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A conditional U5 snRNA mutation affecting pre-mRNA splicing and nuclear pre-mRNA retention identifies SSD1/SRK1 as a general splicing mutant suppressor

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ABSTRACT

A combination of point mutations disrupting both stem 1 and stem 2 of U5 snRNA (U5AI) was found to confer a thermosensitive phenotype *in vivo*. In a strain expressing U5AI, pre-mRNA splicing was blocked before the first step through an inability of the mutant U5 snRNA to efficiently associate with the U4/U6 di-snRNP. Formation of early splicing complexes was not affected in extracts prepared from U5 snRNA mutant cells, while the capacity of these extracts to splice a pre-mRNA *in vitro* was greatly diminished. In addition, significant levels of a translation product derived from intron containing pre-mRNAs could be detected *in vivo*. The SSD1/SRK1 gene was identified as a multi-copy suppressor of the U5AI snRNA mutant. Single copy expression of SSD1/SRK1 was sufficient to suppress the thermosensitive phenotype, and high copy expression partially suppressed the splicing and U4/U6.U5 tri-snRNP assembly phenotypes. SSD1/SRK1 also suppressed thermosensitive mutations in the Prp18p and U1-70K proteins, while inhibiting growth of the cold sensitive U1-4U snRNA mutant at 30°C. Thus we have identified SSD1/SRK1 as a general suppressor of splicing mutants.

INTRODUCTION

The processing of precursor mRNA (pre-mRNA) molecules to mature messenger RNAs (mRNA) requires the large ribonucleoprotein (RNP) complex known as the spliceosome. The spliceosome is assembled from the five small nuclear RiboNucleoProteins (snRNPs) named U1, U2, U4, U5 and U6, and several auxiliary protein factors (for review see 1–5). The intervening sequence (intron) of the pre-mRNA is removed through two sequential transesterification reactions, the first and second step of splicing. The spliceosome is stepwise assembled on pre-mRNAs through interactions of spliceosomal components with exon and intron sequences. The 5' splice site is initially recognised by base pairing with the U1 snRNA (6–8) and later with the U5 and U6 snRNAs at the time of splicing catalysis (9–14). The branch point sequence first binds the protein factors SF1/BBP (Branch point Binding Protein) and U2AF65/Mud2p (15–18), and subsequently a base pairing interaction with the U2 snRNA will define the branch point adenosine (19–23). The catalysis of splicing appears to require only the U2, U5 and U6 snRNPs (for review see 24–26), and it is likely that the U1 and U4 snRNPs are dissociated from the active spliceosome (27–32).

A phylogenetically invariant loop of the U5 snRNA (loop 1, Fig. 1A) has been demonstrated to base pair with exon 1 and exon 2 sequences during both steps of splicing, and is likely to help juxtapose the two exons for the second step (11,13,14,33). Apart from a set of canonical Sm proteins that also assemble on the U1, U2 and U4 snRNPs (for review see 4,34), the yeast and human U5 snRNP contain a number of unique polypeptides, most of which are essential for splicing (for review see 3,4).

A main interaction partner for the U5 snRNP is the U4/U6 di-snRNP. The latter is assembled through an extensive base pairing interaction between the snRNA components (35–39). While the U4 snRNA is likely to be dispensable for the catalysis of splicing (27–32), the highly conserved U6 snRNA is required for both splicing steps and may constitute a part of the spliceosome active site (9–12,40–42). U4 snRNA is associated with a set of canonical Sm proteins. Recently, a corresponding family of Sm-like proteins binding to the U6 snRNA was described (43,44; J.Salgado-Garrido *et al*., manuscript submitted). Several proteins associated with, and specific for, the U4/U6 and/or U4/U6.U5 snRNPs have been found in yeast and mammals. The mammalian U4/U6 and U4/U6.U5 snRNP complexes appear to contain several additional proteins that have no counterparts in yeast (3,45–47).

In this study we have identified a U5 snRNA mutation conferring a thermosensitive (ts) phenotype at 37°C. Characterisation of this mutant demonstrated that it blocks pre-mRNA splicing by preventing the normal association of U5 snRNP with the U4/U6 di-snRNP. Although having no effect on pre-spliceosome formation, the mutation caused severe leakage of intron-containing pre-mRNAs to the cytoplasm. A high copy suppressor was isolated and identified as SSD1/SRK1. This gene suppressed several mutations in splicing factors indicating that it is a general splicing mutant suppressor.

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MATERIALS AND METHODS

Plasmids and yeast strains

pBS682 is a pRS414 derived plasmid (48) harboring a genomic fragment including the gene for U5 snRNA. The U5IA (pBS683), U5A (pBS684), U5B (pBS687) and U5AB (pBS688) mutations were generated by PCR mutagenesis (as described in 49) using pBS682 as template and the following mutagenesis oligos: U5A and U5AI oligo EM98 (5'-ACCCG-GATGCAAGAGGTAAAAG-3'), U5B oligo EM97 (5'-TAA-AAGGCATCTTGCATGTTCGT-3') and U5AB oligo EM99 (5'-CCGGATGCAAGAGGTAAAAGGCATCTTGCATGT-TCGT-3'). PCR fragments were digested with *Bam*HI/*Xho*I and ligated to a pRS414 plasmid digested with the same enzymes. The region amplified by PCR was completely sequenced using T7 DNA polymerase (Pharmacia). Mutant U5I was generated by digestion of pBS683 with *Eco*RI/*Nci*I, and ligation of this fragment together with a *Nci*I/*Xho*I digested pBS682 to an *Eco*RI/*Xho*I digested pRS414 resulting in pBS1583. Mutant U5IAB was generated by digestion of pBS683 with *Eco*RI/*Nci*I, and ligation of this fragment together with a *Nci*I/*Xho*I digested pBS688 to an *Eco*RI/*Xho*I digested pRS414 resulting in pBS1584. pBS1672 contains the SSD1 coding sequence lacking the first two nucleotides, and the first half of the SRP101 gene. pBS1696 and pBS1697 (CEN and 2μ URA3-marked plasmids containing the full length SSD1 gene), were generated by cleaving the pLUC12 plasmid (kind gift of P.Fortes) with *Bgl*II and *Sac*I, and ligating the insert to a *Bam*HI/*Sac*I digested pRS414 or pRS424, respectively. pBS1726 is a 2μ-ARG4 marked plasmid containing SSD1/SRK1. pBS1726 was generated by digestion of the ARG4 containing pt92 plasmid with *Pst*I and ligation of the ARG4-containing fragment to a *Pst*I digested pBS1697.

The synthetic intron for detection of pre-mRNA leakage was constructed as follows. The 5' half was generated by site-directed mutagenesis of pBS7 using oligos ML-13 (5'-GA-TCTCGAGTTCGAACTTAAGGAGTATGTTGCTAGCGA-CTAAAGG-3') and ML-14 (5'-TGGTATGTTGCTAGCGAC-AAAAGGAGGCTTTTCAGGACACGTAATATTGAG-3'), resulting in pBS888 and pBS907, respectively. To generate the intact 5' fragment, a *Nhe*I/*Sac*I fragment of pBS907 was ligated to pBS888 digested with the same enzymes, resulting in pBS916 (pTZIFI-5'). The 3' half of the intron was generated by site-directed mutagenesis of pBS533 using oligo EM96 (5'-CTTT-TATTTTTTATAGGGTAATAATTGGTAC-3') resulting in pBS823. To insert the 3' half in frame with the β-galactosidase reporter gene, a sequenced *Sal*I/*Kpn*I fragment of pBS823 was ligated to a *Sac*I/*Kpn*I digested pBS533 and a *Sal*I/*Sac*I digested pBS1, resulting in pBS917 (pHZIFI-3'). The full-length synthetic intron reporter construct was generated by ligating a *Bam*HI/*Sal*I fragment from pBS916 and a *Sal*I/*Sac*I fragment from pBS917 to a *Bam*HI/*Sac*I digested pBS1, resulting in pBS931 (pHZIFI). In plasmid pBS931 both the pre-mRNA and spliced mRNA is in frame with the β-galactosidase reporter gene (Pin–Min). A 5' frame-shifted construct was generated by digestion of pBS916 with *Bst*BI, filling in with Klenow polymerase and re-ligating, resulting in pBS973. To generate a 3' frame-shifted construct, pBS823 was digested with *Eco*NI, filled in with Klenow polymerase and re-ligated, resulting in pBS972. The frame-shifted 3' half of the intron was put into the β-galactosidase reporter gene by ligating a *Sal*I/*Kpn*I fragment of pBS972 and a *Kpn*I/*Sac*I fragment of pBS533 to a *Sac*I/*Sal*I digested pBS1, generating pBS974. Three different frame-shifted derivatives of pBS931 were generated. A Pin–Mout construct (pBS979) was prepared by ligating a *Sal*I/*Bam*HI fragment from pBS973 and a *Sal*I/*Sac*I fragment from pBS974 to a *Bam*HI/*Sac*I digested pBS1. The Pout–Mout construct (pBS980) was generated by ligating a *Sal*I/*Bam*HI fragment from pBS973 and a *Sal*I/*Sac*I fragment from pBS917 to a *Bam*HI/*Sac*I digested pBS1. Finally, the Pout–Min construct pBS983 was generated by ligating a *Sal*I/*Sac*I fragment of pBS974 to pBS931 digested with the same enzymes. All DNA fragments generated by Klenow polymerase were completely sequenced using T7 polymerase (Pharmacia). All plasmids were propagated in *Escherichia coli* strain MC1066.

All U5 snRNA mutations were introduced into strain BSY415 [MATa, ura3, leu2, trp1, arg4 (RV-), ade2, 112, SNR7::LEU2, pBS662 (CEN-URA3-SNR7)]. Mutant strains were recovered after plasmid shuffling. The following U5 snRNA mutant strains were constructed during this study: BSY426 (U5wt), BSY427 (U5AI), BSY428 (U5A), BSY429 (U5B) and BSY430 (U5AB).

The SSD1/SRK1 gene was tagged using a previously described PCR strategy (50). The TAP tag-K. lactis-URA3 cassette from pBS1539 (G.Rigaut *et al*., manuscript submitted) containing two IgG binding units of *Staphylococcus aureus* protein A was amplified using oligos ML-101 (5'-TTGCCAT-GTTTAACCGTCCGTGCATTAAATCCATTCATGAAGA-GGGTATCCATGGAAAAGAGAAG-3') and ML-102 (5'-AC-GAAAGTGAAAAACAAGAAAAACAGCAATGACGATA-TTGGTAGAAGAGATACGACTCACTATAGGG-3'). Approximately 1 μg of the resulting PCR fragment was transformed into the MDG353-13D yeast strain [MATa, ade2, arg4 (RV-) leu2-3,112, trp1, ura3-52] or FL100 (MATa, ura3) and uracil prototrophs were selected. Positive strains were identified by western blotting. The C-terminally tagged SSD1/SRK1 strain derived from FL100 is named BSY777.

Plasmid shuffling and yeast molecular biology techniques were according to standard procedures. Yeast transformations were according to the protocol of Ito *et al*. (51).

β-galactosidase assay, RNA extraction and primer extension

Cells were grown in 10 ml YPD or liquid minimal glucose medium at 23°C to an OD_{600} of 0.5–1, split into two and diluted to 10 ml. The cultures were grown at either 23 or 37°C for 6 h. Cells containing reporter plasmids were induced for 2 h with 2% galactose. β-gal assays were performed as previously described (23). RNA extractions were performed essentially according to Pikielny and Rosbash (52). Primer extension analysis was performed as described (52), using the RP51A exon 2 primer EM38 or RB1 (52,53), the U3 snoRNA specific EM56 primer (54), or the RPL32 specific exon 2 primer OP74 (55; kind gift of O. M. Puig). Primer extension using a chain termination nucleotide was performed as described by Luukkonen and Séraphin (56).

Multicopy suppressor screen

A yeast library containing genomic fragment from strain FL100 in the pFL44L vector was used for multicopy suppressor screening (kind gift from F.Lacroute). Five micrograms of a genomic DNA library was transformed into 25 OD_{600} units of U5AI cells. The transformed cells were plated on SD –URA

plates and incubated at 23°C. After 24 h the cells were harvested and re-plated either on SD –URA plates or on YPD plates at 37°C. Colonies were picked after 7 and 12 days, and subcloned twice on SD –URA plates at 37°C. The genotypes of all viable colonies were ascertained by replica plating, after which the URA-marked library plasmid was shuffled out by growing cells on media containing 5-floroorotic acid (5-FOA, Sigma). After 5-FOA treatment cells were assayed for thermosensitivity by growth on YPD plates at 37°C. Fourteen colonies re-gained their initial thermosensitive (ts) phenotype after losing the library plasmid. Total genomic DNA was prepared from these strains and the plasmids were recovered in *E.coli*. Plasmids were sequenced using vector primers to map the boundaries of the genomic DNA insert.

RESULTS

Characterisation of a U5 snRNA thermosensitive mutation affecting U4/U6.U5 tri-snRNP assembly

The role of the U5 snRNA stem 1 in 5' splice site selection has been investigated in some detail (13,14; S.Kandels-Lewis and B.Séraphin, manuscript in preparation). When generating a four nucleotide substitution in the stem 1 region just below the invariant loop (loop 1), an extra point mutation (U5I) in the 3' half of stem 2 was fortuitously introduced (Fig. 1A). The U5 snRNA double mutant (U5AI) turned out to be functional but conferred a ts phenotype as a single copy gene *in vivo*. Interestingly, when tested independently, the four base substitution in stem 1 (U5A) and the point mutation in stem 2 (U5I) did not generate a temperature sensitive growth phenotype (Fig. 1B). When the structure of stem 1 was restored in U5AI by introducing the complementary mutations in the 5' half of the stem, cells were able to grow at elevated temperature (mutant U5ABI; Fig. 1A and B). This synergistic effect indicates that perturbation of the stem 2 secondary structure in combination with the disrupted stem 1 was required to cause the ts phenotype.

U5 snRNA is required for both steps of pre-mRNA splicing (for review see 57). To investigate which splicing step the U5AI mutation affected, wt, U5A and U5AI cells were grown at 23 or 37°C, RNA was extracted and splicing of the RP51A and U3 snoRNA precursors was examined by primer extension (Fig. 2A). Cells grown at permissive temperature efficiently spliced both precursor transcripts (Fig. 2A, lanes 1–3 and 7–9). At non-permissive temperature, splicing of the RP51A pre-mRNA was significantly reduced in the U5AI strain as cells accumulated pre-mRNA with a concomitant decrease in mRNA levels (Fig. 2A, lane 6). Significantly, no exon 2–intron lariat intermediate accumulated, demonstrating that the block is prior to the first splicing step. Pre-U3 snoRNA also accumulated at 37°C, but only a slight reduction in mature U3 snoRNA levels was detected after a short exposure of the gel (Fig. 2A, lane 12 and data not shown). This was expected since mature snRNAs have a very long half life (58). Primer extension analysis revealed that the U5AI mutation had little effect on the stability of the U5 snRNA or other spliceosomal snRNAs under these conditions (Fig. 2B). However, when the U5AI strain was grown in minimal media, the levels of U5AI RNA were somewhat reduced at both permissive and non-permissive temperature (Fig. 4).

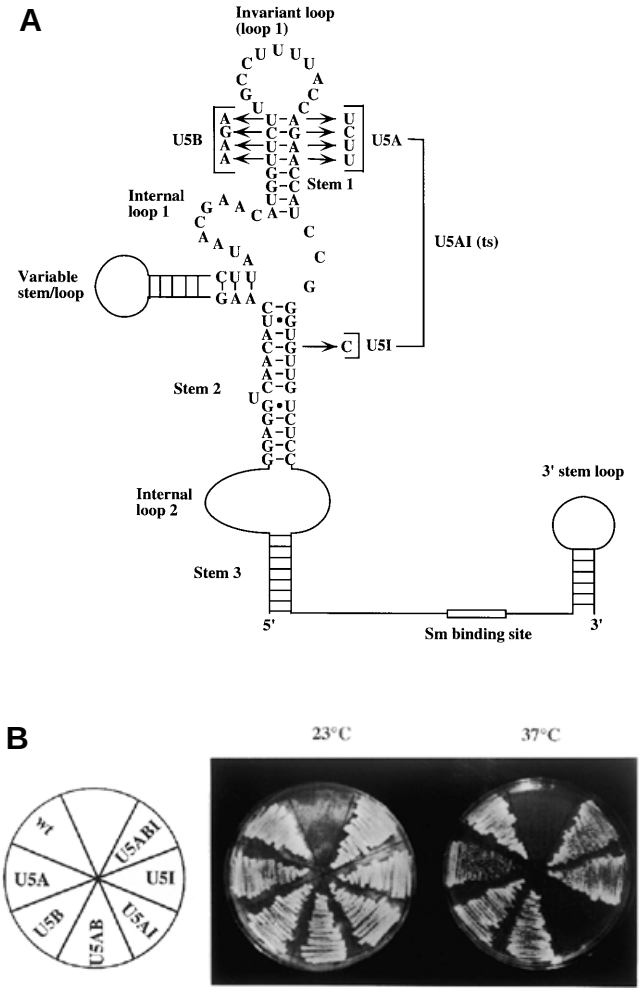

Figure 1. (**A**) Schematic illustration of the U5 snRNA. The nomenclature is as suggested by Frank *et al.* (89). The U5A, U5B and U5I point mutations are indicated with arrows. (**B**) Growth of the wild-type and U5 snRNA mutant strains at 23 and 37°C. Plates were incubated for 5 days.

The invariant loop of U5 snRNA base pairs with exon 1 before the first splicing step and contributes to 5' splice site selection (11,13,14; S.Kandels-Lewis and B.Séraphin, manuscript in preparation). However, in a yeast splicing extract the loop is not essential for catalysis of the first step (59). As the U5AI U5 snRNA mutation affected the first splicing step, we hypothesised that the defect may be in U5 snRNP structure rather than in splicing catalysis. To investigate how efficiently the mutant U5 snRNA was incorporated into U4/U6.U5 tri-snRNP particles, we transformed the wild-type or U5AI mutant strains with a centromeric plasmid expressing the U6 snRNP specific SmX4 gene (G.Rigaut *et al.*, manuscript submitted) fused to two copies of the IgG binding domain of *S.aureus* protein A (kind gift of G. Rigaut). Whole cell splicing extracts were prepared from transformed cells grown at 23 or 37°C, and the SmX4 protein was precipitated using IgG-agarose beads

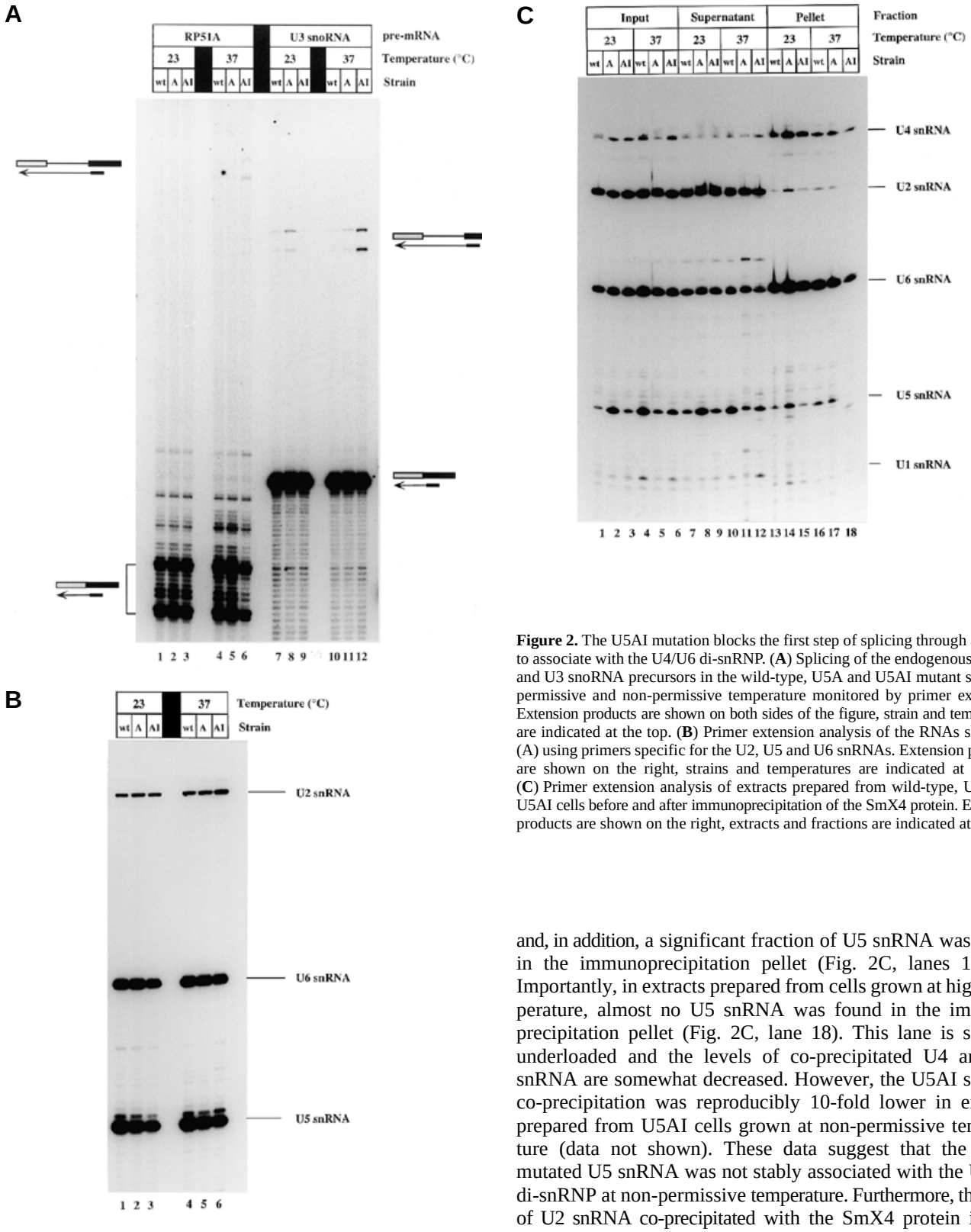

Figure 2. The U5AI mutation blocks the first step of splicing through a failure to associate with the U4/U6 di-snRNP. (**A**) Splicing of the endogenous RP51A and U3 snoRNA precursors in the wild-type, U5A and U5AI mutant strains at permissive and non-permissive temperature monitored by primer extension. Extension products are shown on both sides of the figure, strain and temperature are indicated at the top. (**B**) Primer extension analysis of the RNAs shown in (A) using primers specific for the U2, U5 and U6 snRNAs. Extension products are shown on the right, strains and temperatures are indicated at the top. (**C**) Primer extension analysis of extracts prepared from wild-type, U5A and U5AI cells before and after immunoprecipitation of the SmX4 protein. Extension products are shown on the right, extracts and fractions are indicated at the top.

(Sigma). The U snRNA contents of supernatants and pellets was examined by primer extension analysis (Fig. 2C). The SmX4–protA fusion efficiently precipitated U4 and U6 snRNA, and, in addition, a significant fraction of U5 snRNA was found in the immunoprecipitation pellet (Fig. 2C, lanes 13–17). Importantly, in extracts prepared from cells grown at high temperature, almost no U5 snRNA was found in the immunoprecipitation pellet (Fig. 2C, lane 18). This lane is slightly underloaded and the levels of co-precipitated U4 and U6 snRNA are somewhat decreased. However, the U5AI snRNA co-precipitation was reproducibly 10-fold lower in extracts prepared from U5AI cells grown at non-permissive temperature (data not shown). These data suggest that the U5AI mutated U5 snRNA was not stably associated with the U4/U6 di-snRNP at non-permissive temperature. Furthermore, the level of U2 snRNA co-precipitated with the SmX4 protein is also decreased, suggesting a reduced incorporation of U6 snRNP into the spliceosome or multi-snRNP complexes in the U5AI strain.

The extracts prepared from wt and U5AI strains grown at permissive and non-permissive temperature were next assayed for their ability to form early splicing complexes and to splice a pre-mRNA *in vitro*. Complexes were assembled on a labelled

pre-mRNA substrate and separated by native gel electrophoresis. The U5AI mutation did not prevent assembly of commitment complexes or pre-spliceosomes at non-permissive temperature (data not shown). However, the extracts prepared from mutant cells grown at non-permissive temperature were strongly inhibited in their ability to splice an RP51A-derived pre-mRNA substrate *in vitro* (data not shown). Taken together, these data demonstrate that the U5AI mutation leads to a first step block to splicing, resulting from an inability to assemble into a stable U4/U6.U5 tri-snRNP complex. Consistently, the U5 snRNA mutation did not prevent assembly of early splicing complexes on the same pre-mRNA substrate.

The U5AI mutation causes leakage of unspliced pre-mRNA to the cytoplasm at non-permissive temperature

The U5AI mutation prevents U4/U6.U5 assembly without any apparent block in pre-spliceosome formation. This may result in accumulation of pre-mRNAs assembled into pre-spliceosomes *in vivo*. It has previously been shown that the thermosensitive prp6-1 mutation failed to accumulate U4/U6.U5 tri-snRNPs (60) and, in addition, the prp6-1 mutation was defective in nuclear retention of unspliced pre-mRNA (61). The reporter gene utilised by Legrain *et al*. (61) to monitor pre-mRNA translation was poorly spliced *in vivo*, in contrast to naturally occurring yeast introns. To investigate whether the U5AI mutant also displayed a pre-mRNA leakage phenotype, we therefore constructed an efficiently spliced reporter gene based on a synthetic intron fused to the β-galactosidase coding sequence (Fig. 3A). The synthetic intron and flanking exon sequences were provided with unique restriction sites to facilitate subclonings and introduction of frameshift mutations. In the basic construct shown in Figure 3A, both the pre-mRNA and spliced mRNA is in frame with the β-galactosidase reporter gene [designated Pin–Min (pre-mRNA in frame–mRNA in frame)]. Three frameshifted derivatives of this construct were generated, where either or both the pre-mRNA and mRNA were put out of frame with the β-galactosidase reporter gene (Mout–Pin, Min–Pout and Mout–Pout, respectively, see Materials and Methods). β-Galactosidase assays of wild-type cells transformed with these reporter constructs demonstrated that the Mout constructs did not produce β-galactosidase activity above background levels (data not shown). Importantly, RNA extraction and primer extension analysis showed that all constructs were spliced with an efficiency similar to the endogenous RP51A pre-mRNA (data not shown).

Wild-type, U5A and U5AI mutant cells were transformed with the β-galactosidase reporter constructs Min–Pout and Mout–Pin, or with a β-galactosidase gene lacking the intron. After a 5 h heat shock and 2 h induction with 2% galactose, cells were taken out for determination of β-galactosidase activity (Fig. 3B). Cells grown at permissive temperature efficiently spliced the pre-mRNA reporter, and only background levels of unspliced pre-mRNA were detected (Fig. 3B, 23°C black bars), However, at the non-permissive temperature splicing of the β-galactosidase reporter in U5AI cells decreased more than 80% (Fig. 3B, U5AI/37 black bar) and, significantly, the levels of translated unspliced pre-mRNA were increased more than 30-fold (Fig. 3B, U5AI/37 white bar). In conclusion, these data demonstrate a splicing block in the U5AI mutant strain at non-permissive temperature *in vivo*, confirming the *in vitro* results. Furthermore, the mutation leads to cytoplasmic leakage of unspliced pre-mRNA. The U5AI snRNA mutation does not affect formation of early splicing complexes, suggesting that pre-mRNAs assembled into some splicing complexes are capable of escaping the splicing pathway and entering the cytoplasm. Alternatively, a significant fraction of early splicing factors are sequestered in non-productive complexes, leading to inefficient CC formation and premature export of *de novo* synthesised pre-mRNAs.

Identification of a suppressor of the U5AI thermosensitive phenotype

To identify new gene products potentially involved in U4/U6.U5 snRNP assembly, we performed a multicopy suppressor screen in the U5AI mutant strain. A yeast genomic library inserted in a high-copy number plasmid bearing the URA3 marker was transformed in the mutant strain and clones able to grow at 37°C were recovered. Sequencing analysis of these clones revealed that they originated from seven different genomic loci, one of which corresponded to the wild-type SNR7 locus encoding U5 snRNA. The seven plasmids were transformed into the original U5AI strain, and single colonies were subcloned on minimal –Ura plates at 37°C. One plasmid, in addition to the plasmid containing wild-type U5 snRNA, again rescued the ts phenotype. This plasmid, pBS1672, contained the first 450 nucleotides of the SRP101 gene, and the entire SSD1/SRK1 open reading frame (ORF) except for the first two nucleotides of its start codon.

To investigate which ORF conferred the suppression phenotype, derivatives of pBS1672 lacking either the SRP101 gene fragment or the SSD1/SRK1 gene were generated. After transformation, only the plasmid harboring the SSD1/SRK1 gene fragment was able to confer suppression of the ts phenotype (data not shown). The full length SSD1/SRK1 gene was recovered from plasmid pLUC12 (kind gift of P. Fortes; P.Fortes *et al*., manuscript submitted). SSD1/SRK1 has previously been characterised as a single copy suppressor of a wide variety of mutations. To investigate whether a single gene copy was sufficient to suppress the ts phenotype, the full length SSD1/SRK1 gene was subcloned to either a CEN or a 2μ URA3 marked plasmid. Transformation of these constructs into the U5AI strain revealed that either plasmid enabled the U5AI strain to grow at non-permissive temperature (data not shown). However, cells harboring the single copy plasmid grew with a significantly higher rate, indicating that overexpression of SSD1/SRK1 is slightly toxic (data not shown; P.Fortes, personal communication).

Effects of SSD1/SRK1 expression on pre-mRNA splicing and U5 snRNA stability

As shown above, the U5AI mutation prevented the first splicing step through the incapacity to assemble U5 snRNPs into U4/U6.U5 tri-snRNPs. To investigate if the splicing phenotype of U5AI was suppressed by SSD1/SRK1 expression, the gene was introduced into the wild-type and U5AI strains on low or high copy plasmids alongside empty vector controls. Cells were collected after 0, 2, 4 and 7 h growth in liquid media at 37°C, and total RNA was prepared. Splicing of the endogenous RP51A and RPL32 pre-mRNAs was examined by primer extension. Single copy expression of SSD1/SRK1 had only a modest effect on splicing (data not shown), and only results from multiple copy expression are shown (Fig. 4). Transfer to

A

GGATCCCAAGCTTGGGATCTCGAGTTCGAACTTAAGGAGTATGTTGCTAGCGACAAAAGGAGG
BamHI XhoI BstBI AflII NheI

CTTTTCAGGACACGTAATATTGAGTCGACTCCTGTTTTAGGTAACCGTATACTAACAAGATCTTTTAT
AflIII SalI EcoNI BglII

TTTTTATAGGGTAATAATTGGTACCAAGACCGTCAAGCGTG
KpnI

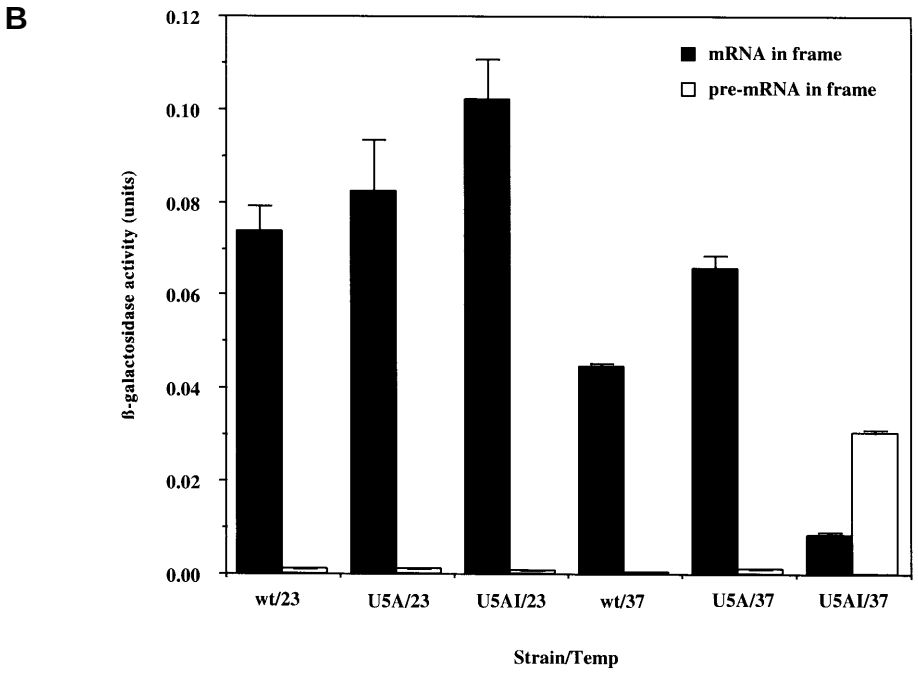

Figure 3. The U5AI mutation leaks unspliced pre-mRNA at non-permissive temperature. (**A**) Schematic illustration of the synthetic intron and flanking exon sequences. Exon sequences are shown in bold face, intron sequences in normal type. Restriction sites used for subclonings and insertion of frameshift mutations are underlined. (**B**) β-Galactosidase assay of wild-type, U5A and U5AI cells transformed with the Min–Pout and Mout–Pin constructs. Strains and temperatures are indicated under the X-axis, the scale on the Y-axis is in arbitrary β-galactosidase units. Black bars, mRNA in frame with the β-galactosidase gene (Min–Pout); white bars, pre-mRNA in frame with the reporter gene (Mout–Pin). β-galactosidase units were calculated as previously described (61).

37°C had no effect on splicing in the wild-type strain, regardless of whether SSD1/SRK1 was overexpressed or not (Fig. 4A, lanes 1–8). In the mutant strain containing empty vector, we observed a progressive accumulation of the RP51A pre-mRNA, with a concomitant decrease in the corresponding mRNA levels after transfer to 37°C (Fig. 4A, lanes 9–12, note that pre-mRNA corresponds to the faint band below the stronger RP51B signal). Comparatively, the U5AI mutant strain overexpressing SSD1/SRK1 accumulated less pre-mRNA and contained significantly higher mRNA levels (Fig. 4A, lanes 13–16). Similar results were obtained for the RPL32 RNA (data not shown). These results were quantitated using a Fuji BAS PhosphorImager and the efficiency of splicing (mRNA/pre-mRNA) was calculated (52) (Fig. 4B). The quantitation showed that, for both pre-mRNAs, overexpression of the SSD1/SRK1 gene increased splicing efficiency more than 5-fold in the mutant strain while having only a weak (1.2-fold) effect in the wild-type strain (Fig. 4B, and data not shown). We conclude that overexpression of SSD1/SRK1 partially suppresses the splicing phenotype of the U5AI mutation.

To investigate whether the increase in splicing efficiency was a result of SSD1/SRK1 affecting the stability or expression of U snRNAs, the level of spliceosomal snRNAs was examined by primer extension (Fig. 4C). The primer extension analysis revealed that neither the wt U5 snRNA nor the U5AI snRNA levels were affected by the shift to non-permissive temperature (Fig. 4C), demonstrating that the conditional ts phenotype was not caused by a depletion of U5AI snRNA (Fig. 2B). However, the U5AI snRNA was less abundant than wild-type U5 snRNA at both temperatures, indicating that the U5AI mutation affected the steady state levels of U5AI snRNA when cells were grown in minimal media. SSD1/SRK1 expression led to

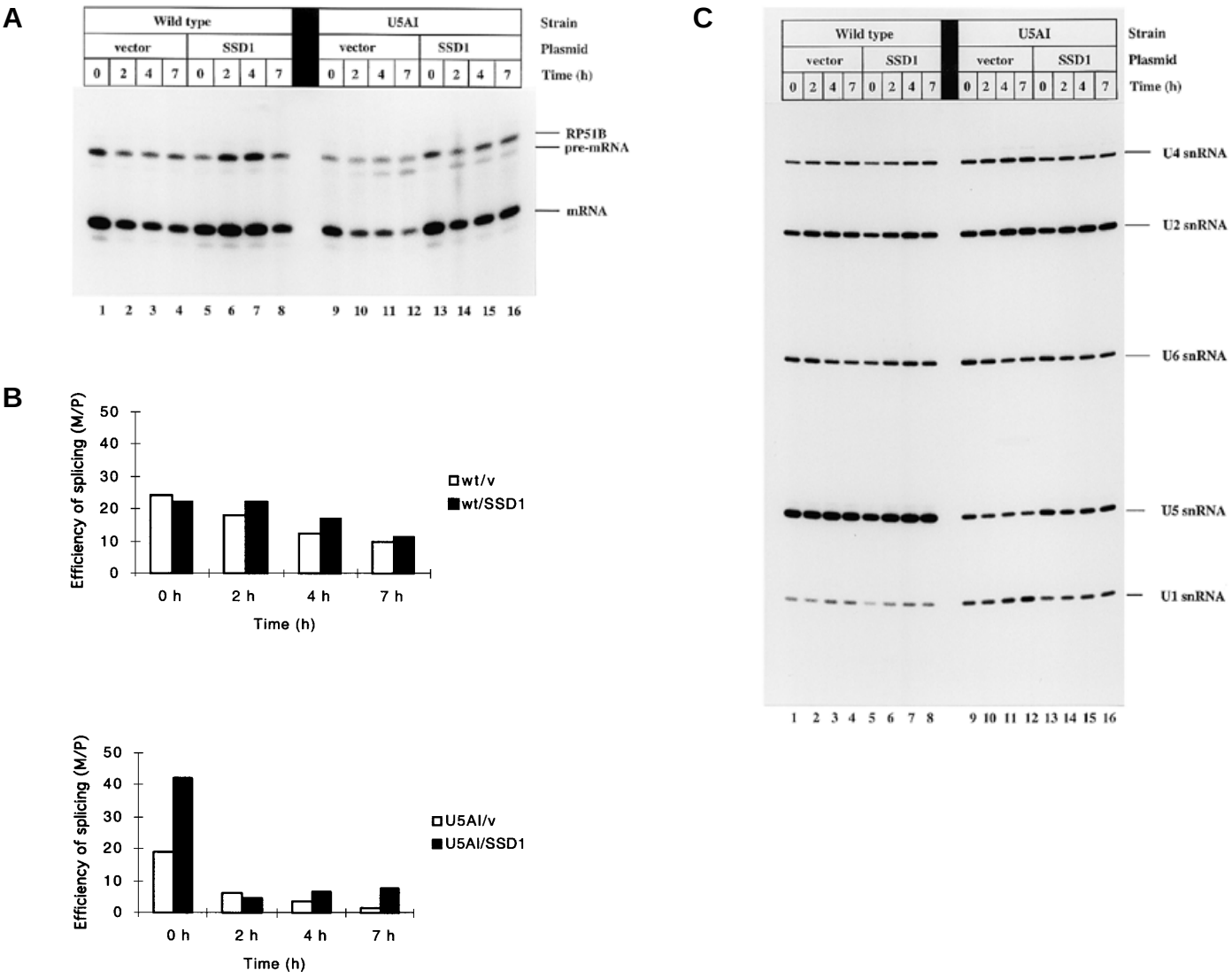

Figure 4. The splicing defect in the U5AI mutated strain is partially suppressed by SSD1/SRK1. (**A**) Primer extension analysis of splicing of the endogenous RP51A pre-mRNA in the wild-type or U5AI mutant strain before or after shift to the non-permissive temperature. Extension products are indicated on the right, strains, plasmids and time points are shown at the top. Primer extension in the presence of ddGTP was performed as previously described (56). (**B**) PhosphorImager quantitation of the gel shown in (A). Efficiency of splicing is calculated as the ratio of mRNA over pre-mRNA as previously described (52). (**C**) Primer extension analysis of the U snRNA contents from the RNA preparation shown in (A). Extension products are shown on the right, strain, plasmid and time points are shown at the top.

a small increase in U5AI snRNA levels at both temperatures, while having no effect on the stability of wild-type U5 snRNA or any other spliceosomal snRNA (Fig. 4C, compare lanes 9–12 with lanes 13–16). The levels of U5AI RNA were similar at permissive and non-permissive temperature, consequently a decrease in stability of the U5AI snRNA cannot account for the conditional growth and splicing phenotype. It is, however, conceivable that the suppression by SSD1/SRK1 at least in part is mediated by an increase in the steady state levels of U5AI snRNA.

Next we wanted to investigate whether the U4/U6.U5 assembly defect could also be suppressed by the SSD1/SRK1 gene product. To allow for concomitant expression of SSD1/SRK1 with the protein A tagged SmX4 gene, the SSD1/SRK1 ORF was transferred to a 2μ-ARG4 marked plasmid. The ARG4-SSD1 plasmid, or a vector control, were co-transformed with the SmX4-protA plasmid into the wild-type or U5AI mutant strains. Whole cell extracts were prepared and the SmX4 protein was precipitated using IgG-agarose beads. The supernatants and pellets were examined by primer extension using U snRNA specific primers. The incubation at high temperature did not affect co-precipitation of U5 snRNA in the wild-type strain, regardless of whether SSD1/SRK1 was expressed or not (data not shown). As shown above, the U5AI mutant snRNA failed to co-precipitate with the U4/U6 snRNP at non-permissive temperature (Fig. 2C, lane 18, and data not

Table 1. SSD1/SRK1 suppression of pre-mRNA splicing mutations

Strain	Allele	37°C	30°C	23°C	16°C	Source
DJY36	prp2-1	–/–	ND	+++/+++	ND	J.Beggs via P.Legrain
SpJ3-33	prp3-1	–/–	ND	+++/+++	ND	J.Beggs via P.Legrain
SpJ4-41	prp4-1	–/–	ND	+++/+++	ND	J.Beggs via P.Legrain
SpJ5-41	prp5-1	–/–	ND	+++/+++	ND	J.Beggs via P.Legrain
SpJ6-66	prp6-1	–/–	ND	+++/+++	ND	J.Beggs via P.Legrain
SpJ8-31	prp8-1	–/–	ND	+++/+++	ND	J.Beggs via P.Legrain
Jm 664	prp9-1	–/–	ND	+++/+++	ND	J.Woolford
PRP17a	prp17-1	–/–	ND	+/+	ND	J.Abelson
PRP18a	prp18-1	+/++	ND	+++/+++	ND	J.Abelson
PRP19a	prp19-1	–/–	ND	+++/+++	ND	J.Abelson
PRP20	prp20-1	–/–	ND	+++/+++	ND	J.Abelson
PRP21a	prp21-1	–/–	ND	+++/+++	ND	J.Abelson
PRP22a	prp22-1	–/–	ND	+++/+++	ND	J.Abelson
PRP24a	prp24-1	–/–	ND	+++/+++	ND	J.Abelson
PRP27a	prp27-1	–/–	ND	+++/+++	ND	J.Abelson
BSY332	U2-41G/42A	+/+	ND	+++/+++	ND	S.Kandels-Lewis, B.G.M.Luukkonen and B.Séraphin, unpublished data
At 216a	sad1-1	–/–	ND	+++/+++	ND	Z.Lygerou and B.S., manuscript submitted
VC682C	prp33-1	–/–	ND	+++/+++	ND	J.Woolford
ESJ35	prp16-2	–/–	ND	+++/+++	ND	C.Guthrie
BSY512	U1-70K^{ts}	–/++	ND	+++/+++	ND	B.Séraphin, unpublished data
BSY589	U6-G52U	–/–	ND	+++/+++	ND	B.G.M.Luukkonen, unpublished data
yDFA7	slu7-1	–/–	+++/+++	+++/+++	++/++	C.Guthrie
BSY287	U1-4U	+/++	++/+	–/–	–/–	B.Séraphin
BSY427	U5AI	–/+++	ND	+++/+++	ND	This study
y149-7B	dbf3-1	–/–	ND	+++/+++	ND	L.Johnston
MGD353-13D	wt	+++/+++	+++/+++	+++/+++	+++/+++	A.Nicolas

Growth was scored after 5 days incubation at 23, 30 and 37°C and after 10 days incubation at 16°C.
The table shows growth with vector plasmid/growth with SSD1/SRK1 plasmid. +++, wild-type growth; ++, reduced growth rate; +, slow growth; –, no growth; ND, not determined.

shown). However, expression of SSD1/SRK1 increased U5 snRNA co-precipitation 3-fold (data not shown).

To investigate if SSD1/SRK1 was stably associated with any spliceosomal snRNA, the IgG binding domain of protein A was fused in-frame with the C-terminus of the SSD1/SRK1 gene. Analysis of the pellet co-precipitated with SSD1/SRK1 by primer extension and pCp labelling failed to reveal any associated RNA (data not shown). Furthermore, depletion of SSD1/SRK1 from the extract to >95% did not affect splicing efficiency *in vitro*. This is consistent with the observation that SSD1/SRK1 is not essential for vegetative growth (62) and further suggests that SSD1/SRK1 does not co-precipitate any essential splicing factor.

SSD1/SRK1 is a general splicing mutant suppressor

SSD1/SRK1 has been shown to be involved in many biological processes, although a function for the protein has not been described so far. In particular, SSD1/SRK1 has been identified as a single copy suppressor of many different mutations. Our data indicated that the protein may be directly or indirectly involved in pre-mRNA splicing. To investigate what role the SSD1/SRK1 gene product may have in RNA processing, we transformed a collection of temperature sensitive pre-mRNA splicing defective yeast strains with the single copy SSD1/SRK1 plasmid or empty vector control (Table 1). Transformed cells were plated on SD –URA plates and grown at 23°C [BSY287 (U1-4U) and yDAF7 slu7-1 (63) were grown at 30°C]. Single colonies were subcloned to –URA plates and incubated at either 23 or 37°C (BSY287 and yDAF7 were in addition incubated at 16 and 30°C, Table 1). SSD1/SRK1 expression was found to affect the growth of three temperature sensitive pre-mRNA splicing defective yeast strains in addition to the U5AI strain (Table 1). The PRP18a strain prp18-1, (64) harbouring a ts allele of the PRP18 gene was found to grow

very slowly at 37°C, while growing significantly faster in the presence of SSD1/SRK1. Interestingly, Prp18p has been shown to be stably associated with the U5 snRNP, but the protein is only required for the second splicing step (64). The ts allele of U1-70K consists of a Leu→Pro substitution in position 123 of the U1-70K ORF, that confers a ts phenotype when combined with a deletion of the A loop (nucleotides 26–34) of the U1 snRNA (B.Séraphin, unpublished data). While the strain is normally not viable at 37°C, SSD1/SRK1 expression allowed for growth at the non-permissive temperature. The U1-4U mutation has a cold sensitive phenotype and the strain cannot grow at low temperatures (7). At 30°C, SSD1/SRK1 expression caused the strain to grow significantly slower than the vector transformed strain, however, at 37°C SSD1/SRK1 allowed the strain to grow slightly faster than the vector control. As these various splicing defective mutations are not involved in the same step of the splicing process, it is unlikely that the suppression by SSD1/SRK1 is direct. However, our data identifies SSD1/SRK1 as a general suppressor of splicing mutations.

DISCUSSION

In this paper we have described the construction and the genetic and molecular characterisation of a thermosensitive U5 snRNA mutation. We have shown that the mutation (U5AI) prevents the first step of pre-mRNA splicing *in vivo*, most likely by inhibiting the formation of U4/U6.U5 tri-snRNPs. The invariant loop (loop I) of U5 snRNA, although being essential for splicing *in vivo*, is not required for the first step *in vitro* (59). However, the invariant U5 snRNA loop was shown to be involved in 5' splice site selection with sensitised mutated reporters (13,14; S.Kandels-Lewis and B.Séraphin, manuscript in preparation). We cannot exclude that the U5AI mutation affects the first splicing step by interfering with the 5' splice site selection process, but we consider this hypothesis unlikely since splicing of sensitised mutated substrates was not affected in the U5AI strain at 30°C (data not shown). Even though the strain does not display a strong splicing phenotype at 30°C, a synthetic negative effect between the 5' splice site mutants and the U5AI mutation was anticipated had the U5AI strain been defective in 5' splice site recognition. This supports our conclusion that the major defect of the U5AI mutant resides in the U5 snRNP structure and its inability to interact with the U4/U6 complex.

Some mutations in splicing factors have been shown to block splicing while allowing nucleo-cytoplasmic transport and translation of unspliced pre-mRNAs (61). Using a reporter pre-mRNA harboring a synthetic intron, we have demonstrated that the U5AI mutant U5 snRNA also allowed for leakage of unspliced pre-mRNA to the cytoplasm. Previously Legrain and Rosbash (61) showed that the prp6-1 and prp9-1 alleles caused translation of unspliced pre-mRNAs at non-permissive temperature. Interestingly, Prp6p is required for accumulation of U4/U6.U5 tri-snRNPs while not affecting the stability of U4/U6 di-snRNPs. Prp9p is a part of the U2 snRNP SF3a complex and is required for pre-spliceosome formation *in vitro* (65–67). Another SF3a component, Prp21p, along with the yeast U2AF65 homolog Mud2p, have also been shown to have defects in nuclear pre-mRNA retention when mutated (67,69). However, mutations in other factors present in Prp6p or Prp9p containing complexes do not lead to pre-mRNA leakage. This suggests that only specific mutations allow for release of the pre-mRNA from splicing complexes. It has been speculated that certain proofreading steps ensure accuracy of the splicing process (5 and references therein). It is possible that some mutations, including U5AI, induce pre-mRNA release by mimicking or disabling proofreading steps.

The SSD1/SRK1 gene was initially characterised as a single copy suppressor of a deletion in the protein phosphatase SIT4 (62) and of a mutation in the cAMP phosphodiesterase PDE2 and the dynein-like gene INS1 (70). Later SSD1/SRK1 has been implicated in many processes and cellular pathways, including the cell cycle (71,72), signal transduction (73,74) and RNA metabolism (75; P.Fortes *et al.*, manuscript submitted). SSD1/SRK1 expression increased the efficiency of splicing 5-fold of both the endogenous RP51A pre-mRNA and RPL32 pre-mRNA, while a 3-fold increase in U5 snRNA co-precipitation was observed. SSD1/SRK1 also suppressed the ts alleles prp18-1 and U1-70K, and affected growth of the cold sensitive U1-4U strain. The non-essential gene product Prp18p is stably associated with U5 snRNP and is involved in the second splicing step (64). In contrast to Prp18p, the U1-70K and U1-4U mutations are acting at very early steps of spliceosome assembly. It is difficult to reconcile the wide array of splicing alleles that SSD1/SRK1 suppresses with a direct role for this factor in splicing. The prp18-1 allele and U5AI may both affect the assembly or function of the U5 snRNP and both can therefore theoretically be suppressed directly by SSD1/SRK1. Ast and Weiner (76) showed that a 2'-O-methyl oligonucleotide targeted to the human U5 snRNA disrupted the U4/U6.U5 tri-snRNP complex and induced a novel U1/U4/U5 tri-snRNP. As this complex interacted with a 5' splice site, it was speculated to constitute a transition stage between U1 snRNP displacement and U5 snRNP addition (76). The existence of such a transition stage in yeast may explain the effects of SSD1/SRK1 on U5 snRNP and U1 snRNP. Recently SSD1/SRK1 was identified in a synthetic lethality screen with the yeast cap binding complex (CBC) (P.Fortes *et al.*, manuscript submitted). In both yeast and mammals, the CBC has been shown to be required for efficient addition of U1 snRNP to a 5' splice site (77–79). This would again indicate a role for SSD1/SRK1 in U1 snRNP function. SSD1/SRK1 appeared to exert an effect on the steady state levels of U5AI snRNA at both permissive and non-permissive temperature under some conditions. As the strains harbouring the U1-4U mutant and the U1-70K ts allele both contain U1 snRNA mutations (Material and Methods), it is possible that the suppression by SSD1/SRK1 is mediated in part by an increase in U1 snRNA levels.

An indirect role for SSD1/SRK1 in snRNP assembly or transport cannot be excluded. Following the initial observations of a genetic interaction between SSD1/SRK1 and SIT4 (62), the ability of SSD1/SRK1 to suppress a hyperactive cAMP-dependent protein kinase and the similarity to the *Schizosaccharomyces pombe* dis3 protein phosphatase (70), it was postulated that SSD1/SRK1 is involved in protein phosphatase function. Protein dephosphorylation has been shown to be important for both steps of pre-mRNA splicing (80), suggesting an indirect way whereby SSD1/SRK1 may affect splicing. SSD1/SRK1 also suppresses mutations in the G1-specific transcription factor SWI4 and the G1 cyclins CLN1 and CLN2 indicating a role in G1→S progression (71,72). A

connection between the cell cycle and splicing has previously been suggested based on the discovery of splicing mutants that display cell cycle defects. For example, an allele of the U5 snRNP associated PRP8 gene (dbf3-1; 81,82) was found to be defective in S-phase progression (83) and an allele of the Prp3p protein was also identified in the screen for mutants defective in DNA synthesis (82). The gene encoding the second step factor Prp17p (84) was recently found to be allelic to the cell division cycle CDC40 gene (85,86). In mammalian cells, cyclin E was found associated with SAP114, SAP145 and SAP155 of the human U2 snRNP SF3b subcomplex, as well as with the SmB and B' proteins (87). SAP155 is phosphorylated prior to the second catalytic step (88) and, interestingly SAP155 was shown to be a target of cyclin E-CDK2 *in vitro* (87).

In conclusion, the SSD1/SRK1 gene product has been shown to be involved in several pathways and processes within the cell. Here we have shown that SSD1/SRK1 is also a general suppressor of splicing mutants. A role for SSD1/SRK1 in a fundamental biological process such as protein dephosphorylation or RNA processing may help to explain the pleiotropic effects of SSD1/SRK1 expression on cell growth and viability.

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