRNA expressed from the *GAL1*::*SMD3* allele is not detectable 30 min after shifting strain JWY2450 to glucose-containing medium (Fig. 3B). No change in the levels of a control RNA, snR189, was observed (data not shown).

The *GAL1*::*SMD3* allele is fully functional since it can complement the lethality of the *smd3Δ1*::*LEU2* null allele; strain JWY2450 grows in galactose medium with a doubling time of 4 h (Fig. 3A). When shifted to glucose medium, this strain continues to grow logarithmically for about 15 h, after which the rate of growth decreases rapidly and ceases by about 30 h. We infer that this delayed decrease in growth rate results from overproduction of Smd3 in the galactose-grown cells, such that cessation of growth does not occur until subsequent dilution of Smd3 following several cell divisions in glucose medium.

To assess the effect of depletion of Smd3 on pre-mRNA splicing, we assayed the amounts of unspliced *CRY1* pre-mRNA and spliced *CRY1* mRNA at various times after the shift in carbon source (Fig. 3B). Spliced *CRY1* mRNA, but not unspliced *CRY1* pre-mRNA, was detected in cells grown in galactose medium or up to 4 h after shifting to glucose medium. At 15 h after the shift to glucose medium, concomitantly with the decrease in growth rate, *CRY1* pre-mRNA accumulated; the ratio of unspliced *CRY1* pre-mRNA to spliced *CRY1* mRNA increased in glucose medium as the growth rate of the strain decreased. However, even at the latest time points, some *CRY1* mRNA was detected. Identical results were obtained for a second intron-containing transcript, *ACT1* (data not shown). In both cases, no splicing intermediates were observed upon shifting to glucose medium. Thus, Smd3 is necessary for efficient pre-mRNA splicing in vivo at a step or steps prior to the first cleavage and ligation reactions of splicing.

Depletion of Smd3 affects 5′ cap modification and results in a decrease in the amounts of the spliceosomal U snRNAs. snRNP assembly in metazoans is dependent on association of

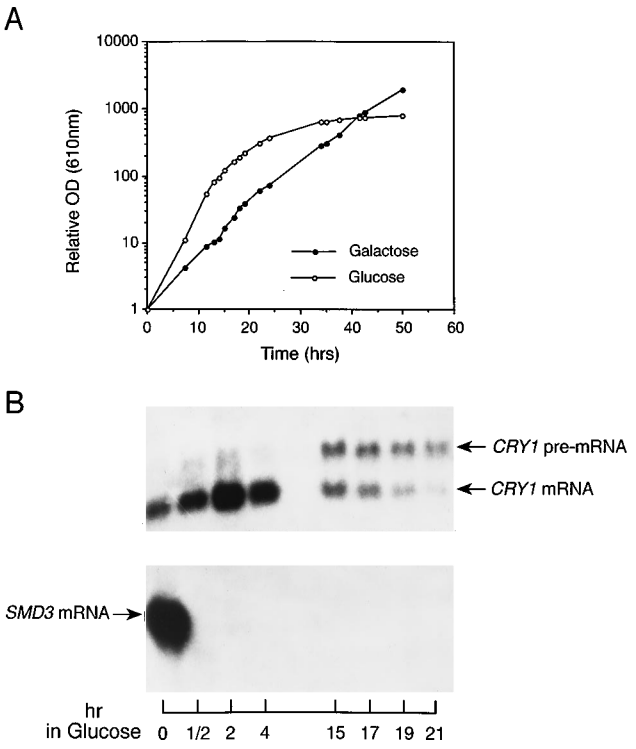

FIG. 3. Depletion of Smd3 from yeast cells results in cessation of growth and accumulation of unspliced pre-mRNA. (A) The rate of growth of strain JWY2450 remains unchanged in galactose-containing medium but decreases in glucose-containing medium after seven generations and ceases completely by 30 h. The zero time point represents the point at which the cultures grown in galactose-containing medium were shifted to glucose-containing medium. Relative OD (optical density recorded at 610 nm) was measured as $OD_t/OD_{t=0}$ plotted on a logarithmic scale. (B) RNA was extracted from strain JWY2450 grown in galactose-medium and at different times after shifting to glucose-containing medium (as indicated). RNA blot analysis shows that *SMD3* mRNA is overexpressed in galactose medium and completely shut off in glucose medium. No *SMD3* mRNA was detectable 30 min after shifting the cultures to glucose medium. The RNA blot was also probed with a 370-nt PCR fragment internal to the *CRY1* gene showing that *CRY1* pre-mRNA begins to accumulate in strain JWY2450 after 15 h in glucose medium and continues to accumulate up to 21 h in glucose medium.

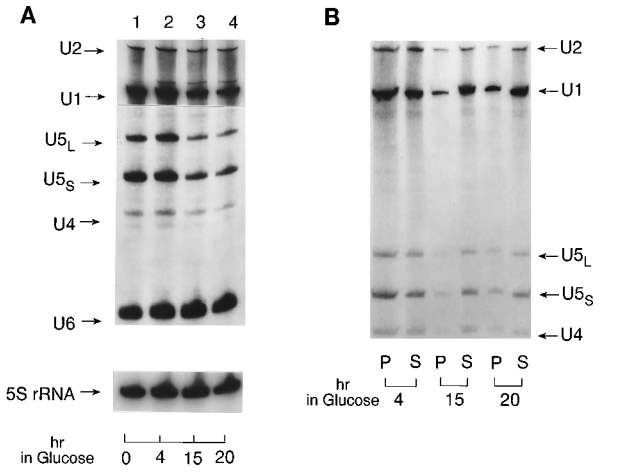

FIG. 4. Depletion of Smd3 results in decreased accumulation and decreased 5′ cap hypermethylation of spliceosomal U snRNAs. (A) RNA blot analysis of strain JWY2450, grown in galactose medium (lane 1) or 4 (lane 2), 15 (lane 3), or 20 (lane 4) h after shifting to glucose medium. Total RNA from an equivalent number of yeast cells was used for each time point. The U5 snRNA is represented by two bands corresponding to a long and a short form. The bottom panel shows the same blot hybridized with a probe for 5S rRNA. (B) RNA blot analysis after immunoprecipitation with anti-TMG antibody. Strain JWY2450 was shifted to glucose medium for the indicated times, and total RNA was extracted and immunoprecipitated with the anti-TMG antibody. Supernatants and pellets were subjected to gel electrophoresis. For the later time points in glucose, the immunoprecipitations were scaled up approximately fivefold to compensate for the decrease in U snRNA levels (see Materials and Methods). P, pellet; S, supernatant.

the core snRNP proteins with the Sm-binding region of the U snRNAs (2). Disruption of this association can lead to decreased stability of the snRNAs (31, 51). If the pathway for snRNP biogenesis in *S. cerevisiae* is similar to that in metazoans, then depletion of a yeast core snRNP protein might result in decreased levels of U snRNAs. To ascertain the effect of depletion of Smd3 on the accumulation of the yeast U snRNAs, we assayed levels of the U1, U2, U4, U5, and U6 snRNAs in the *GAL1*::*SMD3* strain (JWY2450) at various times after shifting to glucose medium. We also determined the levels of 5S rRNA to serve as a control for loading. No effect on snRNA levels was observed 4 h after the medium shift, when pre-mRNA splicing is apparently unaffected; the levels of snRNAs are identical to those in this strain grown in galactose medium (Fig. 4A; compare lanes 1 and 2). However, we observed a reduction in the levels of the U1, U2, U4, and U5 snRNAs 15 and 20 h after shifting to glucose-containing medium (Fig. 4A, lanes 3 and 4). There was little or no change in the levels of U6 snRNA and 5S rRNA at either time point. Quantification of the levels of the snRNAs, normalized with respect to 5S rRNA levels, showed that U5 snRNA levels decreased by 80%, U4 snRNA levels decreased by 60%, and U1 and U2 snRNA levels decreased by 50 and 40%, respectively. Thus, depletion of Smd3 results in a decreased accumulation of the U1, U2, U4, and U5 snRNAs. The levels of the different U snRNAs are reproducibly affected to different extents; levels of U5 snRNA are reduced to the greatest extent. The decreased accumulation could be due to a decrease in either the stability of the U snRNAs or else their synthesis. Currently, our experiments do not differentiate between these two possibilities.

We determined whether depletion of Smd3 affects the 5′ guanosine cap modification of the spliceosomal snRNAs. Total RNA extracted from the cells before and after Smd3 depletion was immunoprecipitated with antibodies that specifically recognize the modified TMG cap. The amounts of U snRNAs in the immunoprecipitate and supernatant fractions were assayed by RNA blot analysis (Fig. 4B). In cells expressing Smd3 protein, approximately 50% of U1 snRNA, 40% of U2 snRNA, and 60% of the U4 and U5 snRNAs were immunoprecipitated by the anti-TMG antibody. We do not know whether the snRNAs remaining in the supernatant are not hypermethylated or whether this reflects the limited efficiency of the immunoprecipitation by the anti-TMG antibody. After depletion of Smd3, however, less than 5% of the U4 and U5 snRNAs and only 10% of U1 and U2 snRNAs were immunoprecipitated with the anti-TMG antibody. The ratio of snRNAs in the immunoprecipitate versus the supernatant decreases markedly after depletion of Smd3. Therefore, after Smd3 depletion, the majority of the remaining snRNAs are no longer immunoprecipitable with the anti-TMG antibody, suggesting that they do not contain hypermethylated 5′ guanosine caps. Alternatively, depletion of Smd3 may result in decreased accessibility of the hypermethylated 5′ guanosine caps to the anti-TMG antibody. However, we believe that the former possibility is more likely.

Immunological detection of Smd3 and Smd1. To further characterize the Smd3 protein, we tagged Smd3 with the in-

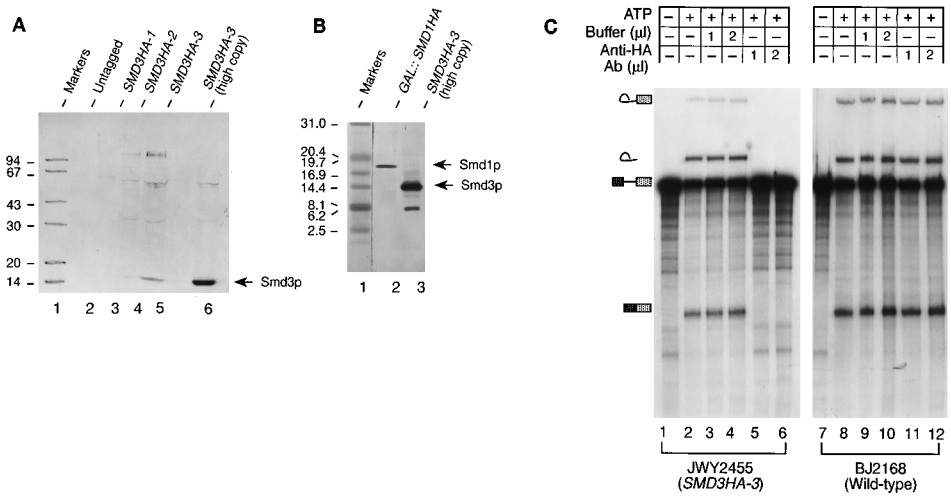

FIG. 5. (A) Detection of epitope-tagged Smd3. Cell extracts containing approximately equal amounts of protein, as judged by Ponceau S staining, were loaded in each lane. Lanes: 1, molecular weight marker; 2, strain JWY2449 expressing untagged Smd3; 3, JWY2451 expressing amino terminus-tagged Smd3HA-1; 4, JWY2454 expressing carboxy-terminus-tagged Smd3HA-2; 5, JWY2455 expressing doubly tagged Smd3HA-3; 6, JWY2456 expressing Smd3HA-3 from a high-copy-number plasmid. (B) Smd1HA and Smd3HA-3 are distinguishable from each other. Western immunoblot analysis of strain BZY1-16D expressing Smd1HA (lane 2) and strain JWY2456 expressing Smd3HA-3 (lane 3). The 6.0-kDa band in lane 3 probably represents a degradation product of Smd3HA-3. (C) Antibodies against Smd3 inhibit splicing in vitro. Splicing extracts prepared from strain BJ2168 containing untagged Smd3 or strain JWY2455 containing epitope-tagged Smd3HA-3 were tested for splicing in vitro in the presence of increasing amounts of purified anti-HA antibody or buffer. ATP was added to the splicing reactions as indicated. mRNA precursors, splicing intermediates, and products are indicated by the symbols shown. Ab, antibody.

fluenza virus HA epitope. Smd3 containing the nine-amino-acid HA epitope at either the amino or carboxy terminus could not be detected by immunoblot analysis of whole-cell extracts or splicing extracts of yeast strains expressing these tagged alleles. However, Smd3 containing the HA epitope at both positions (Smd3HA-3) was identifiable as an approximately 14-kDa polypeptide (Fig. 5A, lane 5). This size is similar to those observed for the human D proteins (1, 27). The identity of this polypeptide as Smd3HA-3 was confirmed by the fact that the amount of this polypeptide is greatly increased in extracts from a strain carrying *SMD3HA-3* on a high-copy-number plasmid (Fig. 5A, lane 6). This protein is fully functional; yeast cells containing the *smd3Δ1*::*LEU2* null allele and *SMD3HA-3* on a low-copy-number CEN plasmid (strain JWY2455) grow at wild-type rates and splice *CRY1* pre-mRNA as efficiently as wild-type cells (data not shown).

The three human D proteins are similar in size to each other but can be distinguished from each other by SDS-polyacrylamide gel electrophoresis (26). To determine whether epitope-tagged Smd3HA-3 could be distinguished from Smd1, we constructed a *GAL1*::*SMD1HA* allele that expresses Smd1 tagged with the HA epitope at its carboxy terminus. Immunoblot analysis of cell extracts from strain BZY1-16D expressing Smd1HA and strain JWY2456 expressing Smd3HA-3 indicates that the two proteins are readily distinguishable from each other (Fig. 5B). Smd1HA migrates in SDS-polyacrylamide gels as an 18.2-kDa polypeptide.

Antibodies against Smd3 inhibit splicing in vitro. We assayed whether anti-HA antibodies could inhibit splicing of extracts prepared from strain JWY2455 expressing only the epitope-tagged allele of *SMD3* (*SMD3HA-3*). Extracts prepared from this strain are able to support splicing of an in vitro-transcribed *ACT1* pre-mRNA in the presence of ATP (Fig. 5C, lanes 1 and 2). Splicing was almost completely abolished if the extracts were preincubated with 1 or 2 μl of anti-HA antibody (Fig. 5C, lanes 5 and 6). Addition of identical volumes of buffer alone had no effect on the splicing reaction (Fig. 5C, lanes 3 and 4). An identical experiment carried out with extracts from an untagged wild-type strain, BJ2168, showed that preincubation with the anti-HA antibodies did not affect splicing (Fig. 5C, lanes 7 to 12).

Smd1 and Smd3 are associated with the spliceosomal U snRNAs. We tested whether Smd1 and Smd3 were core snRNP proteins associated with spliceosomal U snRNAs by assaying whether the snRNAs were coimmunoprecipitated with the anti-HA antibodies directed against epitope-tagged Smd1HA and Smd3HA-3. Splicing extracts prepared from strain BZY1-16D carrying the *SMD1HA* allele, strains JWY2455 and JWY2456 carrying the doubly tagged *SMD3HA-3* alleles, and

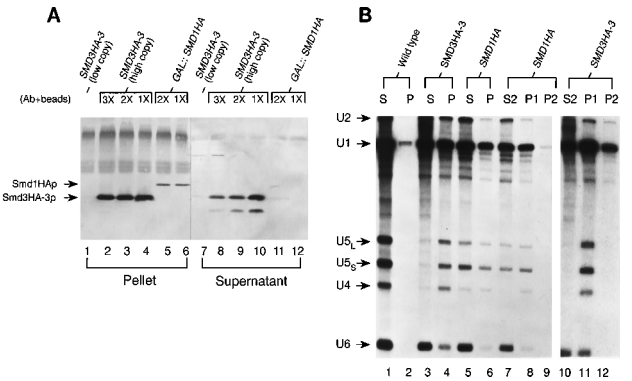

FIG. 6. Smd3 and Smd1 are associated with the spliceosomal U snRNAs. Splicing extracts from strains JWY2455 and JWY2456 expressing epitope-tagged Smd3HA-3, strain BZY1-16D expressing epitope-tagged Smd1HA, or strain BZY1-7A containing untagged Smd3 and Smd1 were immunoprecipitated with anti-HA antibody (Ab). A total of 1 μl of antibody plus 30 μl of beads (or as indicated, twice or three times these amounts) was used for immunoprecipitation. (A) Western immunoblot analysis of proteins from immune pellet and supernatant fractions; (B) Northern blot analysis of U snRNAs in the immune pellet and supernatant fractions of strains BZY1-7A (wild-type), BZY1-16D (*SMD1HA*), and JWY2455 (*SMD3HA-3*). P, pellet; S, supernatant. P2 and S2, pellet and supernatant fractions of a second consecutive immunoprecipitation, respectively.

wild-type strain BZY1-7A were subjected to immunoprecipitation with the anti-HA antibody. Immunoblot analysis of the pellet and supernatant fractions (Fig. 6A) indicates that the Smd1HA and Smd3HA-3 proteins are immunoprecipitated from the respective epitope-tagged extracts. In a parallel experiment, RNA was extracted from the immune complexes and subjected to gel electrophoresis and RNA blot analysis. The U1, U2, U4, U5, and U6 snRNAs were specifically coimmunoprecipitated with the anti-HA antibody from extracts carrying the tagged alleles of *SMD1* and *SMD3* but not from untagged wild-type extracts (Fig. 6B). Thus, both Smd1HA and Smd3HA-3 are associated with the U1, U2, U4, and U5 snRNAs, indicating that Smd1 and Smd3 are core components of these snRNPs. The coimmunoprecipitation of U6 snRNA may be indirect, since the majority of the U6 snRNAs in these extracts are associated with U4 snRNAs in U4/U6 snRNPs (8).

The U1 to U5 snRNAs were not completely immunoprecipitated by antibodies to either Smd3 or Smd1 (Fig. 6B). This could be explained if a fraction of the SmD proteins associated with the snRNAs is not accessible to the antibody. Alternatively, the antibody-bead mixture may be saturated with the extracts. However, these possibilities seem unlikely, because almost all of the Smd1 protein as well as the Smd3 protein is immunoprecipitated with this antibody (Fig. 6A). To determine whether the snRNAs remaining in the supernatant were capable of being further immunoprecipitated with the anti-HA antibody, we subjected the supernatants to a second identical immunoprecipitation. The majority of the U snRNAs remained in the supernatant of this second immunoprecipitation (Fig. 6B, lanes 7 to 12). Therefore, it is likely that the snRNAs in the supernatant represent those that are not associated with Smd1 or Smd3.

***SMD1* and *SMD3* encode proteins with nonidentical functions.** The *SMD1* and *SMD3* genes each encode essential proteins with significant sequence similarity that are both associated with the spliceosomal snRNAs. Smd1 and Smd3 may have identical functions; deletion of either gene may reduce the amount of SmD protein below a threshold required for viability. If such is true, then overexpression of one gene should compensate for the absence of the other. Alternatively, each gene product may perform unique essential functions, in which case overexpression of one will not compensate for the deletion of the other. To distinguish between these possibilities, we determined whether the lethality of a null allele of *SMD1* could be overcome by overexpression of *SMD3* and whether the lethality of a *SMD3* null allele could be suppressed by overexpression of *SMD1*. Haploid yeast strain BRY100, which contains a *smd1*::*LEU2* null allele at the *SMD1* locus and p*GAL1*::*SMD1* (40), was transformed with plasmids containing *SMD3* on low- and high-copy-number plasmid vectors. Transformants were tested for their abilities to lose the *URA3*-marked p*GAL1*::*SMD1* plasmid by plating on selective medium containing 5-FOA. No 5-FOA-resistant colonies were obtained, indicating that the presence of low- or high-copy-number plasmids containing *SMD3* does not suppress the *smd1*::*LEU2* lethal phenotype.

To test whether overexpression of *SMD1* could suppress the lethality of the *smd3Δ1*::*LEU2* null allele, diploid strain BJ3434 (*SMD3*/*smd3Δ1*::*LEU2*) was transformed with p*GAL1*::*SMD1*. The resulting strain was sporulated, and tetrads were dissected on galactose-containing medium. No more than two viable spore clones per tetrad were obtained, all of which were Leu$^-$. Thus, the p*GAL1*::*SMD1* plasmid was unable to suppress the lethality of the *smd3Δ1*::*LEU2* null allele. A null allele of *SMD1* can be suppressed by expression of the human D1 gene under the transcriptional control of the yeast *GAL1* promoter (41). We determined whether overexpression of the human D1 protein could compensate for the loss of Smd3. Strain BJ3434 was transformed with p*GAL1*::*HD1* and sporulated. Upon tetrad dissection, as described above, we failed to recover more than two viable spore clones per tetrad or any meiotic offspring that were leucine prototrophs. Thus neither overexpression of *SMD1* nor the human D1 gene can suppress the lethality of the *smd3Δ1*::*LEU2* null allele. We conclude that the proteins encoded by the *SMD1* and *SMD3* genes have qualitatively distinct functions in vivo.

There is a cytoplasmic pool of Smd3. snRNP assembly is a multistep process that begins in the cytoplasm. Cytoplasmic snRNA-free pools of several of the snRNP proteins are found in mammalian cells (44) as well as in *Xenopus* oocytes (13, 53). To ascertain whether this is also the case in *S. cerevisiae*, the intracellular location of Smd3 was determined by indirect immunofluorescence microscopy utilizing a strain expressing the Smd3HA-3 epitope-tagged protein. We were unable to detect a signal above background from a strain that contained *SMD3HA-3* on a centromeric plasmid. However, a strong signal was obtained in a strain expressing *SMD3HA-3* from a high-copy-number plasmid (Fig. 7). The Smd3HA-3 protein is localized both to the cytoplasm and the nucleus. The cytoplasmic staining is particulate in nature; however, we do not know whether this is biologically significant or is an artifact of the immunostaining procedure. The variation in the intensity of the signal between cells is probably a reflection of the differences in the copy numbers of the high-copy-number plasmid.

Overexpression of proteins can in some cases lead to their mislocalization. Therefore, we sought to confirm the above result by Western immunoblot analysis of nuclear and cytoplasmic fractions from a strain expressing *SMD3HA-3* on a centromeric plasmid (Fig. 8). Approximately half (55%) of the tagged Smd3 protein was found in the cytoplasmic fraction (Fig. 8A, lanes 3 and 4). Identical results were obtained with a strain containing *SMD3HA-3* on a high-copy-number plasmid (Fig. 8A, lanes 1 and 2). Immunoblot assays with antibodies to the cytoplasmic proteins ribosomal protein L1 (5) and phosphoglucokinase (24) and the 200-kDa nuclear subunit of RNA polymerase II (49) were also carried out. For both yeast strains, the majority of the ribosomal protein L1 and phosphoglucokinase was found in the cytoplasmic fractions as expected (Fig. 8A). RNA polymerase II was found predominantly in the nuclear fraction (85% was in the nuclear fraction and 15% was in the cytoplasmic fraction).

To determine the localization of the U snRNAs, we extracted RNA from equivalent amounts of nuclei and cytoplasm and performed RNA blot analysis for detection of the U1, U2, U4, U5, and U6 snRNAs (Fig. 8B). Quantification of these results showed that 75% of U1 and U2 snRNAs were in the nuclear fraction and 80% of the U4 and U5 snRNAs were in the nuclear fraction and that the U6 snRNA was almost exclusively found (>90%) in the nuclear fraction. The small amount of the U snRNAs found in the cytoplasmic fractions may represent the steady-state levels of these snRNAs in the cytoplasm or may be due to experimental artifacts. We conclude that although there is possibly some leakage of nuclear components into the cytoplasm during our fractionation procedure, this cannot account for the amount of Smd3 that we find in the cytoplasmic fractions. Taken together with the immunofluorescence results, these data indicate that there is a cytoplasmic pool of yeast Smd3, as observed for metazoan core snRNP proteins.

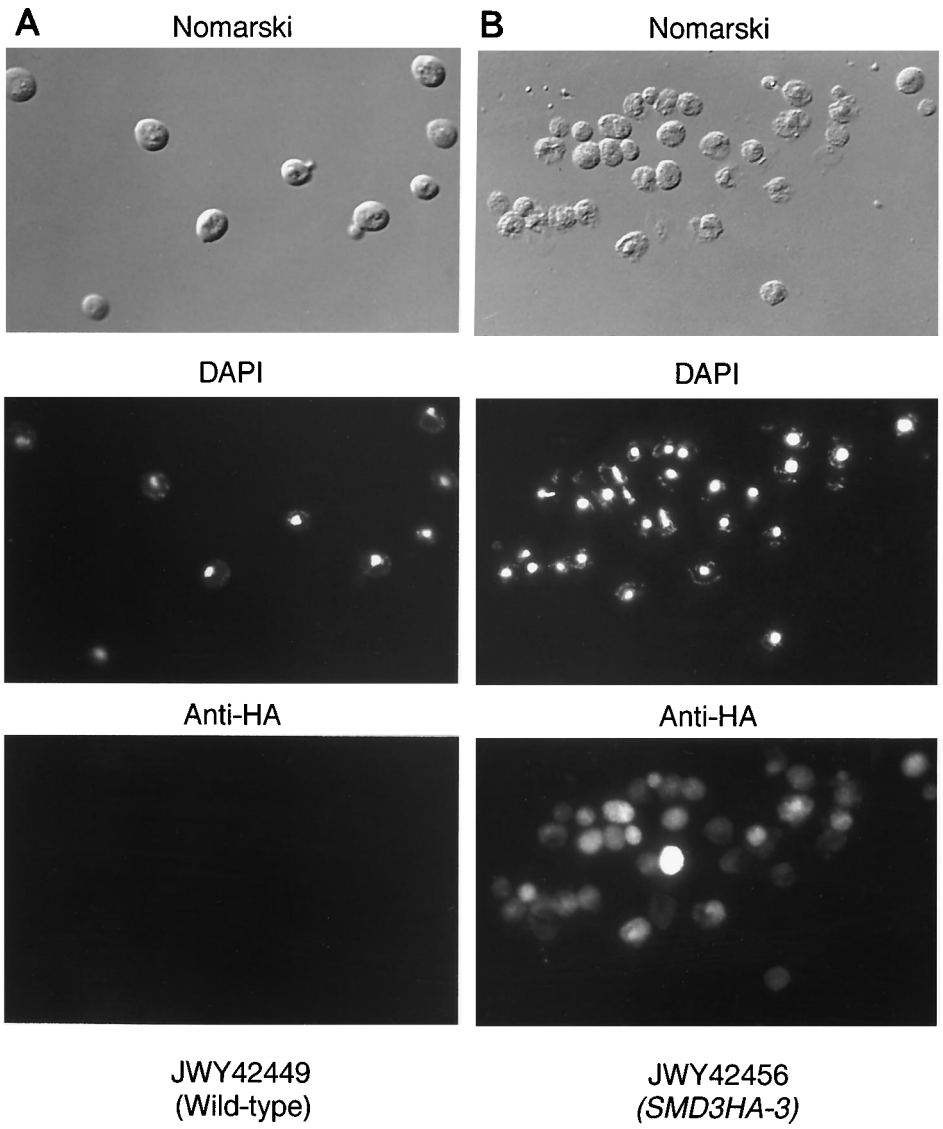

FIG. 7. Smd3 is localized in both the cytoplasm and nucleus of *S. cerevisiae*. Indirect immunofluorescence microscopy of strains JWY2449 (expressing untagged Smd3) (A) and JWY2456 (expressing Smd3HA-3 from a high-copy-number plasmid) (B) is shown. The panels depict yeast cells that are viewed by Nomarski optics; that are stained with 4′,6-diamidino-2-phenylindole (DAPI), which visualizes nuclear and mitochondrial DNA; and that are stained with anti-HA antibody.

DISCUSSION

We have characterized a gene, *SMD3*, encoding a core snRNP protein in *S. cerevisiae*. Like the previously identified yeast D1 core protein Smd1 (40), the Smd3 protein is essential for pre-mRNA splicing and shares sequence similarity with the human D1 core snRNP protein. The three human D snRNP proteins D1, D2, and D3 are similar in sequence to each other; the sequence of Smd3 is most similar to that of the human D3 protein (29b). Therefore, it is highly likely that Smd3 is the homolog of the metazoan D3 core snRNP protein. A third yeast gene that encodes a protein similar in sequence to those of the human D proteins has been identified (3a). These three yeast proteins possibly encode the yeast counterparts of metazoan core D snRNP proteins.

SMD3 is essential for vegetative growth, as is the case for the majority of yeast genes whose products are involved in pre-mRNA processing (for a review, see reference 42). Depletion of Smd3 from yeast cells, using a *GAL1*::*SMD3* conditional allele, results in accumulation of *CRY1* and *ACT1* pre-mRNAs. This suggests that Smd3 is required for splicing in vivo. However, splicing is not completely abolished even after shifting yeast cells bearing the *GAL1*::*SMD3* allele to glucose medium for 21 h. At this time point, the growth rate of these cells is decreased considerably but some mature mRNA is still detectable. Similar results have been observed after depletion of U5 snRNA (33) or Smd1 (40) using *GAL1* conditional alleles. It is likely that a decrease in growth rate occurs when expression (splicing) of one or more essential yeast genes becomes limiting. The residual splicing observed probably occurs in cells that still contain residual amounts of Smd3.

Antibodies against epitope-tagged Smd3 inhibit splicing in vitro, suggesting that Smd3 is required during spliceosome assembly or the *trans*-esterification reactions. However, previous studies with mammalian U4 snRNA have suggested that association of U4 snRNA with Sm proteins is dispensable for splicing in vitro (51). Therefore, it is possible that Smd3 is

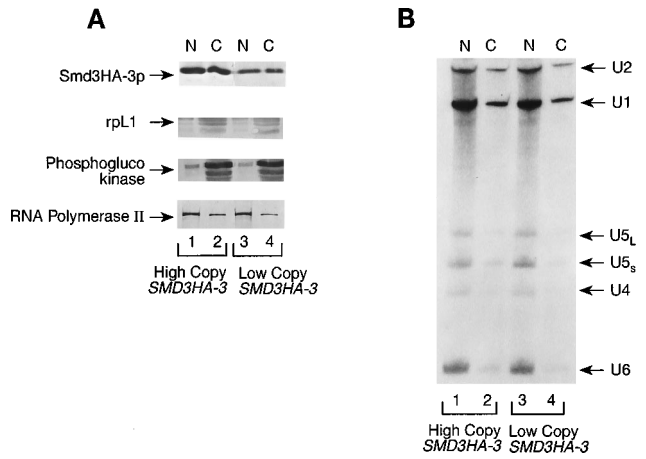

FIG. 8. Localization of Smd3 by cellular fractionation. Strain JWY2456 (lanes 1 and 2), containing *SMD3HA-3* on a high-copy-number plasmid, and strain JWY2455 (lanes 3 and 4), containing *SMD3HA-3* on a low-copy-number (centromeric) plasmid, were partitioned into cytoplasmic and nuclear fractions. (A) Equivalent amounts of each fraction were subjected to immunoblot analysis and probed with anti-HA antibodies, polyclonal antisera against ribosomal protein L1 (rpL1) or phosphoglucokinase, and a monoclonal antibody against RNA polymerase II. (B) RNA from equivalent amounts of nuclei and cytoplasm was extracted and subjected to RNA blot analysis for detection of U1, U2, U4, U5, and U6 snRNAs. N, nuclear fraction; C, cytoplasmic fraction.

present in the active spliceosome but is not absolutely required for pre-mRNA splicing in vitro. The association of the antibody with the Smd3HA-3 antigen could interfere with spliceosome assembly or function by steric hindrance rather than by direct inactivation of Smd3 function. We assume that Smd3 is necessary for splicing in vivo because it is required for snRNP biogenesis. However, our data do not rule out the possibility that Smd3 also plays a direct role in the splicing process.

Smd3 migrates on SDS-polyacrylamide gels as a 14-kDa protein, very similarly to the human D3 protein (1, 27). Unlike Smd1, the Smd3 protein does not appear to have a yeast-specific insertion in its sequence (41). It does contain a highly charged carboxy-terminal tail which is also found in Smd1 and the human D1 protein (41). It is possible that this highly charged region mediates binding to the snRNAs during snRNP assembly. The remainder of the protein could be involved in interactions with other core snRNP proteins or have functions in pre-mRNA splicing that have thus far not been tested for core snRNP proteins. Future studies of mutant alleles of *SMD3* will help to investigate these possibilities.

Although the three core snRNP D proteins have similar sequences, Smd1 and Smd3 clearly do not have identical functions in *S. cerevisiae*. The lethal null allele *smd3Δ1*::*LEU2* cannot be complemented by overexpression of *SMD1*, nor does the overexpression of *SMD3* complement the *smd1*::*LEU2* null mutation. The human D1, D2, and D3 proteins are associated with the U1, U2, U4/U6, and U5 snRNAs (27). We observe coimmunoprecipitation of the U1, U2, U4/U6, and U5 snRNAs with antibodies against either Smd1 or Smd3. However, it is unclear whether all three D proteins, in either metazoans or *S. cerevisiae*, are components of the same snRNPs, i.e., whether each snRNP contains D1, D2, and D3, or whether there are separate populations of snRNPs containing only D1, D2, or D3. The former possibility seems more likely but has yet to be rigorously tested. In this regard, we note that the different U snRNAs are immunoprecipitated to different extents with antibodies against Smd1 and Smd3. For example, the U5 snRNA is reproducibly coimmunoprecipitated to a greater extent by antibodies to Smd3 than by antibodies to Smd1. This could merely be a reflection of the different extents to which the two epitopes are exposed to the antibody. More interestingly, however, this could indicate that different populations of snRNPs consisting of either Smd1 or Smd3 or neither coexist in *S. cerevisiae*. The isolation of genes encoding the yeast counterparts of the metazoan D core proteins not only provides a genetic tool for determining the differences in D protein functions but also can be used for the identification of yeast genes encoding other core snRNP proteins.

snRNP assembly in metazoans is mediated in the cytoplasm by association of the core snRNP proteins into an RNA-free 6S complex (2). The D core proteins are among the first snRNP proteins to begin assembly, together with the E, F, and G polypeptides. This 6S complex of core snRNP proteins then associates with the B and B′ proteins and newly transcribed U1, U2, U4, and U5 snRNAs that have been exported to the cytoplasm to form a pre-snRNP complex. However, the U6 snRNA is not exported to the cytoplasm but associates with the U4 snRNP in the nucleus during the final stages of snRNP assembly (2). One might predict that the cytoplasmic snRNAs would be unstable in the absence of proper formation of the RNA-free core snRNP protein complex. This can be achieved in *S. cerevisiae* by depletion of one or more of the components of such a putative complex by the use of conditional alleles of core protein genes. Indeed, the depletion of Smd1 results in decreased levels of U1, U2, U4, and U5 snRNAs but does not affect the levels of U6 snRNA (40). We observe identical results upon depletion of Smd3, suggesting that snRNP assembly in *S. cerevisiae* may occur by a pathway that is very similar to that in metazoans. Upon depletion of Smd1 or Smd3, we reproducibly observe that the levels of the different U snRNAs are reduced to different extents. Furthermore, the patterns are not identical for Smd1 and Smd3. It is not clear whether these differences reflect different rates of assembly of snRNPs or other mechanisms.

Our results also show that the amount of snRNAs immunoprecipitable by anti-TMG antibodies is greatly reduced upon depletion of Smd3. In metazoans, hypermethylation of the 7-methyl cap occurs in the cytoplasm after assembly of the snRNAs with the core snRNP proteins (30). The modified cap provides one of the signals that targets the pre-snRNP complex to the nucleus (11, 16). Upon depletion of Smd3 in *S. cerevisiae*, a pre-snRNP complex might not form, resulting in incomplete, inappropriate, or no snRNA cap modification. We suggest that lack of cap modification and/or lack of binding to a core snRNP protein targets the snRNAs for degradation. An alternative explanation is that Smd3 functions in the stabilization of snRNPs after they have formed. However, given the similarity of Smd3 to human D proteins and the demonstration that the D proteins are directly involved in snRNP biogenesis in higher eukaryotes (10), it is more likely that Smd3 has a similar role in *S. cerevisiae.*

Studies of mouse fibroblast cells and fully mature *Xenopus* oocytes have shown that unlike other splicing factors, the core snRNP proteins, especially the B, B′, and D proteins, are stored in the cytoplasm as large RNA-free pools (13, 14, 44). Synthesis of snRNAs and assembly of snRNPs in mouse fibroblasts occur normally for over 1 h after inhibition of protein synthesis (13, 14, 44). Pulse-chase experiments with these cells have determined that the half-life of radiolabeled B, B′, and D proteins in the cytoplasm is about 2 h. Upon chasing with unlabeled proteins, these radiolabeled proteins move rapidly to the nucleus, presumably as a part of the pre-snRNP complex.

We determined the intracellular location of Smd3 by both

indirect immunofluorescence microscopy and Western immunoblot analysis of nuclear and cytoplasmic fractions. We observed a strong fluorescent staining in the cytoplasm and a weaker nuclear staining. Cell fractionation determined that about half of the Smd3 protein is localized in the cytoplasmic fractions; a substantially smaller fraction of U snRNAs was present in the cytoplasmic fractions. This evidence strongly suggests that a large pool of Smd3 exists in the cytoplasm. The majority of this pool of Smd3 may not be associated with snRNAs but rather may represent free Smd3 protein or complexes of RNA-free core proteins. This hypothesis is consistent with the immunoprecipitation results in Fig. 6, in which most of the SmD proteins are immunoprecipitated with the anti-HA antibody but only a portion of the snRNAs are coimmunoprecipitated. With the availability of antibodies against the other yeast D core proteins, future experiments can test whether the cytoplasmic pool of Smd3 is associated with them. Such studies will provide further insights into the mechanism of yeast snRNP biogenesis.

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REFERENCES

1. **Anderson, J., R. J. Feeny, and G. W. Zieve.** 1990. Identification and characterization of the small nuclear ribonucleoprotein particle D′ core protein. Mol. Cell. Biol. **10:**4480–4485.
2. **Anderson, J., and G. W. Zieve.** 1991. Assembly and intracellular transport of snRNP particles. BioEssays **13:**57–64.
3. **Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.

3a. **Cooper, M., L. Johnston, and J. Beggs.** Personal communication.

4. **Couto, J. R., J. Tamm, R. Parker, and C. Guthrie.** 1987. A *trans*-acting suppressor restores splicing of a yeast intron with a branch point mutation. Genes Dev. **1:**445–455.
5. **Deshmukh, M., Y.-F. Tsay, A. Paulovich, and J. L. Woolford, Jr.** 1993. Yeast ribosomal protein L1 is required for the stability of newly synthesized 5S rRNA and the assembly of 60S ribosomal subunits. Mol. Cell. Biol. **13:**2835–2845.
6. **Elledge, S. J., and R. W. Davis.** 1988. A family of versatile centromeric vectors designed for use in the sectoring shuffle mutagenesis assay in *Saccharomyces cerevisiae*. Gene **70:**303–312.
7. **Fabrizio, P., S. Esser, B. Kastner, and R. Lührmann.** 1994. Isolation of *S. cerevisiae* snRNPs: comparison of U1 and U4/U6.U5 to their human counterparts. Science **264:**261–264.
8. **Fabrizio, P., D. S. McPheeters, and J. Abelson.** 1989. *In vitro* assembly of yeast U6 snRNP: a functional assay. Genes Dev. **3:**2137–2150.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
10. **Fisher, D. E., G. E. Conner, W. H. Reeves, R. Wisniewolski, and G. Blobel.** 1985. Small nuclear ribonucleoprotein particle assembly *in vivo*: demonstration of a 6S RNA-free core precursor and post-translational modification. Cell **42:**751–758.
11. **Fisher, U., and R. Lührmann.** 1990. An essential signalling role for the m_3G-cap in the transport of U1 snRNP to the nucleus. Science **249:**786–789.
12. **Fisher, U., V. Sumpter, M. Sekine, T. Satoh, and R. Lührmann.** 1993. Nucleo-cytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m_3G cap. EMBO J. **12:**573–583.
13. **Forbes, D. J., T. B. Kornberg, and M. W. Kirschner.** 1983. Small nuclear RNA transcription and ribonucleoprotein assembly in early *Xenopus* development. J. Cell Biol. **97:**62–72.
14. **Fritz, A., R. Parisot, D. Newmeyer, and E. M. De Robertis.** 1984. Small nuclear U-ribonucleoproteins in *Xenopus laevis* development. J. Mol. Biol. **178:**273–285.
15. **Futcher, B., and J. Carbon.** 1986. Toxic effects of excess cloned centromeres. Mol. Cell. Biol. **6:**2213–2232.
16. **Hamm, J., E. Darzynkiewicz, S. M. Tahara, and I. W. Mattaj.** 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. Cell **62:**569–577.
17. **Heinrichs, V., W. Hackl, and R. Lührmann.** 1992. Direct binding of small nuclear ribonucleoprotein G to the Sm site of small nuclear RNAs. J. Mol. Biol. **227:**15–28.
18. **Hieter, P., and R. S. Sikorski.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
19. **Hodges, P. E., M. Plumpton, and J. D. Beggs.** Splicing factors in the yeast *Saccharomyces cerevisiae*. *In* A. Krainer (ed.), Eukaryotic mRNA processing: frontiers in molecular biology, in press. Oxford University Press, Oxford.
20. **Hoet, R. M., B. Kastner, R. Lührmann, and W. J. Venrooij.** 1993. Purification and characterization of human autoantibodies directed to specific regions on U1 RNA; recognition of native U1RNP complexes. Nucleic Acids Res. **21:**5130–5136.
21. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
22. **Johnston, M., and R. Davis.** 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:** 1440–1448.
23. **Jones, M. H., and C. Guthrie.** 1990. Unexpected flexibility in an evolutionarily conserved protein-RNA interaction: genetic analysis of the Sm binding site. EMBO J. **9:**2551–2561.
24. **Kuchler, K., H. G. Dohlman, and J. Thorner.** 1993. The a-factor transporter (*STE6* gene product) and cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. **120:**1203–1215.
25. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. Methods Enzymol. **154:** 367–382.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lehmeier, T., K. Foulaki, and R. Lührmann.** 1990. Evidence for three distinct D proteins, which react differentially with anti-Sm autoantibodies, in cores of the major snRNPs U1, U2, U4/U6 and U5. Nucleic Acids Res. **18:**6475–6484.
28. **Liautard, J.-P., J. Sri-Widada, C. Brunel, and P. Jeanteaur.** 1982. Structural organization of ribonucleoproteins containing small nuclear RNAs from Hela cells. J. Mol. Biol. **162:**623–643.
29. **Lin, R.-J., A. J. Newman, S.-C. Cheng, and J. Abelson.** 1985. Yeast mRNA splicing *in vitro*. J. Biol. Chem. **260:**14780–14792.

29a. **Lührmann, R.** Personal communication.

29b. **Lührmann, R.** Unpublished data.

30. **Mattaj, I.** 1986. Cap trimethylation of U snRNAs is cytoplasmic and dependent on U snRNP protein binding. Cell **46:**905–911.
31. **Mattaj, I. W., and E. M. De Robertis.** 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell **40:**111–118.

31a. **Moritz, M.** 1991. Ph.D. thesis. Carnegie-Mellon University, Pittsburgh, Pa.

32. **Moritz, M., A. G. Paulovich, Y.-F. Tsay, and J. L. Woolford, Jr.** 1991. Assembly of 60S ribosomal subunits is perturbed in temperature-sensitive yeast mutants defective in ribosomal protein L16. Mol. Cell. Biol. **11:**5681–5692.
33. **Patterson, B., and C. Guthrie.** 1987. An essential yeast snRNA with a U5-like domain is required for splicing *in vivo*. Cell **49:**613–624.

33a. **Preston, R., and E. Jones.** Personal communication.

34. **Preston, R., M. F. Manolson, K. Becherer, E. Weidenhammer, D. Kirkpatrick, R. Wright, and E. W. Jones.** 1991. Isolation and characterization of *PEP3*, a gene required for vacuolar biogenesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**5801–5812.
35. **Pringle, J. R., A. E. M. Adams, D. G. Drubin, and B. K. Haarer.** 1991. Immunofluorescence methods for yeast. Methods Enzymol. **194:**565–602.
36. **Riedel, N., S. Wolin, and C. Guthrie.** 1987. A subset of yeast snRNAs contains functional binding sites for the highly conserved Sm antigen. Science **235:**328–331.
37. **Rokeach, L. A., J. A. Haselby, and S. Hoch.** 1988. Molecular cloning of a cDNA encoding the human Sm-D autoantigen. Proc. Natl. Acad. Sci. USA **85:**4832–4836.
38. **Rokeach, L. A., J. A. Haselby, and S. O. Hoch.** 1992. Overproduction of a human snRNP-associated Sm-D autoantigen in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene **118:**247–253.
39. **Rothstein, R. J.** 1983. One step gene disruption in yeast. Methods Enzymol. **101:**202–211.

40. **Rymond, B. C.** 1993. Convergent transcripts of the yeast *PRP38-SMD1* locus encode two essential splicing factors including the D1 core snRNP polypeptide. Proc. Natl. Acad. Sci. USA **90:**848–852.
41. **Rymond, B. C., L. A. Rokeach, and S. O. Hoch.** 1993. Human snRNP polypeptide D1 promotes pre-mRNA binding in yeast and defines non-essential yeast Smd1p sequences. Nucleic Acids Res. **21:**3501–3505.
42. **Rymond, B. C., and M. Rosbash.** 1992. Yeast pre-mRNA splicing, p. 143–192. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast *Saccharomyces cerevisiae*: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
44. **Sauterer, R. A., R. J. Feeny, and G. W. Zieve.** 1988. Cytoplasmic assembly of snRNP particles from stored proteins and newly transcribed snRNAs in L929 mouse fibroblasts. Exp. Cell Res. **176:**344–359.
45. **Sauterer, R. A., A. Goyal, and G. W. Zieve.** 1990. Cytoplasmic assembly of small nuclear ribonucleoprotein particles from 6S and 20S RNA-free intermediates in L929 mouse fibroblasts. J. Biol. Chem. **265:**1048–1058.
45a. **Schmidtt, M.** Personal communication.
46. **Schneider, C., R. A. Newman, D. R. Sutherland, U. Asser, and M. F. Greaves.** 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. J. Biol. Chem. **257:**10766–10769.
47. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics, p. 166–167. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Siliciano, P. G., M. H. Jones, and C. Guthrie.** 1987. *Saccharomyces cerevisiae* has a U1-like small nuclear RNA with unexpected properties. Science **237:** 1484–1487.
49. **Thompson, N. E., D. B. Aronson, and R. R. Burgess.** 1990. Purification of eukaryotic RNA polymerase II by immunoaffinity chromatography. J. Biol. Chem. **265:**7069–7077.
50. **Tollervey, D., and I. Mattaj.** 1987. Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. EMBO J. **6:**469–476.
51. **Wersig, C., and A. Bindereif.** 1992. Reconstitution of functional mammalian U4 small nuclear ribonucleoprotein: Sm protein binding is not essential for splicing in vitro. Mol. Cell. Biol. **12:**1460–1468.
52. **Wise, J. A.** 1991. Preparation and analysis of low molecular weight RNAs and small ribonucleoproteins. Methods Enzymol. **194:**405–415.
53. **Zeller, R., T. Nyffenegger, and E. M. De Robertis.** 1983. Nucleocytoplasmic distribution of snRNPs and stockpiled snRNA-binding proteins during oogenesis and early development in *Xenopus laevis*. Cell **32:**425–434.