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Identification and characterization of Uss1p (Sdb23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins

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The *SDB23* gene of *Saccharomyces cerevisiae* was isolated in a search for high copy-number suppressors of mutations in a cell cycle gene, *DBF2*. *SDB23* encodes a 21 276 Da protein with significant sequence similarity to characterized mammalian snRNP core proteins. Examination of multiple sequence alignments of snRNP core proteins with Sdb23p indicates that all of these proteins share a number of highly conserved residues, and identifies a novel motif for snRNP core proteins. Sdb23p is essential for cell viability and is required for nuclear pre-mRNA splicing both *in vivo* and *in vitro*. Extracts prepared from Sdb23p-depleted cells are unable to support splicing and have vastly reduced levels of U6 snRNA. The stability of U1, U2, U4 and U5 spliceosomal snRNAs is not affected by the loss of Sdb23p. Antibodies raised against Sdb23p strongly co-immunoprecipitate free U6 snRNA and U4/U6 base-paired snRNAs. These results establish that *SDB23* encodes a novel U6 snRNA-associated protein that is essential for the stability of U6 snRNA. We therefore propose the more logical name *USS1* (*U-Six SnRNP*) for this gene.

Key words: core protein/RNA splicing/snRNP/U6 snRNA/yeast

Introduction

Nuclear pre-mRNA splicing involves two distinct, sequential *trans*-esterification reactions that occur within a large, highly dynamic complex termed the spliceosome. Splicing requires ATP and is dependent upon multiple *trans*-acting factors that interact and associate with one another and with conserved *cis*-elements within the pre-mRNA. The mechanism of the two-step splicing pathway is highly conserved from yeast to mammals (reviewed in Green, 1991; Moore *et al.*, 1993), as are at least some splicing factors. The major subunits of the spliceosome are five small nuclear ribonucleoprotein particles (snRNPs); U1, U2, U4, U5 and U6. These snRNPs, in conjunction with other protein factors, play critical roles in defining introns and folding pre-mRNAs into a conformation suitable for catalysis, and may play catalytic roles in the splicing reaction (reviewed in Moore *et al.*, 1993). As characterized in metazoans each snRNP, with the exception of U6, is composed of a single small nuclear RNA (snRNA) with a tri-methylguanosine (TMG) cap, a set of core proteins (B, B′, D1, D2, D3, E, F and G; in order of decreasing apparent molecular weight) and a variable number of snRNP-specific proteins (reviewed in Lührmann *et al.*, 1990). Unlike the others, U6 snRNA is transcribed by RNA polymerase III, has a γ-monomethyl guanosine cap structure and does not directly bind the core proteins since it lacks the appropriate structural motif, the Sm site (Branlant *et al.*, 1982).

The U6 snRNA associates with U4 snRNP and enters the spliceosome as a U4/U6.U5 tri-snRNP complex. A number of yeast proteins associated with U6-containing particles have been identified. Prp24 protein has three RNA recognition motifs and is associated with U6 snRNA (Shannon and Guthrie, 1991). Prp3 and Prp4 proteins are components of the U4/U6 snRNP (Banroques and Abelson, 1989; Abovich *et al.*, 1990; Bordonné *et al.*, 1990; Xu *et al.*, 1990) and Prp6p is a U4/U6.U5 tri-snRNP-associated protein required for the stability and accumulation of the tri-snRNP complex (Gallisson and Legrain, 1993). All these proteins are required for RNA splicing and their *in vivo* inactivation influences the cellular levels of U6 snRNA (Blanton *et al.*, 1992 and references therein; Hu *et al.*, 1994).

The binding of the core proteins to the Sm site is an important step in the biogenesis of snRNPs: cytoplasmic cap tri-methylation and nuclear localization of snRNPs are two core protein-dependent functions (Mattaj and De Robertis, 1985; Hamm *et al.*, 1990; Fischer *et al.*, 1993; Plessel *et al.*, 1994). In addition, the core proteins (individually or as a core complex) provide a flexible platform with which the snRNP-specific proteins can associate, adding stability to the snRNP (Nelissen *et al.*, 1994). Focusing on the U1 snRNP, Nelissen *et al.* (1994) detected direct protein–protein interactions between the U1-specific proteins (70K, A and C), and the core proteins B, B′ and D2. These interactions occur in addition to established protein–RNA interactions between U1 snRNA and the U1–70K and A proteins (Query *et al.*, 1989; Scherly *et al.*, 1989).

With yeast, the ability to combine modern molecular biology and genetics with biochemistry has led to the relatively rapid identification and characterization of new factors involved in the nuclear pre-mRNA splicing reaction (reviewed in Ruby and Abelson, 1991; Rymond and Rosbash, 1992). However, conventional genetic screens for splicing mutants have been far from saturating. Most notable is the failure of these screens to isolate mutants affecting the homologues of the metazoan snRNP core proteins. All the evidence available suggests that yeast homologues of the Sm-binding core proteins do exist. First, the yeast U1, U2, U4 and U5 snRNAs, although

not closely conserved in primary sequence, have retained Sm sites (Riedel *et al.*, 1987). These sites are biologically functional (Riedel *et al.*, 1987) and required for snRNP stability (Jones and Guthrie, 1990). Secondly, the yeast snRNAs can be immunoprecipitated (albeit weakly) by human anti-Sm antisera (Siliciano *et al.*, 1987; Tollervey and Mattaj, 1987), indicating that epitopes of the core proteins have been conserved to some degree. Thirdly, large scale immunoaffinity purification of spliceosomal snRNPs from yeast has detected a number of candidates for snRNP core proteins (Fabrizio *et al.*, 1994). Two yeast genes, *SMD1* and *SMD3*, that encode polypeptides homologous to the human D1 and D3 proteins have now been identified fortuitously as neighbours of *PRP38* and *PEP3* respectively (Rymond, 1993; Lehmeier *et al.*, 1994; Roy *et al.*, 1995).

A third yeast gene *SDB23* has been identified (Parkes and Johnston, 1992) that encodes a polypeptide with low but significant homology to the snRNP core proteins. Sdb23p is only 25.1 and 26.8% identical to the yeast Smd1 and Smd3 proteins respectively, but significantly, all three proteins share a number of highly conserved residues within their N-terminal domains. All the snRNP core proteins sequenced to date contain these N-terminal conserved amino acids. We propose that these residues form a signature motif for snRNP core proteins. This paper describes the *in vivo* and *in vitro* characterization of *SDB23*, establishing a role for Sdb23p early in the splicing process, and demonstrating an absolute requirement for Sdb23p for U6 snRNA stability. This is the first identification, in any organism, of a U6 snRNP-associated core-like protein.

Results

Isolation of the SDB23 gene

SDB23 was one of three genes isolated in a search for high copy-number suppressors of a mutation in *DBF2*, which encodes a cell cycle protein kinase (Parkes and Johnston, 1992). The suppressing activity of the complementing plasmid was localized by Tn*1000* insertion mutagenesis to a 1.4 kb *Hin*dIII fragment. This region was subjected to DNA sequence analysis and found to contain a single 561 nucleotide uninterrupted open reading frame (ORF) beginning with an ATG codon at position 1 (Figure 1A) and terminating with TAA at positions 562–564. Sequences resembling the yeast transcriptional control consensus TATAA are located at positions −178 (TAATTA) and −78 (TATTA) 5′ of the ORF. Potential 3′ processing sequences are located immediately downstream of the presumed termination codon (Figure 1).

When the nucleotide sequence of the 1.4 kb *Hin*dIII fragment was compared with sequences in the GenBank database, nucleotides −290 to −374 were identified as the *SWI4* promoter sequence (Foster *et al.*, 1993). Thus, the DNA fragment that complements the temperature-sensitive defect of *dbf2-1* contains a single ORF (database accession number X82649) whose transcriptional control elements are adjacent to (if not overlapping) the *SWI4* promoter sequence (Figure 1B). Chromosome mapping of the *SDB23* locus was performed by hybridizing ^{32}P-labelled *SDB23* DNA to a yeast chromosome OFAGE blot. A single band corresponding to chromosome V

A

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-374 TCATAGTGATAAGTAATTTTAAATGTGTTTAAAGTACTACTTTTCCTTTACCGCCAGTT  315
-314 CCTGTACTATGAAAAAGGCAAATTCCGCATTGTAGCCGCCCACACGGCATTTTGATCATC 255
-254 AATTACGAAATTTGCCGCACACGTGTCACGTGATAAGCACTCTTACTATCATGTTTTACG 195
-194 GAGTAGCAATGATGTTTAATTATTGCAGCTTTCTTTCGTGAAATCGTAGTATCATAGACC 135
-134 TTCCTAATGATGGAAGCGGTAAAGAAGGAAATCGTAAAAGTAAATTAACGAAGTAGTATT -75
 -74 AGTAAAACAGAGTTGAAAAACTGATAAATCTTCAACTCGAACTGAAAAGAAACACAATAG -15

 -14 AATATTTTTCCCTCAATGCTACCTTTATATCTTTTAACAAATGCGAAGGGACAACAAATGC  46
                M  L  P  L  Y  L  L  T  N  A  K  G  Q  Q  M  Q
  47 AAATAGAATTGAAAAACGGTGAAATTATACAAGGGATATTGACCAACGTAGATAACTGGA 106
     I  E  L  K  N  G  E  I  I  Q  G  I  L  T  N  V  D  N  W  M
 107 TGAACCTTACTTTATCTAATGTAACCGAATATAGTGAAGAAAGCGCAATTAATTCAGAAG 166
     N  L  T  L  S  N  V  T  E  Y  S  E  E  S  A  I  N  S  E  D
 167 ACAATGCTGAGAGCAGTAAAGCCGTAAAATTGAACGAAATTTATATTAGAGGGACTTTTA 226
     N  A  E  S  S  K  A  V  K  L  N  E  I  Y  I  R  G  T  F  I
 227 TCAAGTTTATCAAATTGCAAGATAATATAATTGACAAGGTCAAGCAGCAAATTAACTCCA 286
     K  F  I  K  L  Q  D  N  I  I  D  K  V  K  Q  Q  I  N  S  N
 287 ACAATAACTCTAATAGTAACGGCCCTGGGCATAAAAGATACTACAACAATAGGGATTCAA 346
     N  N  S  N  S  N  G  P  G  H  K  R  Y  Y  N  N  R  D  S  N
 347 ACAACAATAGAGGTAACTACAACAGAAGAAATAATAATAACGGCAACAGCAACCGCCGTC 406
     N  N  R  G  N  Y  N  R  R  N  N  N  N  G  N  S  N  R  R  P
 407 CATACTCTCAAAACCGTCAATACAACAACAGCAACAGCAGTAACATTAACAACAGTATCA 466
     Y  S  Q  N  R  Q  Y  N  N  S  N  S  S  N  I  N  N  S  I  N
 467 ACAGTATCAATAGCAACAACCAAAATATGAACAATGGTTTAGGTGGGTCCGTCCAACATC 526
     S  I  N  S  N  N  Q  N  M  N  N  G  L  G  G  S  V  Q  H  H
 527 ATTTTAACAGCTCTTCTCCACAAAAGGTCGAATTTTAAACAAATTTTGTATTATAATAAT 586
     F  N  S  S  S  P  Q  K  V  E  F

 587 TATGTACATATATAAATATATTGGTACATATGTACTGTGTGTGATGTGAATGTTGATTAC 646
 647 CGTTTTCTTT
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B

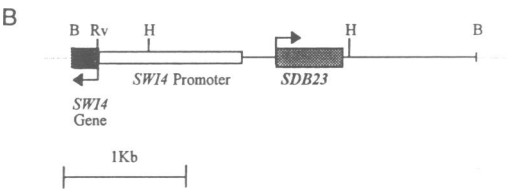

Fig. 1. Sequence of *SDB23* and a physical map of the *SDB23* chromosomal locus. (**A**) *SDB23* nucleotide and predicted polypeptide sequences (database accession number X82649). Potential TATAA elements are underlined, the presumed initiation codon is in bold. The region corresponding to part of the *SWI4* promoter sequence is italicized. (**B**) Restriction map of the *SDB23* chromosomal locus isolated on a *Bam*HI fragment showing the *SDB23* gene (hatched box), *SWI4* promoter (open box) and gene (black box). Arrows indicate direction of transcription. Restriction sites: B, *Bam*HI; H, *Hin*dIII; Rv, *Eco*RV.

was detected (data not shown). Complete sequencing of chromosome V has identified the precise location of *SDB23* on the right arm of this chromosome (database accession number Sc9781).

SDB23 encodes a polypeptide with low but significant homology to snRNP core proteins

The *SDB23* gene encodes a predicted 187 amino acid protein with a calculated molecular weight of 21 276 Da. An initial comparison of the *SDB23*-encoded ORF with protein sequences in the Gen/EMBL database revealed that Sdb23p is an uncharacterized protein with low but significant sequence similarity to two snRNP core proteins, human D1 polypeptide (Rokeach *et al.*, 1988) and its yeast homologue Smd1p (Rymond, 1993).

A more extensive search of protein databases available on the EMBL-Heidelberg and NCBI network file-servers, using the BLITZ (Sturrock and Collins, 1993) and BLAST (Altschul *et al.*, 1990) search programs respectively with varied search-sensitivity levels, found that the proteins most closely related to the predicted yeast polypeptide are snRNP core proteins, known to play a critical role in the structure and function of the spliceosome (reviewed in Lührmann *et al.*, 1990). The amino-terminal halves of

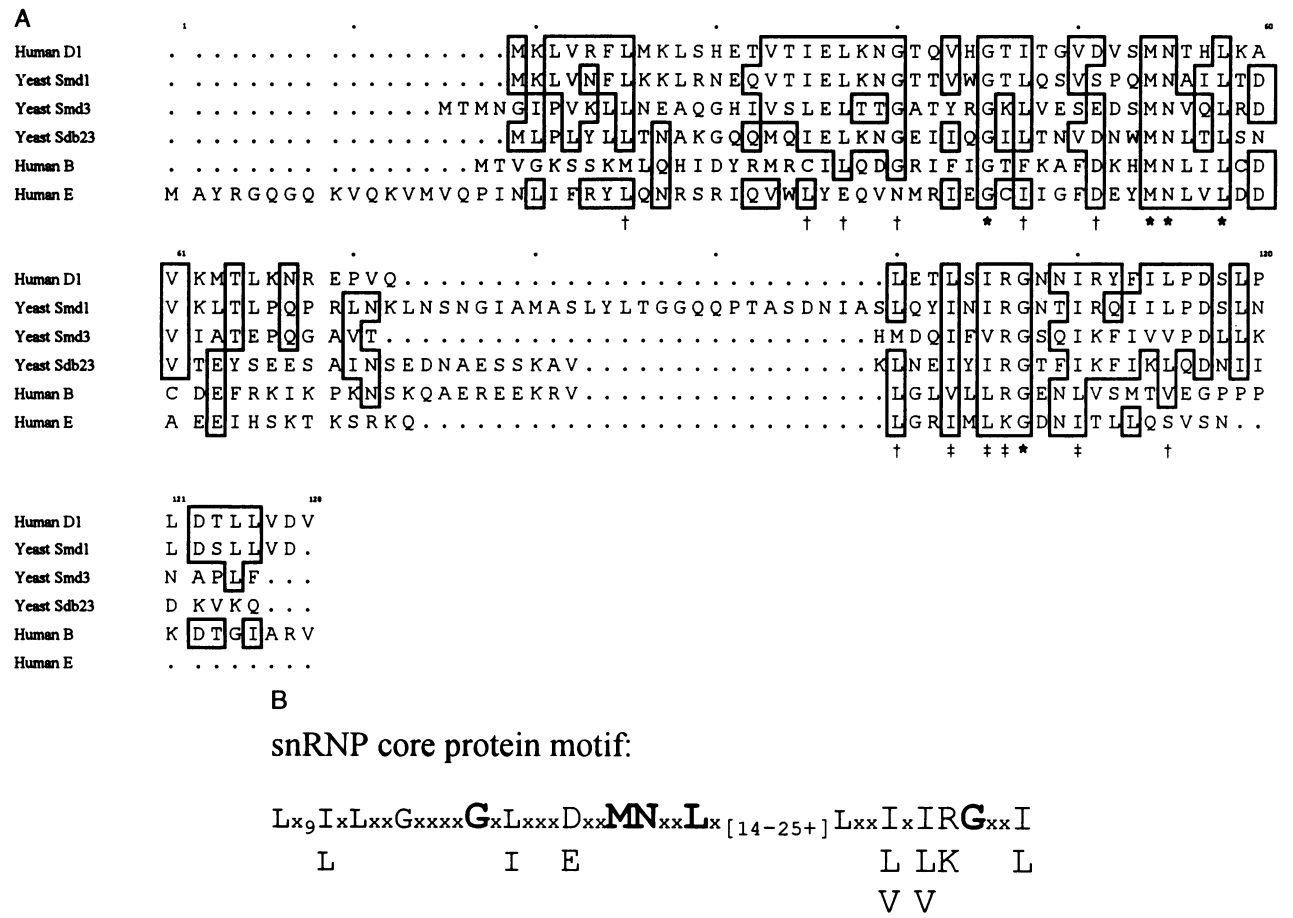

Fig. 2. Alignment of N-terminal domains of multiple snRNP core proteins identifying a novel snRNP protein motif. (**A**) snRNP proteins were aligned using the program PILEUP in the GCG7 suite of sequence analysis programs. The snRNP motif was identified visually. Alignment gaps are indicated by dots. The alignment is numbered above using the methionine codon of human E protein as position 1. Protein names are given alongside the sequence. Boxed text indicates positions in which residues belonging to a single conservative grouping are present in at least 50% of the sequences. The degree of conservation is indicated by the following symbols: * , indicates positions which are absolutely invariant; ‡, indicates positions where a single conservative grouping represents 100% of the sequences; ↑, indicates positions where the nature of the residue is conserved in at least 83% of the sequences. Accepted conservative groupings were I = V = L, D = E, R = K, T = S, N = Q, F = Y = W. The Swissprot, EMBL or Genbank accession number for the protein sequences, or for the nucleotide sequences from which the peptide sequence was derived, are as follows: human D1, P13641; yeast Smd1, L04669; yeast Smd3, M65144; yeast Sdb23, X82649; human B, P14678; human E, P08578.
(**B**) Consensus snRNP motif derived from the alignment of multiple published snRNP core protein sequences. During the revision of this manuscript the sequences of core polypeptides D2 and D3 from HeLa were published (Lehmeier *et al.*, 1994). Incorporation of these sequences into Figure 2A supports the consensus derived from this alignment with the exception of the C-terminal conserved hydrophobic residue at position 115. This has been considered in deriving the snRNP core protein motif shown in B. The font size reflects the degree of conservation at any position with absolutely invariant residues highlighted in enlarged, bold text. At positions where alternative residues within a conserved grouping do occur, these residues are listed in order of preference below the most commonly used residue. x represents any residue and the spacing parameters are shown by numbers; + indicates that this number is variable.

these proteins share the greatest degree of sequence similarity (Figure 2A), the carboxy-terminal portions diverging considerably.

The amino-terminal conserved sequence of all the snRNP core proteins and Sdb23p can be split into two highly conserved regions. The first contains four invariant residues; Gly45, Met54, Asn55 and Leu58 (numbered according to alignment, Figure 2A). In addition, there appears to be a preference for an acidic residue, either aspartate or glutamate, two positions upstream of the conserved methionine residue. The first conserved region is separated from the second by a variable number of amino acids, typically 14–25. The yeast Smd1p is atypical in its longer amino-terminal sequence and introduces the only major gap in the alignment (Figure 2A, residues 72–98). The second region consists of only one invariant residue, glycine at position 107. However, immediately prior to the conserved glycine, there is a clear preference for a basic residue (Arg/Lys106), which is preceded by a hydrophobic residue, either isoleucine, leucine or valine. At three additional positions hydrophobic residues are preferred (Leu100, Ile/Leu/Val103 and Ile/Leu110). A novel motif characteristic of the snRNP core protein family has been derived from the alignment of published snRNP core protein sequences (Figure 2B).

Sdb23 is a small hydrophilic protein that has an overall positive charge (pI = 10.31) in common with the majority of snRNP proteins. However, the nature of this charge distribution is uneven; the amino-terminal half of the protein is essentially neutral with an approximately equal number of basic and acidic residues. In contrast the carboxy-terminal half contains a preponderance of basic amino acids, its most notable feature being the presence of a particularly asparagine-rich segment; 85% of the

carboxy-terminal 97 amino acids are hydrophilic with 35% of these being asparagine residues. This asparagine-rich segment distinguishes Sdb23p from all the other snRNP core proteins, which tend to have an abundance of arginine and glycine residues.

SDB23 is an essential gene

To determine whether *SDB23* encodes an essential function, a heterozygous diploid strain (MCY5) was constructed in which one *SDB23* locus had *LEU2* integrated at the presumed initiation codon, disrupting read-through from the promoter sequence. After sporulation of the *sdb23::LEU2/SDB23* diploid, 10 independent tetrads were dissected, all of which contained only two viable spores; in each case the viable cells were auxotrophic for leucine, indicating that disruption of the *SDB23* gene results in loss of viability. Genomic Southern blot analyses confirmed the chromosomal configurations of the *sdb23::LEU2/SDB23* diploid and of the haploid progeny (data not shown). Thus *SDB23* encodes a novel activity essential for cell viability.

In vivo depletion of Sdb23p arrests cell growth

To address the potential function of Sdb23p, a haploid strain, MCY4, was constructed in which the sole chromosomal copy of *SDB23* was under the control of the inducible *GAL1* promoter (*GAL1−SDB23*; see Materials and methods). The growth of strain MCY4 is galactose-dependent; the cells grew on galactose as the sole carbon source but not on glucose (Figure 3A). A plasmid bearing the entire *SDB23* gene with its own promoter (pSDB23-1) was capable of rescuing lethality on glucose medium (Figure 3A).

When the growth rate of MCY4 cells was monitored following a shift to non-permissive glucose medium, the doubling times in galactose or glucose media were almost indistinguishable for the first 10 h (Figure 3B). However, growth began to slow after approximately 11–12 h following the shift to glucose medium and stopped after 15–17 h in glucose. It is possible that over-production of Sdb23p was toxic to MCY4 cells since they grew slower than wild-type (MCY2) cells in galactose medium (data not shown).

Sdb23p is required for nuclear pre-mRNA splicing

To investigate the effect on pre-mRNA splicing of depleting cells of Sdb23p, total RNA was extracted from wild-type and MCY4 cells grown continuously in galactose or shifted to glucose medium for various lengths of time. Splicing efficiency was determined by probing Northern blots with the yeast ribosomal protein gene *RP28* (Figure 4). In the wild-type control cells splicing of the RP28 transcript was efficient and carbon source independent. Interestingly, unspliced RP28 pre-mRNA was detected in MCY4 cells grown continuously in galactose medium (lanes 4–6), although mature mRNA was the predominant species. This partial splicing defect was no longer apparent when MCY4 cells had been in glucose medium for 5 h; only mature mRNA was detected at this time (lane 10). This indicates that over-production of Sdb23p is in some way detrimental to splicing and could explain the poor growth noted for this strain in galactose medium. However, 10 h following the shift to glucose medium, immediately prior to the onset of reduced growth rate, the ratio of spliced RP28 mRNA to unspliced RP28 pre-mRNA had

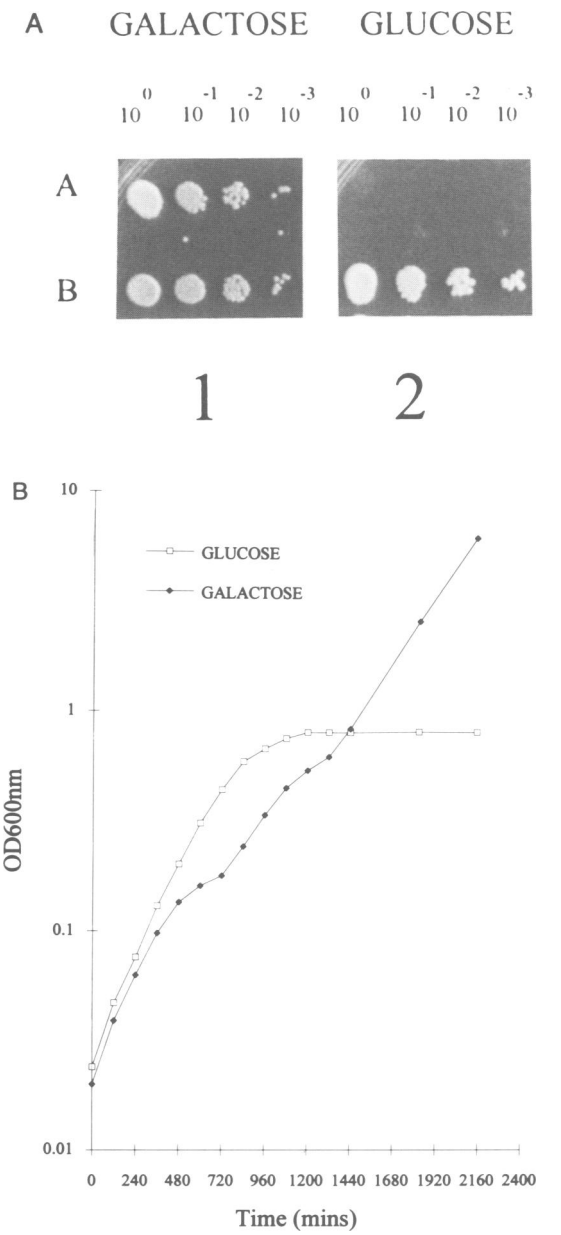

Fig. 3. Depletion of Sdb23p leads to growth arrest. (**A**) Ten-fold serial dilutions of galactose-grown, mid-logarithmic cultures of MCY4 cells, transformed with either pFL39 (row A) or pSDB23-1 (row B) were spotted onto selective galactose (1) and glucose (2) media and incubated at 30°C for 3 days. (**B**) A culture of untransformed MCY4 cells was grown to mid-logarithmic phase in galactose medium, harvested, washed and resuspended in sterile water. Half of this suspension was used to inoculate either fresh pre-warmed galactose or glucose media to an initial OD_{600} of 0.05. Cultures were diluted to keep all OD_{600} readings below 0.6, maintaining the cells in logarithmic growth.

decreased 18-fold (lane 11; data quantified by PhosphorImager analysis). By 20 h, when the cells were no longer capable of further growth, the ratio was dramatically reduced although mature RP28 mRNA could still be detected (lane 12). *ACT1* transcripts, which also contain an intron, behaved similarly, whereas the level of transcripts of the intronless *URA3* gene was unaffected by repression of *SDB23*, indicating that this effect was specific to intron-containing transcripts (data not shown).

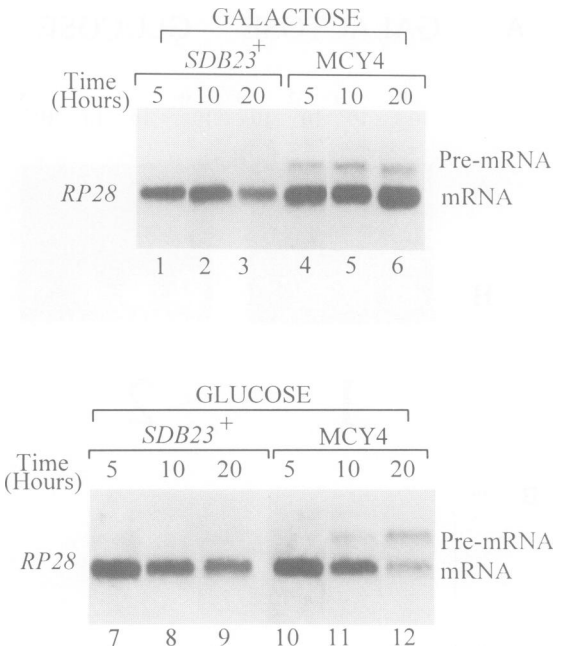

Fig. 4. Sdb23p is required for nuclear pre-mRNA splicing. Total RNA was extracted from wild-type and MCY4 cells grown continuously in galactose or at various times following a shift to glucose medium, resolved on a 1.4% denaturing agarose gel, blotted and probed with radiolabelled *RP28* DNA. The positions of pre-mRNA and mature mRNA are indicated.

Sdb23p is required prior to step 1 of splicing in vitro

The *in vivo* depletion of Sdb23p demonstrated a role for this protein in nuclear pre-mRNA splicing. To determine whether Sdb23p is also required for splicing *in vitro*, extracts prepared from MCY4 cells either grown continuously in galactose or transferred to glucose for 13 h (i.e. just prior to the point of complete growth arrest) were tested for their ability to splice RP28 transcript. Extracts derived from cells grown in galactose efficiently spliced RP28 transcript to mature mRNA (Figure 5A, lanes 1–3). In contrast, Sdb23p-depleted extracts failed to support splicing (lanes 4–6).

The ability of Sdb23p-depleted extract to form spliceosome complexes was analysed by non-denaturing gel electrophoresis (Pikielny *et al.*, 1986). This gel system resolves three spliceosome complexes, termed I, II and III, from yeast splicing reactions on the basis of their electrophoretic mobility. These complexes assemble in the order III, I and II; splicing reaction intermediates and products are detected in complex II. Figure 5B shows that complex II rapidly formed in extract derived from galactose-grown MCY4 cells. The formation of complex II in these extracts coincided with the detection of mature mRNA and splicing intermediates in the corresponding *in vitro* splicing reaction (Figure 5A, lane 3, compared with Figure 5B, lane 3). In the Sdb23p-depleted extract, the complex assembly stalled following the formation of the pre-spliceosome, complex III (Figure 5B, lanes 4–6). The absence of complex I in the Sdb23p-depleted reaction is consistent with Sdb23p being required for the addition of the U4/U6.U5 tri-snRNP to the pre-spliceosome.

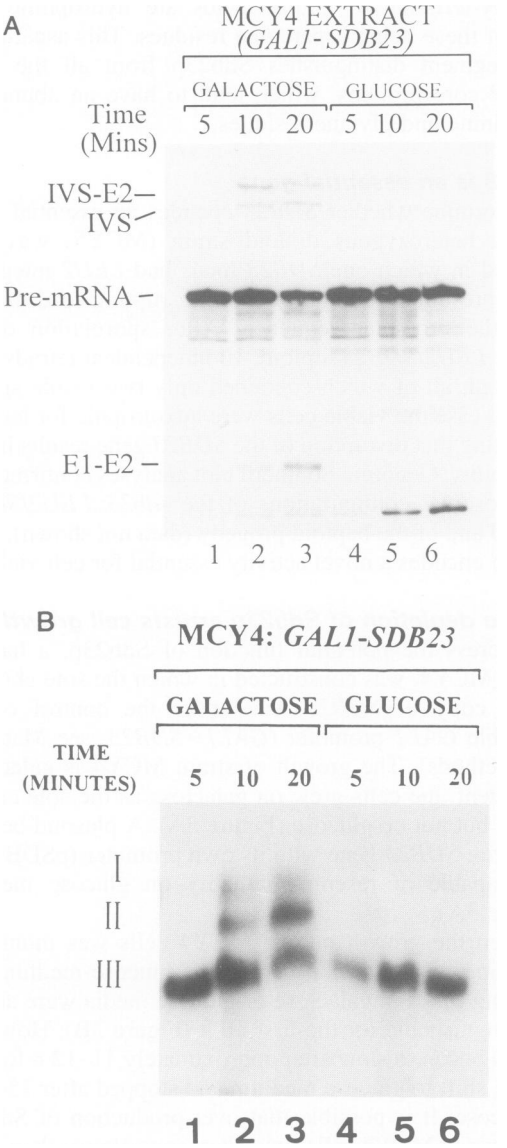

Fig. 5. *In vitro* splicing inactivation following depletion of Sdb23p. (**A**) Extracts prepared from MCY4 cells grown in galactose or in glucose for 13 h were assayed for their ability to splice *RP28* transcript and form spliceosome complexes. Splicing reactions (10 µl) containing radiolabelled *RP28* pre-mRNA were incubated at 25°C for 5, 10 and 20 min, RNA recovered and analysed on a 6% (w/v) denaturing polyacrylamide gel. IVS-E2, lariat intron–exon 2; IVS, lariat excised intron; pre-mRNA, *RP28* substrate RNA; E1-E2, spliced exons. (**B**) For analysis of splicing complex formation, 5 µl aliquots of the splicing reactions shown in (A) were fractionated on a non-denaturing gel; splicing complexes I, II and III as defined by Pikielny *et al.* (1986).

Sdb23p is essential for the stability of U6 snRNA

To investigate the possibility that the stability of the spliceosomal snRNAs was affected by *in vivo* depletion of Sdb23p, total RNA was extracted at various times from MCY4 cells grown in glucose medium. Northern analysis showed that depletion of Sdb23p resulted in a dramatic reduction in the level of U6 snRNA (Figure 6). After 10 h growth in glucose medium, when growth still appeared to be normal, U6 snRNA was reduced to 40–50% of its original level. After 20 h, the level of U6 snRNA was further reduced to 10–20% of the original level. In a control experiment, the level of U6 snRNA in a wild-type

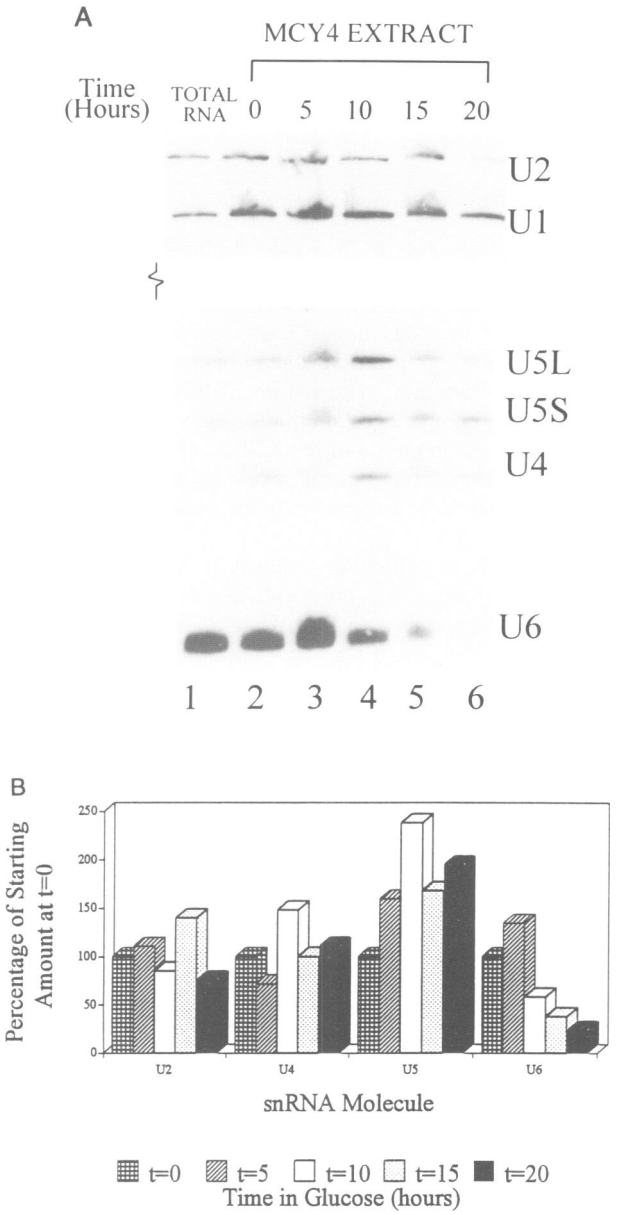

Fig. 6. Effect of *in vivo* depletion of Sdb23p on snRNA stability. (**A**) Total RNA was extracted from MCY4 cells grown for various lengths of time in glucose medium, fractionated on a 6% denaturing polyacrylamide gel, blotted and probed with oligonucleotides specific for the snRNAs (Materials and methods). (**B**) Levels of snRNAs were quantified, values normalized against U1 snRNA and plotted as a percentage of the starting amount as 100%. U5L and U5S data were combined and plotted as a single value. Probing with the intron-less *URA3* validated using U1 snRNA as a standard (data not shown). Note: the variability observed in this experiment for the level of U2 snRNA is not due to depletion of Sdb23p. In several identical experiments the only reproducible significant effect observed was the reduction in the level of U6 snRNA.

strain was not affected by carbon source (data not shown). Although the levels of U1, U2, U4 and U5 snRNAs in both wild-type and mutant cells fluctuated, this was not significant compared with the reproducible effect on U6 of depleting Sdb23p (Figure 6 and data not shown).

Immunoprecipitation of the spliceosome-associated snRNAs with antibodies raised against either the TMG-cap of the snRNAs, or the U5 snRNP-specific protein

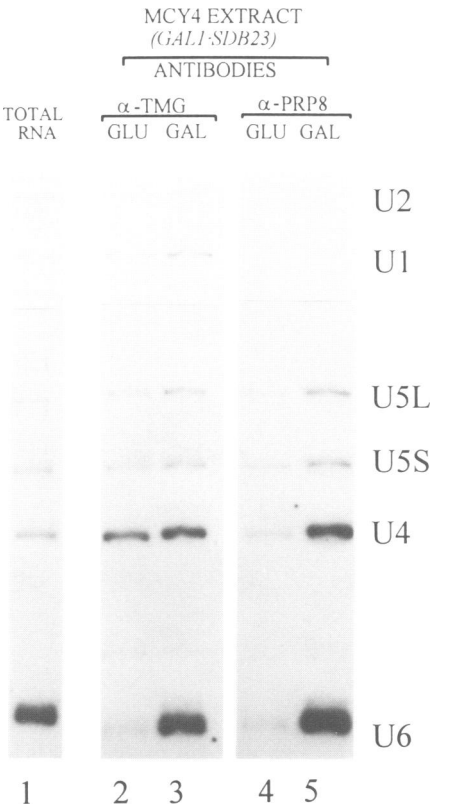

Fig. 7. Immunoprecipitation of snRNAs with antibodies against TMG or Prp8p. Extract derived from cells grown either continuously in galactose (GAL) or in glucose (GLU) for 13 h was subjected to immunoprecipitation with either anti-TMG (lanes 2 and 3) or Prp8 (lanes 4 and 5) antibodies. Incubation of the extract with antibodies and subsequent washes were in 150 mM salt. The RNAs were recovered and analysed as described for Figure 6A. Lane 1 shows total RNA from galactose-grown MCY4 extract.

Prp8 confirmed that Sdb23p is not required for the maintenance of U1, U2, U4 and U5 snRNA stability (Figure 7). However, the amount of U4 snRNA associated with U5 snRNA (and thereby co-precipitated by anti-Prp8 antibodies) was significantly reduced in Sdb23p-depleted extracts (compare lanes 4 and 5), suggesting that the association of U4 with U5 is affected by the reduced level of U6 snRNA.

Association of Sdb23p with U6 snRNA

To facilitate the further characterization of Sdb23p, polyclonal antibodies were generated against a β-galactosidase−Sdb23 fusion protein. Immunoblot analysis of wild-type yeast splicing extracts probed with immune serum specifically recognized a polypeptide with an apparent molecular weight of 21–22 kDa. This polypeptide was not detected by pre-immune serum, nor by immune serum when probing cell extracts depleted of Sdb23p (data not shown). To determine whether any spliceosomal snRNAs were associated with Sdb23p, RNAs co-immunoprecipitated with Sdb23p polyclonal antibodies were analysed by Northern blotting. Pre-immune serum did not immunoprecipitate any RNA species above background level, whereas at low salt concentrations U1, U2 (albeit weakly), plus U4, U5 and U6 snRNAs were detected in the immune pellet (Figure 8A, lanes 2–5). After extensive washing at moderate to high salt concentrations (500 and 750 mM

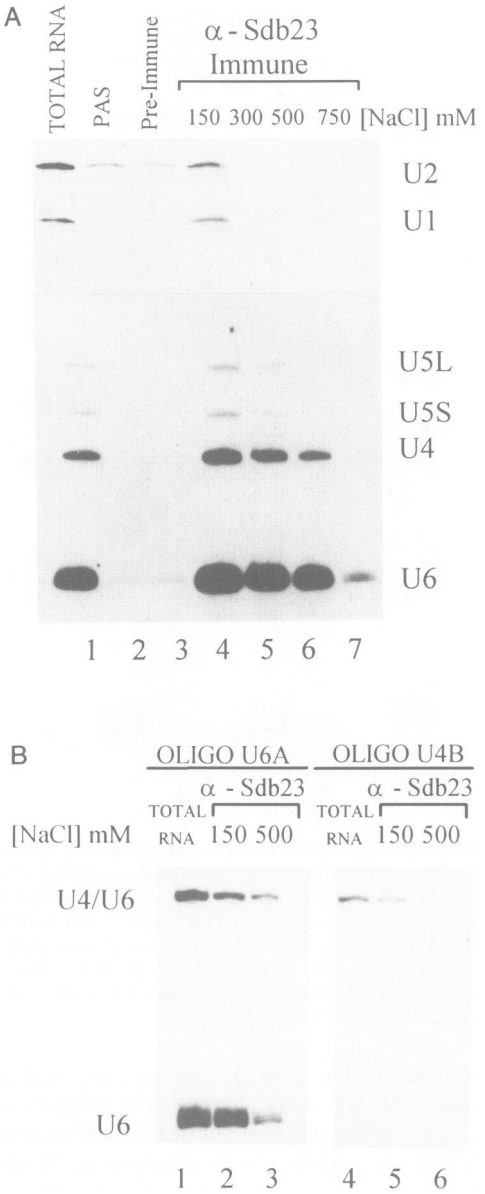

Fig. 8. Co-immunoprecipitation of U4, U5 and U6 snRNAs with Sdb23p. (**A**) Wild-type splicing extract was subjected to immunoprecipitation with pre-immune serum (lane 3) or anti-Sdb23 antibodies (lanes 4–7). Incubation of the extract with antibodies was in 150 mM salt; washes were performed using various concentrations of salt (lane 4, 150 mM; 5, 300 mM; 6, 500 mM; 7, 750 mM). The RNAs were recovered and analysed as described for Figure 6A. Lane 1 shows total RNA from the same extract. Lane 2 indicates background precipitation due to non-specific binding of the snRNAs to protein A–Sepharose (PAS). (**B**) For analysis of free U6 and U4/U6 snRNPs, half the RNA samples immunoprecipitated from wild-type splicing extract with anti-Sdb23 antibodies were resolved on a non-denaturing polyacrylamide gel (Zairong and Brow, 1993), blotted and probed successively with oligonucleotides complementary to U6 snRNA (lanes 1–3) and U4 snRNA (lanes 4–6). Lanes 2 and 5 correspond to samples shown in lanes 4 and 6 respectively in (A).

NaCl) the precipitate with the immune serum contained U4 and U6 snRNA (lanes 6 and 7). These results indicated an association between Sdb23p and the U4/U6 snRNP and perhaps to a lesser extent the U4/U6.U5 tri-snRNP.

To determine whether Sdb23p was associated with free U6, the snRNAs co-immunoprecipitated by Sdb23 antibodies were fractionated on a non-denaturing polyacrylamide gel (Figure 8B) and probed successively with oligonucleotides complementary to U6 or U4 snRNAs. The result clearly demonstrates that Sdb23p associates with free U6 snRNA as well as with the base-paired U4/U6 snRNAs.

Discussion

SDB23 was isolated as a weak suppressor of a *dbf2* mutation that has a cell cycle phenotype, being defective in late mitosis and in DNA synthesis (Parkes and Johnston, 1992). *DBF2* encodes a cell cycle protein kinase that functions late in the mitotic cycle and is probably required for the G_1/S transition. The nature of this suppression is not understood nor is it certain that suppression is mediated by *SDB23*. Cells depleted of the *SDB23*-encoded polypeptide do not have an obvious cell cycle phenotype and their primary defect is a reduction in the cellular content of U6 snRNA.

On the basis of sequence similarity to human and yeast snRNP core proteins, this polypeptide is a core-like snRNP protein. Unlike the known core proteins, however, it only associates with free U6 and U6-containing particles. We therefore propose the more logical name *USS1* (U Six SnRNP) for this gene, as its original name *SDB23* was based on its uncharacterized suppressor activity (Parkes and Johnston, 1992).

USS1/SDB23 has been shown to be essential for viability. The long lag after shifting cells to glucose medium before a substantial effect is seen on the growth may indicate slow turnover of this protein. Typically, shutting off the synthesis of essential, stable proteins or snRNAs results in a lag before growth rate slows, since dilution to limiting levels requires several generations (e.g. Strauss and Guthrie, 1991).

The decreased growth rate of MCY4 cells depleted of Uss1p/Sdb23p corresponded with reduced splicing efficiency in these cells, as evidenced by a decrease in the mRNA:pre-mRNA ratio for the two transcripts examined. The partial splicing defect observed with MCY4 cells grown continuously in galactose could be a consequence of Uss1p/Sdb23p overproduction. Excess Uss1p/Sdb23p could potentially interact with and titrate out other factors (possibly U6-associated proteins) that are required for nuclear pre-mRNA splicing.

In vitro, extracts depleted of Uss1p failed to support splicing. Spliceosome complex assembly in these extracts was blocked at an early stage, after formation of the U1/U2 snRNP-containing pre-spliceosome complex. These data are consistent with Uss1p being required for the assembly of the U4/U6.U5 tri-snRNP into the spliceosomes, or for the stable formation of the U4/U6.U5 tri-snRNP itself. Indeed, the level of U4/U6.U5 tri-snRNP complex is reduced in the Uss1p-depleted extract.

Point mutations and deletions in the Sm site of yeast spliceosomal snRNAs can have a marked effect on snRNP assembly and snRNA stability, and may block splicing both *in vivo* and *in vitro* (McPheeters *et al.*, 1989; Jones and Guthrie, 1990; Siliciano *et al.*, 1991). In yeast, the spliceosomal snRNAs are extremely sensitive to *in vivo* depletion of Smd1p or Smd3p. The removal of either of these proteins causes a dramatic reduction in the levels of U1, U2, U4 and U5 snRNAs but leaves the level of

U6 snRNA relatively unaffected (Rymond, 1993; Roy *et al.*, 1995). U6 snRNA differs from the other spliceosomal snRNAs in that it lacks an Sm site and, by itself, does not associate with core proteins. In contrast to the *in vivo* depletion of Smd1p or Smd3p, depletion of Uss1p resulted in a vast reduction in the level of U6 snRNA without causing significant changes to the levels of U1, U2, U4 and U5 snRNAs. Thus, although Uss1p resembles the core proteins in terms of sequence similarity and in being required for the stability of an snRNA molecule, it differs in terms of its functional specificity. This is the first identification of a U6-specific protein that is related to the snRNP core proteins.

Depletion of Uss1p could affect the abundance of U6 snRNA through decreased U6 snRNA synthesis or by rendering the RNA more susceptible to decay. U6 snRNA is unique amongst the spliceosomal snRNAs in that it is transcribed by RNA polymerase III (RNA Pol III). Alterations in the intracellular content of Uss1p could specifically affect RNA Pol III transcription without affecting the transcription, and hence levels, of snRNAs produced by RNA polymerase II. Both ethidium bromide staining of RNA fractionated in polyacrylamide gels and 3′ end-labelling of total RNA isolated from Uss1p-depleted extracts showed that the steady state levels of 5S rRNA and of tRNAs (also produced by RNA Pol III) were not affected by the reduction in cellular content of Uss1p (data not shown). This implies that in the absence of Uss1p, the rate of decay of U6 snRNA is accelerated. Uss1p may influence the rate of U6 snRNA decay directly by associating with and stabilizing this RNA. Alternatively, Uss1p could affect other factors required for U6 stability.

Anti-Uss1p antibodies strongly co-immunoprecipitated U4 and U6 snRNAs from a wild-type splicing extract which suggests that Uss1p is associated with the U4/U6 snRNP. U1, U2 and U5 snRNAs were only weakly co-immunoprecipitated with Uss1p at low salt concentrations. The inability to detect U5 snRNA above 300 mM NaCl may reflect the sensitivity of the U4/U6.U5 tri-snRNP complex to high salt concentrations (Abovich *et al.*, 1990; Banroques and Abelson, 1990). However, bearing in mind the high degree of sequence similarity between Smd1p, Smd3p and Uss1p, polyclonal anti-Uss1p antibodies may recognize epitopes shared by all three proteins. Thus, weak immunoprecipitation of U1, U2 and U5 snRNAs could be attributed to the immunoprecipitation of low amounts of Smd1p and Smd3p.

Non-denaturing gel electrophoresis demonstrated a clear association between Uss1p and free U6, in addition to its association with U4/U6 snRNP. These data indicate a direct or indirect interaction between Uss1p and U6 snRNA that is maintained following the physical association of U4 and U6 snRNAs in the U4/U6 snRNP (Madhani and Guthrie, 1994). At present, however, it is not known whether Uss1p is a component of spliceosomes, since antibodies to Uss1p do not detectably interact with the pre-mRNA-containing spliceosome complexes (data not shown). This could be a consequence of masking of the Uss1p epitopes or the absence of Uss1p in these complexes.

U6 snRNA is normally present in excess over U4 snRNA. This excess is believed to be a major factor in driving the formation of U4/U6 snRNP, the favoured state of U6 snRNA. The intracellular content of U6 snRNA drops in response to heat inactivation of *prp3*, *prp4*, *prp6* and *prp24* temperature-sensitive strains (Blanton *et al.*, 1992 and references within). All these factors are essential for nuclear pre-mRNA splicing and are components of U6-containing particles. Prp4p is a component of the U4/U6 snRNP, thought to associate with the 5′ stem–loop of U4 snRNA, although no direct RNA–protein interaction has been demonstrated (Bordonné *et al.*, 1990; Xu *et al.*, 1990). Prp4p is not required for maintaining the stability of U4 snRNA since heat inactivation of a temperature-sensitive *prp4-1* strain significantly reduced the level of U6 but not U4 snRNA (Galisson and Legrain, 1993). Glycerol gradient analysis of *prp4-1* snRNP fractions showed the presence of a free U4 snRNP particle in the absence of functional Prp4 protein and U6 snRNA. Immunoprecipitation of snRNPs from Uss1p-depleted extracts, using antibodies against the TMG-cap, similarly showed that the total cellular level of U4 snRNA was not affected by reduced levels of U6 snRNA. The association of U4 snRNP with U5 snRNP was affected however, as indicated by immunoprecipitations with antibodies raised against the U5 snRNP-specific protein, Prp8.

It is not known whether Uss1p interacts with U6 snRNA directly, or associates indirectly through protein–protein interactions possibly involving Prp24p or other (as yet unidentified) U6-associated proteins. Unlike Prp24p, which has three RNA recognition motifs (RRM) (Shannon and Guthrie, 1991), Uss1p does not contain a good match to any of the characterized RNA-binding motifs (Birney *et al.*, 1993 and references within; Burd and Dreyfuss, 1994). The amino-terminal 80 amino acids of Uss1p do include a potential RNP2 motif, but have a very poor match to the degenerate RNP1 consensus motif (Birney *et al.*, 1993).

USS1 encodes a small, basic protein with an apparent pI value of 10.31. This value is in common with most of the snRNP core proteins which have apparent pI values ranging between 8 and 10. The multiple alignment of published snRNP core protein sequences with Uss1p revealed that the amino-terminal regions of these proteins share a significant degree of similarity and identified a signature motif for snRNP core proteins. This motif consists of five invariant residues, one residue where the consensus is either arginine or lysine, and another position where the consensus is split between aspartate, glutamate and serine, with a clear preference for an acidic residue. There are a number of other highly conserved residues, and in particular three positions are always occupied by hydrophobic residues, either isoleucine, leucine or valine.

Compared with the human D1 protein, yeast Smd1p contains an additional 27 amino acids. Rymond *et al.* (1993) demonstrated that Smd1p lacking the yeast D1-specific sequence (residues 80–99, Figure 2A) is fully functional. However, extending this deletion by six amino acids (residues 79–105, Figure 2A) knocks out Smd1p function (Rymond *et al.*, 1993). The larger deletion encompasses three highly conserved positions in the core protein motif, amino acids Leu100, Ile103 and Ile105, which indicates that residues within this motif are indeed essential for core protein function.

The conserved residues may be required for the correct folding, stability or function of the individual core protein.

In addition, or alternatively, they may mediate protein−protein interactions between individual core proteins. In mammalian cells, snRNP assembly begins with the interaction of core proteins to form an snRNA-free protein complex (Fisher *et al.*, 1985; Lehmeier *et al.*, 1994; R.Lührmann, personal communication) which then associates with the Sm site of the snRNA molecule. The ability of snRNP core proteins to form stable complexes in the absence of an snRNA molecule implies that protein−protein interactions are dominant over RNA−protein interactions in the snRNA−core protein complex. To date, a direct interaction between a core protein and an snRNA within a snRNP has been demonstrated only for the snRNP-G polypeptide and the Sm site of U1 snRNA (Heinrichs *et al.*, 1992).

The amino-terminal domain alignment (Figure 2A) identifies two small sub-motifs that are very highly conserved among the D proteins (VTIELKNG, residues 33–40; ILPD, residues 114–117 of human D1). On the basis of extremely similar regions to these in Uss1p, Uss1p would appear to be structurally most similar to the D proteins. However, the homology between Uss1p and the mammalian D1 (25.1% identical) is much less than that observed between mammalian D1 and the yeast functional homologue, Smd1p (48.7% identical). Uss1p differs most noticeably from the D proteins in two ways. First, the carboxy-terminal domain of Uss1p is extended by 41 and 68 amino acids compared with the human D1 and yeast Smd1 proteins, respectively. Secondly, the carboxy-terminal domain is extremely asparagine-rich. At present, the functional importance of the Uss1p asparagine-rich carboxy-tail is unclear. It is possible that this domain of Uss1p is required for the U6-specificity of this protein.

Conceivably, U6 snRNA could be complexed with as many 'core' proteins as the other spliceosomal snRNAs. Indeed, another yeast gene, *SMX*4, has now been identified which encodes a core protein-like polypeptide that also associates with U6 snRNA particles (Séraphin, 1995). As rapid sequencing of the yeast genome reveals novel open-reading frames, the proposed consensus should aid in the identification of other members of this apparently expanding family of proteins.

Materials and methods

Strains, plasmids and RNA substrates

The *Escherichia coli* strain DH5αF′ (GIBCO BRL) was used for cloning and propagation of plasmid DNAs. The following *Saccharomyces cerevisiae* strains were used: MCY1 (MAT**a**/α *ade1-101/ADE1*, *leu2-3,-112/leu2 his3*-Δ1/*HIS3*, *trp1-289/TRP1*, *ura3-52/ura3-52*); MCY2 (MAT**a**, *ade1-101*, *leu2-3,112*, *trp1-289*, *ura3-52*); MCY3 (MAT**a**/α *ade1-101/ADE1*, *leu2-3,-112/leu2 his3*-Δ1/*HIS3*, *trp1-289/TRP1*, *ura3-52/ura3-52 SDB23/LEU2-GAL1-SDB23*); MCY4 (MATα, *ade1-101*, *his3*-Δ1, *trp1-289*, *ura3-52*, *LEU2-GAL1-SDB23*); MCY5 (MAT**a**/α *ade1-101/ADE1*, *leu2-3,-112/leu2 his3*-Δ1/*HIS3*, *trp1-289/TRP1*, *ura3-52/ura3-52*, *sdb23::LEU2/SDB23*). Yeast cells were grown on rich YPGalA [1% (w/v) yeast extract, 2% (w/v) Bacto-tryptone and 2% (w/v) galactose, 0.003% (w/v) adenine] or selective YMGalCas [0.67% (w/v) yeast nitrogen base w/o amino acids, 1% (w/v) vitamin-free casamino acids, 2% (w/v) galactose] inducing media; non-inducing media were as for YPGalA and YMGalCas except that galactose was replaced by 2% (w/v) glucose. Yeast transformations were performed as described by Gietz *et al.* (1992).

Plasmids YEp24-SDB23 and pBS-SDB23 contain the entire *dbf2-1*-complementing *Bam*HI fragment (Figure 1B) subcloned into the *Bam*HI site of YEp24 and pBluescript$^-$ (Stratagene) respectively. Plasmid pSDB23-1 is the 1.4 kb *Hin*dIII fragment encoding *SDB23* and *SWI4* promoter cloned into the *Hin*dIII of pFL39 (Bonneaud *et al.*, 1991). The *GAL1−SDB23* conditional allele of *SDB23* was constructed as follows: a 648 nt fragment encoding the *SDB23* gene was generated by PCR using pBS-SDB23 as template, incorporating *Bam*HI and *Sal*I restriction sites 5′ and 3′ of the gene respectively. This fragment was cloned between the *Bam*HI and *Sal*I restriction sites of pBM125, placing *SDB23* under control of the *GAL1* promoter. The *Eco*RI site 5′ of the *GAL1* promoter in pBM125 was converted to *Xho*I by linker insertion. The *GAL1−SDB23* fusion was isolated on an *Xho*I−*Sal*I fragment and subcloned into the unique *Sal*I site of a pUC19-based vector containing the yeast *LEU2* gene and 5′ non-transcribed *SDB23* sequence: the 1 kb fragment containing 5′ non-transcribed *SDB23* sequence was generated by PCR amplification of pBS-SDB23. MCY4 was derived from MCY3 which has one wild-type *SDB23* locus replaced with the *LEU2−GAL1−SDB23* (one-step gene disruption of MCY1 by the method of Rothstein, 1983). Sporulation and tetrad dissection (Rose *et al.*, 1990) of MCY3, followed by a screen for galactose-dependent growth identified strain MCY4 as containing the chromosomal *LEU2−GAL1−SDB23* allele. Southern blot analysis was used to confirm the correct chromosomal configurations of MCY3 and MCY4.

Plasmid pT7rp28 was linearized by digestion with *Eco*RI to provide a template for *in vitro* transcription (Lossky *et al.*, 1987) with T7 RNA polymerase. The transcripts were uniformly ^{32}P-labelled by using 60 μCi [α-^{32}P]UTP (Amersham, UK) in a 18 μl transcription reaction containing a total concentration of 8 μM UTP, according to the protocol of the manufacturer (Pharmacia LKB).

Growth curves

Growth of MCY4 cells in galactose-containing medium or after a shift to glucose medium was assayed as follows: cells were grown to mid-logarithmic phase in galactose medium, pelleted, washed and resuspended in sterile water. Half of this suspension was used to inoculate fresh pre-warmed galactose or glucose media to an initial OD_{600} of 0.05. Cultures were diluted to keep all OD_{600} readings below 0.6, maintaining the cells in logarithmic growth.

Nucleic acid methods

Nucleotide sequence of *USS1* was determined by using the Sequenase Version 2.0 DNA sequencing system (United States Biochemical). For Northern blot analysis of mRNA, total RNA was extracted by the method of Hopper *et al.* (1978), followed by denaturing agarose gel electrophoresis and transfer onto Hybond N nylon membrane according to the manufacturer's instructions (Amersham). Hybridizations were performed at 60°C using SES1 buffer [0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 7% (w/v) SDS; Church and Gilbert, 1984]. Filters were washed three times for 30 min at 60°C in SES2 buffer [40 mM sodium phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 5% (w/v) SDS]. DNA fragments of cloned genes were radiolabelled by the random priming method (Feinberg and Vogelstein, 1984). For denaturing Northern analysis of snRNAs, RNA samples were fractionated on 6% (w/v) polyacrylamide−8 M urea gels. For the detection of U4/U6 snRNA complex, RNA was resolved on a 9% non-denaturing acrylamide gel (30:1 acrylamide to bisacrylamide) in 50 mM Tris−borate pH 8.3 and 1 mM EDTA according to Zairong and Brow (1993). The gels were transferred electrophoretically to Hybond N nylon membrane at 60 V for 30 min. All hybridizations were performed as described for mRNA Northerns in SES1 buffer, except that the hybridization temperature was adjusted to 5°C below the calculated melting temperature of the oligonucleotide/snRNA hybrid. Filters were washed three times for 30 min at 35°C in SES3 buffer [0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 5% (w/v) SDS]. snRNA probes were prepared by end-labelling the following snRNA oligonucleotides with 60 μCi [γ-^{32}P]ATP (Amersham, UK) using T4 polynucleotide kinase (New England Biolabs): U1, CACGCCTTCCGCGCCGT; U2, CTACACTTG-ATCTAAGCCAAAAGGC; U4,CCGTGCATAAGGAT; U5, AATATG-GCAAGCCC; U6, TC(A/T)TTCTCTGTATTG (obtained from D.Brow); U4B, AGGTATTCCAAAAATTCCC; U6A, AAAACGAAATAAAT-CTCTTTG. The sequences for U4B and U6A oligonucleotides were as in Zairong and Brow (1993) where U6A corresponds to oligonucleotide U6D. Unless stated otherwise, all oligonucleotides were synthesized by Oswell DNA services, Edinburgh University. Blots were analysed by autoradiography, and on a PhosphorImager (Molecular Dynamics, UK) for quantification.

Splicing extract preparation and in vitro splicing reactions

Yeast whole cell extracts were prepared as described by Lin *et al.* (1985). Extracts from strain MCY4 were depleted of Sdb23p by growth in

glucose medium for 13 h at 30°C, maintaining the cells in logarithmic growth by diluting the cells to keep all OD600 readings below 0.6. *In vitro* splicing reactions were performed as described (Lin *et al.*, 1985). The reaction products were fractionated on a 6% (w/v) polyacrylamide–8 M urea gel and visualized by autoradiography. Non-denaturing gel electrophoresis of splicing reactions was performed as described (Pikielny *et al.*, 1986), except that 10 mM EDTA was present in both the electrophoresis buffer and the acrylamide/agarose gel.

Immunoprecipitations

Antibodies against the TMG-cap were a kind gift from R.Lührmann. Anti-Prp8 antibodies were from anti-8.6 serum against a 35 amino acid synthetic peptide corresponding to the amino-terminal sequence of Prp8. Rabbit polyclonal anti-Sdb23p antibodies (sera 756 and 763) were raised against a β-galactosidase fusion protein containing the entire 187 amino acid Sdb23 polypeptide. Antibodies were bound to protein A–Sepharose beads (PAS; Sigma) in NTN buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.1% v/v Nonidet P-40). For snRNA analysis, PAS-bound antibodies were then incubated for 1 h in NTN-B (NTN plus 100 μg/ml each of glycogen, *E.coli* tRNA and BSA) before washing four times with NTN. Splicing extracts were incubated at 4°C with PAS-bound antibodies for 2 h with rotation. The antibody complexes were washed three times with NTN, varying the salt concentration according to the stringency of the wash required, and once with NT (NTN without Nonidet P-40). Immunoprecipitated snRNAs were recovered by treatment with proteinase K (Boehringer Mannheim, prepared according to manufacturer's instructions) for 30 min at 37°C, followed by successive extractions with phenol and phenol/chloroform, then precipitated with ethanol in the presence of 10 μg *E.coli* tRNA as carrier.

Accession number

The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X82649.

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