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Srp2, an SR protein family member of fission yeast: *in vivo* characterization of its modular domains

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ABSTRACT

We isolated *srp2*, a gene encoding a protein composed of two RNA binding domains (RBDs) at the N-terminus followed by an arginine-rich region that is flanked by two short SR (serine/arginine) elements. The RBDs contain the signatures RDADDA and SWQDLKD found in RBD1 and RBD2 of all typical metazoan SR proteins. *srp2* is essential for growth. We have analyzed *in vivo* the role of the modular domains of Srp2 by testing specific mutations in a conditional strain for complementation. We found that RBD2 is essential for function and determines the specificity of RBD1 in Srp2. Replacement of the first RBD with RBD1 of Srp1 of fission yeast does not change this specificity. The two SR elements in the C-terminus of Srp2 are also essential for function *in vivo*. Cellular distribution analysis with green fluorescence protein fused to portions of Srp2 revealed that the SR elements are necessary to target Srp2 to the nucleus. Furthermore, overexpression of modular domains of Srp2 and Srp1 show different effects on pre-mRNA splicing activity of the *tfIId* gene. Taken together, these findings are consistent with the notion that the RBDs of these proteins may be involved in pre-mRNA recognition.

INTRODUCTION

Nine SR (serine/arginine-rich) protein family members have been detected in mammalia. Four, including SC35 (SRp30b), contain one RNA binding domain (RBD1) with the RNA-binding motifs RNP-2 and RNP-1, respectively, at the N-terminus. RBD1 is followed by the RS domain, consisting of RS/SR dipeptides of variable length. The other SR proteins, including ASF/SF2 (SRp30a), consist of two RBDs, whereas in the second (RBD2), the RNA-binding motifs are not conserved. The two RBDs are also followed by an RS domain of different length (1–7).

The RBD of SC35 binds to RNA *in vitro*. In the case of ASF/SF2, both RBDs can bind to RNA, whereas the two together seem to have distinct binding specificity (8–13). *In vitro* studies revealed that the mammalian SR proteins can act in constitutive and alternative pre-mRNA splicing, playing a major role in the assembly and formation of a catalytic spliceosome (6,12,14,15). It appears that SR proteins are regulators of cell and tissue-specific differential splicing and are involved in the selection of splice sites (13,16–18).

Exonic splicing enhancers have been identified in differentially spliced exons. These RNA sequences bind SR proteins which then might recruite the splicing machinery and activate splicing. Specificity may be conferred by binding individual SR proteins to unique splicing enhancers (19–21). Transient overexpression of different SR proteins in cell culture led in many cases to a shift to the proximal 5' splice site of the reporter gene. This is consistent with data obtained *in vitro*. However, the situation may be more complex, since with SC35 and ASF/SF2 using the SV40 early region as reporter, the *in vitro* and the *in vivo* data yielded conflicting results (5,16–19). Nevertheless, changing concentrations of the SR proteins in the cell appears to influence splice site selection (19,22,23).

Mammalian SR proteins are nuclear phosphoproteins and are found in sub-nuclear structures called speckles. These structures are discussed as sub-nuclear concentrations of SR splicing factors used to supply actively transcribed genes with the appropriate set of SR proteins for splicing. Several protein kinases have been identified which phosphorylate SR proteins *in vitro* and co-localize with the SR proteins in speckles (24–28). It has recently been shown that ASF/SF2, Srp20 and 9G8 shuttle between the nucleus and the cytoplasm. Based on these observations additional roles, possibly in mRNA transport, have been suggested for SR proteins (29,30). In *Drosophila melanogaster* a mutation in an SR protein, called B52, caused a dominant phenotype altering the splicing of *doublesex*, thus indicating that SR proteins also play a role in developmental processes of invertebrates (31). B52 is closely related to the mammalian SR-family members Srp75, Srp55 and Srp40 containing two RBDs. SR proteins also have been detected in plants. The SR-family member most closely related to the mammalian Srp30a (ASF/SF2) is capable of complementing a HeLa S100 splice extract (32,33).

Comparative analysis of the complete genome of *Saccharomyces cerevisiae* revealed that budding yeast does not contain typical SR proteins. This finding raised the possibility that SR

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+AF012278, D89163 and U66833

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
D1	*h*$^{-S}$/*h*$^{+N}$ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*
SL11	*h*$^{-S}$ *ura4-294 prp4-73*^{ts}
SL1	*h*$^{-S}$ *leu1-32 ura4-D18 prp4-73*^{ts}
SL97	*h*$^{-S}$ *leu1-32 his7-366 ura4-D18*
SL97a	*h*$^{-S}$ *leu1-32 int::pJK148-srp2 his7-366 ura4-D18*
Srp2ths	*h*$^{-S}$ *leu1-32 int::pJK148-srp2 ura4-D18 his7-366 srp2::his7*

proteins and regulated pre-mRNA splicing may be an invention specific to metazoans (6,15,19).

Recently, we have isolated Srp1, a protein containing one typical RBD at the N-terminus, followed by an RS domain from the fission yeast *Schizosaccharomyces pombe*. Srp1 contains a C-terminal domain for which no homologous sequences were found in the database. We showed *in vivo* that Srp1 is likely to be involved in pre-mRNA splicing (34).

Here we report the identification and characterization of Srp2 from fission yeast. Srp2 is essential for growth. The deduced amino acid sequence revealed two RBDs that are most closely related to the two RBDs of Srp75, Srp55 and Srp40 of the mammalian SR protein family. However, Srp2 does not contain a typical SR domain. Instead, there are two short SR elements, called SR1 and SR2, which are essential for targeting the protein to the nucleus. Studies with overexpressed domains and mutations of Srp1 and Srp2 revealed that RBD1 and RBD2 of both proteins potentially mediate and specify RNA binding.

MATERIALS AND METHODS

Strains and media

Schizosaccharomyces pombe strains used in this study are listed in Table 1. Cells were grown at 30 or 26°C on rich medium YEA and YEL (35) or on Edinburgh minimal medium (36) supplemented with 2 mM thiamine, indicated +Thi.

Recombinant DNA and RNA methodology

Restriction endonucleases were purchased from Gibco BRL and MBI Fermentas and used according to the manufacturer's instructions. All PCR reactions were performed with *Pfu* polymerase (Stratagene) using an OmniGene thermocycler (MWG-Biotech). All PCR products were gel-purified with Nucleotrap (Machery-Nagel), digested with *Bam*HI and cloned into pUC19. All cloned PCR products were checked for PCR errors by sequencing. Bacterial transformations were performed as described by Sambrook et al. (37). Yeast transformations were performed using the LiOAc method as described (38,39). Plasmid preparations and sequence analysis were performed as previously described (40). Yeast DNA for Southern hybridization analysis was isolated as described (41). Total RNA preparation and northern analysis were as described previously (42).

Isolation of *srp2*

The primers 5'-ATATGGATCCAGAGAAAGTGCTGCTTC-TAA-3' and 5'-ATATGGATCCTTACCATTCAGCAGCGA-CCT-3', derived from a partial cDNA sequence (GenBank accession no. D89163), were used to isolate a 0.8 kb probe by PCR using a *S.pombe* cDNA library from Clontech (Match-maker). Full-length *srp2* cDNA was isolated by colony hybridization (37) from the same library. *Bam*HI linkers were added to the ends of the full-length cDNA. A 3.3 kb genomic fragment containing *srp2*$^+$ was isolated from a *S.pombe* genomic library (43) by colony hybridization using the 1.1 kb *srp2* cDNA as a probe.

DNA sequencing

The 3.3 kb *Eco*RI/*Hin*dIII fragment containing the *srp2* gene was cloned into pUC19. Both strands were sequenced by the dideoxy chain termination method using Sequenase 2.0 (USB). Sequence analysis was performed with the GCG package (Genetics Computer Group, WI, USA). Multiple alignments were made with ClustalW (44) and Boxshade v3.31 C.

Constructs for gene disruption

The *srp2::LEU2* null allele was produced using the one-step gene disruption method originally developed by Rothstein (45). Constructs were made in pUC19 with the 3.3 kb genomic fragment containing *srp2*$^+$. The 1.6 kb *Nru*I/*Eco*47III fragment encompassing the region from exon 2 to 8 was replaced with a 2.2 kb *Xho*I/*Sal*I fragment containing the *S.cerevisiae LEU2* gene. A diploid strain (D1, see Table 1) was transformed with the *srp2::LEU2* DNA excised from pUC19-*srp2::LEU2* with *Bam*HI and *Pst*I. Stable leucine prototrophic transformants were selected, and the proper integration of this construct was analyzed by Southern blot hybridization using the 0.5 kb genomic *Eco*RV fragment of *srp2*$^+$ as a probe. Transformants in which one *srp2* allele was disrupted were induced to undergo sporulation. Tetrads were dissected on YEA using a Singer MSM 200 micromanipulator (Singer Instruments, UK).

Construction of the conditional *srp2* allele

The 1.1 kb fragment containing the full-length *srp2* cDNA was cloned into pREP81 (46). From pREP81-*srp2*, a 2.3 kb *Pst*I/*Sst*I fragment consisting of the *nmt1* promotor fused to the *srp2* cDNA was excised and ligated into pJK148 (47). This vector was linearized with *Sun*I and transformed into strain SL97. The *srp2::his7* null allele was generated by replacing the 1.6 kb *Nru*I/*Eco*47III fragment of the 3.3 kb genomic *srp2* fragment containing *srp2*$^+$, encompassing the region from exon 2 to 8, with the 1.9 kb *Xho*I/*Eco*RI fragment from pEA2 (48) containing the *S.pombe his7*$^+$ gene. Strain SL97a was transformed with the *srp2::his7* DNA excised from pUC19-*srp2::his7* with *Bam*HI and *Pst*I. Stable *his*$^+$ transformants were selected on

EMM without thiamine. Transformants which did not grow on EMM with thiamine after replica plating were analyzed by Southern blot hybridization with the 0.5 kb genomic *Eco*RV fragment of *srp2*$^+$ as a probe.

Construction of the expression vector containing the authentic *srp2* promotor

The *srp2* promotor was amplified by PCR using the primers 5'-ATATCTGCAGTCGATGAAATTATCATCGCC-3' and 5'-ATATGGATCCCATTTTTTTGGGTAGTTGG-3'. The 0.3 kb PCR product was gel-purified, digested with *Bam*HI and *Pst*I, and inserted into pREP2 (46) replacing the *nmt1* promotor.

Expression of *srp2*

For expression of Srp2 in *S.pombe* the shuttle vectors pREP1, pREP81 and pREP2 were used (46). Vectors were modified by a Klenow fill-in of the *Sal*I site and religated in order to obtain the correct reading frame for expression of *srp2* constructs when the construct was ligated into the *Bam*HI site.

Cloning of modular domains of *srp2* and *srp1*

Modular domains of *srp2* were produced by PCR with the following primers: (i) 5'-ATATGGATCCATGTCGGAGACTAGATTGTTTG-3', (ii) 5'-ATATGGATCCCTGCAGAGAAGCAGCACTTTCTCT-3', (iii) 5'-ATATGGATCCCTGCAGAAATACCCCCGTCCCCGTCGC-3', (iv) 5'-ATATGGATCCCTGCAGGATGCCGCTAATGAGCCCCTT-3', (v) 5'-ATATGGATCCTTAACGACGGCGAGCAGGTGG-3' and (vi) 5'-ATATGGATCCTTACCATTCAGCAGCGACCT-3'. RBD1 was isolated using the primer pair 1+2, RBD1+RBD2 using 1+5, RBD2+RRD (RR-rich domain) using 3+6 and RRD using 4+6. Primers 1, 5 and 6 have *Bam*HI linkers. Primers 2, 3 and 4 were linked with *Bam*HI and *Pst*I.

RBD1 of *srp1* was amplified using the primers 5'-ATATGGATCCATGAGTCGCAGAAGCCTTCGTACCC-3' and 5'-ATATGGATCCCTGCAGTAGCCGTCCAGAATCTCCG-3'. Domain swap constructs were made by fusion of RBD2+RRD, RBD2 and RRD of *srp2* to RBD1 of *srp1* using *Pst*I.

Mutagenesis of *srp2* cDNA

Site-directed mutagenesis of the *srp2* cDNA was performed as described by Kunkel et al. (49) using the Muta-Gene 2 kit (Bio-Rad). The 1.1 kb *Bam*HI fragment containing the full-length cDNA of *srp2* was cloned into M13mp19. Primers for mutagenesis were: 5'-ACGATATCTTGAACATTTTGAACGTTAACAACCTCAACAAAGCC-3', 5'-TACGCATTACATTCCGCACGTTCTGCAGAGCGACATCTTC-3', 5'-ACGTCTAGGAGCAGGTGCCCGGGCATAAGCGTCACGTCGATACTC-3' and 5'-TGGAGCACGTCGACGGAAGCGGGCGGGTACTTCGGGAAGGGGCTC-3'. For the mutations of the RR domain, changing all 14 tyrosines into phenylalanines in the RRD between the SR elements, the following oligonucleotides were used: Y1–Y4: 5'-GCTCGCCGTCGTTTTCGTGATGATTTTCGTAGAGGAGGCGATTTCCGTCGCGACGCTTTTCGTCCCGGCAGAGATGATGAA-3'; Y5–Y8: 5'-CGTCGTTTTGCTCCTCGTGGTGAATTCAGGCGTAATAATCGTGATGAATTTCGTCGTGGTGGTCGCGATGAGTTTAGA-3'; Y9–Y11: 5'-CGTAATTCCAGAAGTGATTTTCGTCGACCTCATGATGATGAGTTTCGTCGTCCTAGAGGTGATGAATTTCGCCCTGGA-3'; Y12–Y14: 5'-CGAGACGAATTTCGTCGTTCTCGTGATGATGGACGCCCTTCCCATGATGATGAGTTTCGACGTGACGCGTTTAGCCGTAGTCCT-3'. All mutations were checked by restriction and sequencing analysis.

GFP fusions

GFP fusions were made by insertion of the *srp2* cDNA and the modular domains into the *Bam*HI site of pTG11 and pTG13 *GFP* fusion vectors (T.Groß, unpublished), containing wild type *GFP*. Expression of the *GFP* fusion constructs was driven by the thiamine repressible *nmt1* promotor. GFP (green fluorescence protein) was fused to the N-terminus of the constructs. pTG13 vectors were integrated into the *ura4* locus of strain SL11. pTG11 constructs were transformed into SL1. GFP fluorescence was visualized using a Zeiss Axioplan 2 fluorescence microscope, equipped with filter set 09/487909-0000. Images were taken with a Zeiss Axiophot 2 camera, using Kodak Elite II 400 film.

RESULTS

Identification and isolation of *srp2*

Each of the RBDs of the mammalian SR proteins contain a signature sequence: RDAEDA or RDADDA is found in all N-terminal RBD1 of known metazoan SR splicing factors and appears adjacent to the conserved RNP-1 motif of RBD1 (Fig. 1A). A comparison of the five mammalian SR proteins consisting of two RBDs show the signature sequence of RBD1 and an invariable sequence SWQDLKD in RBD2 (Fig. 1A) (2). We compared this short sequence with the database and found it in a protein sequence deduced from a partial cDNA of *S.pombe* (accession no. D89163). With this sequence information we produced a PCR product from a *S.pombe* cDNA library. The PCR product was used as a probe to screen a genomic and the cDNA library. The isolated DNAs were sequenced and further characterized. Analysis of the genomic fragment revealed a complex gene structure containing nine exons and eight introns (Fig. 2A) (50). The open reading frame (ORF) deduced from the nine exons is consistent with the ORF of the cDNA. We call this gene *srp2*. The amino acid sequence revealed two RBDs at the N-terminus. RBD1 contains the signature sequence discussed above and RBD2 displays the SWQDLKD motif. The RBDs are followed by a short SR element (SR1), whereas serine alternates with arginine (Fig. 1B). This SR element shows similarity in sequence with the beginning of the RS domains of the mammalian SR proteins (Fig. 1A). Then, however, the fission yeast protein changes over 100 amino acids into an arginine-rich region in which the motif YRR preceded by the acidic amino acids glutamate and aspartate is found 11 times (Fig. 1B). This arginine-rich domain, termed RRD, is followed by a second SR element (SR2) in which serine alternates with arginine or proline (Fig. 1B). Thus, the fission yeast protein Srp2 contains two RBDs with the signature sequences found in RBDs of metazoan SR proteins (51); it does not, however, display a typical RS domain. The deduced amino acid sequence consists of 365 amino acids yielding a molecular mass of 40 kDa.

srp2 is essential for growth

We used a 3.3 kb genomic *Eco*RV/*Hin*dIII fragment encompassing the complete *srp2* locus for a gene disruption construct

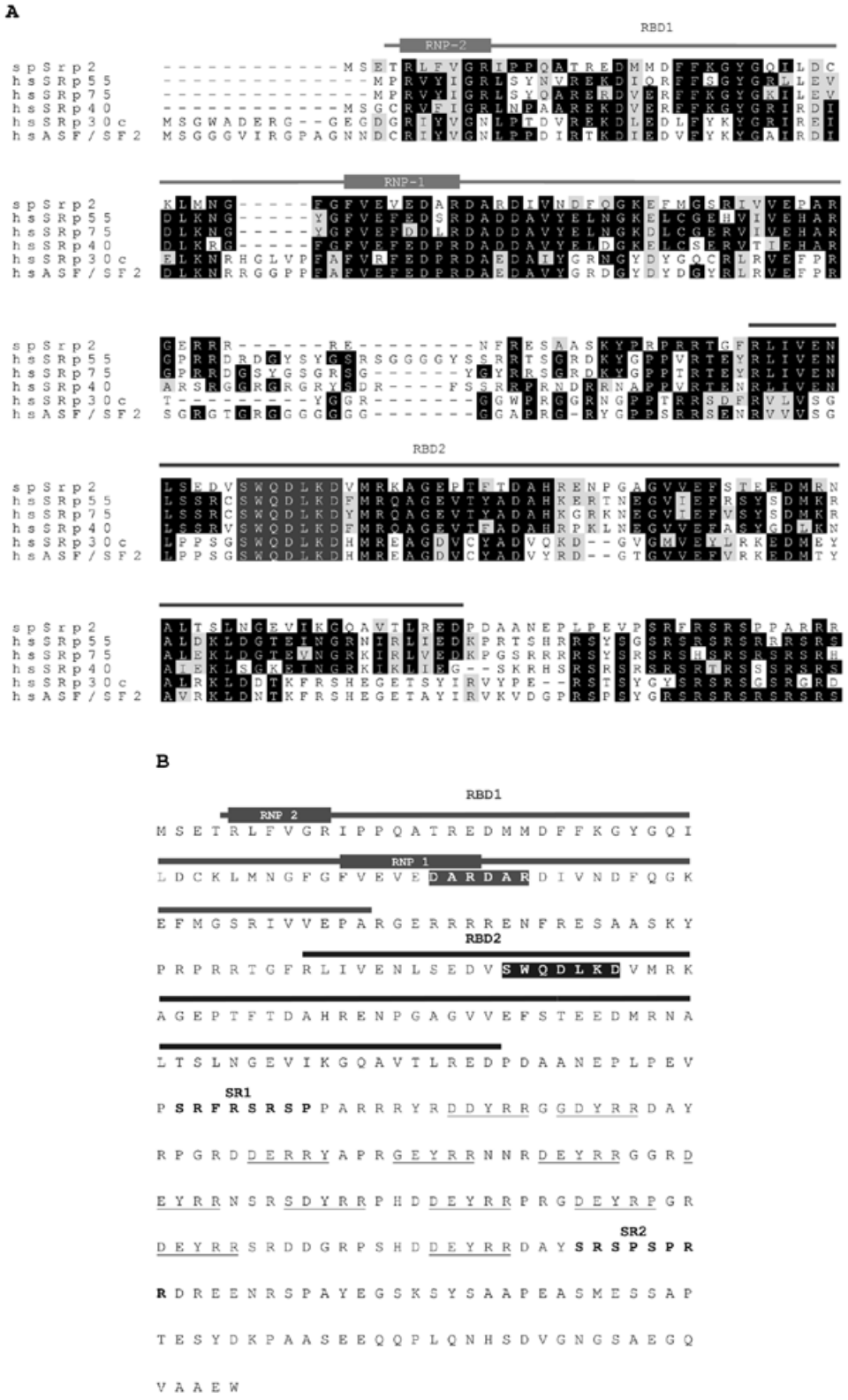


Figure 1. (**A**) Alignment of the RBD1 and RBD2 modules found in the five mammalian SR proteins with the RBDs of Srp2 (sp, *S.pombe*; hs, *Homo sapiens).* The positions of RNP-2 and RNP-1, respectively, are indicated. The positions of conserved residues are highlighted. The EMBL accession number of Srp2 is AF012278. SwissProt (SP) accession numbers of the SR proteins shown are: Q13242 (SRp30c), Q13243 (SRp40), Q13247 (SRp55), Q08170 SRp75 and Q07955 (ASF/SF2). (**B**) Deduced amino acid sequence of Srp2. The positions of the RNP-2 and RNP-1 submotifs in RBD1 are indicated. The signature sequence of RBD1 is highlighted in gray; the signature sequence of RBD2 is highlighted in black. The SR1 and the SR2 elements, which flank the so-called RRD, are in bold. The YRR repeats in the RRD are underlined.

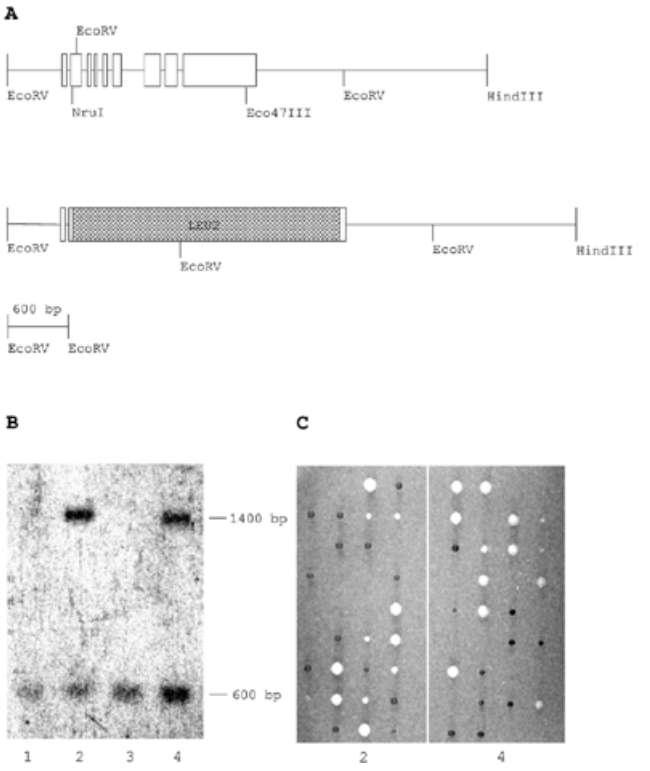


Figure 2. (**A**) A schematic representation of the chromosomal *srp2* gene and of the strategy to replace the genomic copy with the *LEU2* gene of *S.cerevisiae.* Squares represent exons, lines between the squares represent introns. Restriction sites are indicated. For details see Materials and Methods. (**B**) Southern analysis of two diploid strains demonstrating that one *srp2*$^{+}$ allele has been replaced by *LEU2*. Isolated genomic DNA was digested with *Eco*RV and probed with the radiolabeled 600 bp *Eco*RV fragment shown in (A). Lanes 1 and 3 contain DNA of wild type strains. Lanes 2 and 4 contain DNA of two growing diploid strains after the gene disruption procedure. (**C**) Tetrad analysis of the two strains harboring one interrupted allele (panels 2 and 4, respectively). Sporulation was induced on malt extract agar (MEA). The smaller and dark (red) colonies contain the *ade6-M210* allele, which was used as a marker gene.

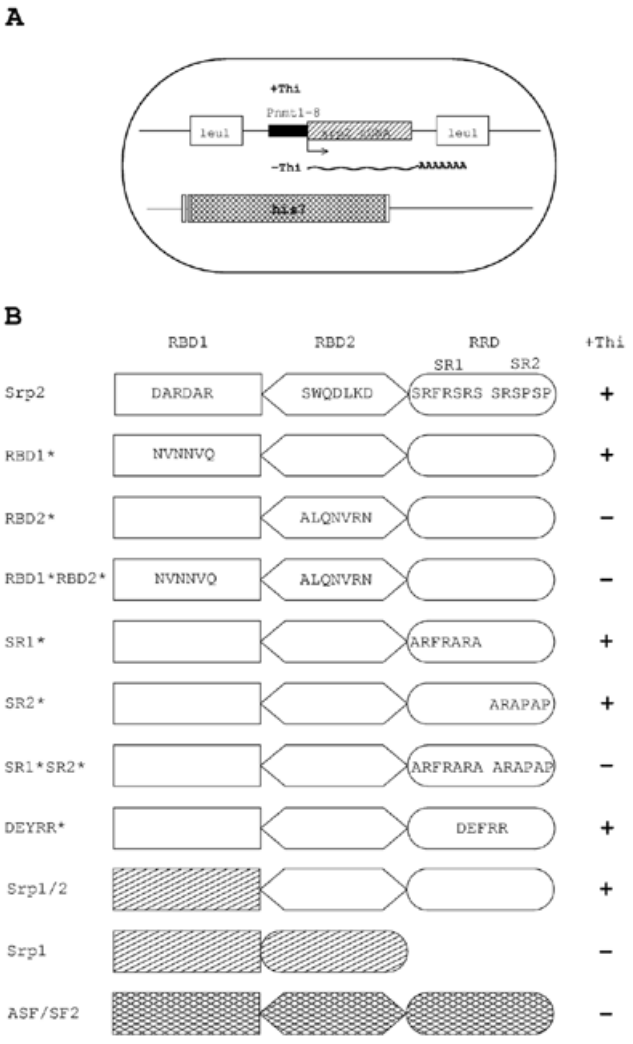


Figure 3. Complementation studies with *srp2*. (**A**) Schematic representation of the conditional *srp2* strain used. The authentic *srp2* gene was replaced by the *his7* gene and the *nmt1-8* promoter driven cDNA was integrated into the *leu1* locus. For details see Materials and Methods. When thiamine is added (+Thi) the promoter is off. (**B**) The indicated constructs were transformed into the conditional strain without thiamine in the medium. All constructs were driven by the authentic *srp2* promoter. Column +Thi indicates the growth behavior on plates supplemented with thiamine, +, growth; –, no growth. Symbols: square, RBD1; hexagon, RBD2; oval, RRD; hatched square, RBD1 of Srp1; hatched oval RS domain of Srp1; srp2, reveals the wild type motifs in the modular domains. The sequences of the mutated motifs are shown in the particular module and indicated by asterisks. ASF/SF2 contains the cDNA encoding the mammalian SR protein.

(Fig. 2A). The exon and intron sequences of *srp2* spanning 1.6 kb were replaced with a 2.1 kb DNA fragment containing the *LEU2* gene of *S.cerevisiae* as shown in Figure 2A. The resulting fragment was transformed into a diploid strain homozygous for *leu1-32* and screened for leucine prototrophic colonies. Two growing colonies were tested for proper integration of the construct (Fig. 2B, lanes 2 and 4). Tetrad analysis was performed after sporulation (Fig. 2C, panels 2 and 4), revealing that two out of the four spores always grow out healthy colonies that are leucine auxotroph. The other two spores grow out cells which stop mitotic growth after a few divisions. Interestingly, the number of division cycles after outgrowth seems in each case quite different. (Fig. 2C). These results suggest that *srp2* is essential for mitotic growth.

In vivo functional analysis of mutations in domains of Srp2

For *in vivo* analysis of *srp2*, we constructed a strain with a conditional *srp2* allele. For this purpose we fused cDNA encoding Srp2 with the *nmt1-8* promoter. This promoter is repressible with thiamine (46). Moreover, *nmt1-8* contains a mutation in the TATA box, which leads to a 1000-fold reduction in activity when a plasmid borne *lacZ* is used as the reporter gene (52,53). Under repressed conditions (+Thiamine, +Thi), very little activity was measured. To reduce promoter activity under repressed conditions to a negligible amount, we integrated the *nmt1-8* driven *srp2* gene via homologous recombination in the *leu1* locus (Fig. 3A). We subsequently interrupted the wild type allele of *srp2* using a construct in which we had replaced the ORF of *srp2* with the *his7*$^{+}$ gene. After transformation of the linear construct, we selected under derepressing conditions

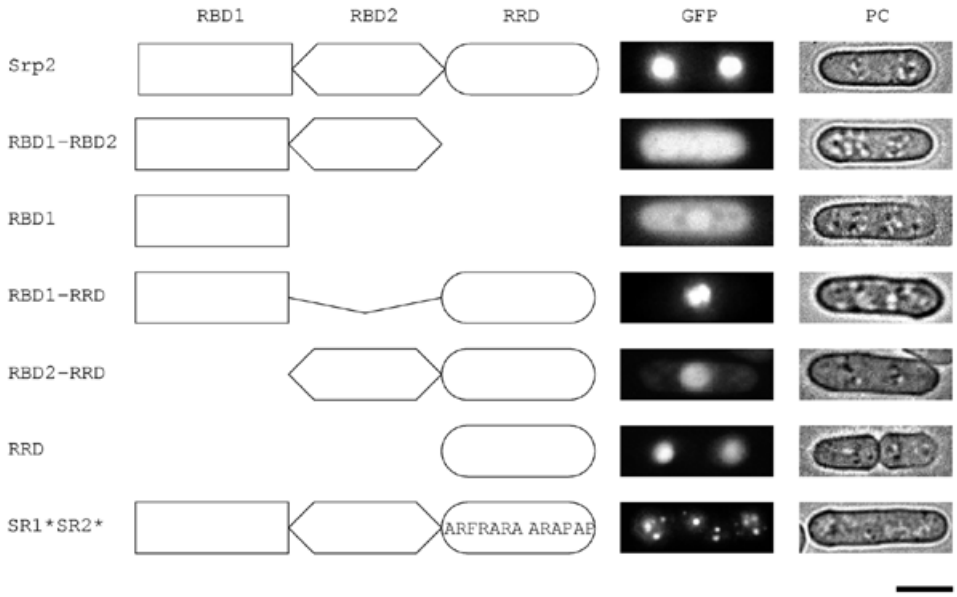


Figure 4. Cellular distribution of Srp2. Srp2 and the indicated modular domains of Srp2 were fused to the GFP. GFP fluorescence was visualized and photographed directly under a Zeiss fluorescence microscope (GFP); PC, phase contrast light microscopy photograph of the same cell. Srp2, complete gene; SR1*SR2* contains both mutated SR elements; black bar, 5 μm.

(–Thi) for histidine prototrophic colonies (Fig. 3A). Growing colonies were replica-plated on plates with Thiamine (+Thi) and selected for those which did not grow. With this approach we isolated a strain which grows when the *srp2* gene was derepressed, but does not grow when the gene is repressed (Fig. 3A). The strain was rescued under repressed conditions (+Thi) when we introduced a wild type *srp2* gene (Fig. 3B). The strain containing this conditional *srp2* allele was used for complementation analysis of site-specific mutations in *srp2*. It is important to note here that all constructs tested for complementation were plasmid borne and contained the authentic *srp2* promoter.

We tested first whether mutations in the signature motifs of RBD1 and RBD2 complement when the conditional *srp2* allele was switched off. For that purpose, we changed DARDAR in RBD1 to NVNNVQ, and SWQDLKD in RBD2 to ALQNVRN (Fig. 3B). We also constructed a gene containing both mutations (Fig. 3B). The mutation in the signature sequence of RBD1 complemented the conditional *srp2* allele, whereas the mutation in the signature sequence of RBD2 as well as the double mutation did not rescue the conditional allele. This suggests that the SWQDLKD motif in RBD2 is essential for proper function of Srp2 (Fig. 3B).

Srp2 does not contain a typical RS domain as found in Srp1 (34) and in the mammalian SR proteins. The protein displays two short SR elements beginning at position 182 (SR1) and 294 (SR2), respectively (Fig. 1C). We replaced the serines with alanines, changing SRFRSRS (SR1) to ARFRARA and SRSPSP (SR2) to ARAPAP (Fig. 3B). We also constructed a gene containing both mutated elements. The mutation in SR1 and the mutation in SR2 rescue the conditional allele, showing no negative effect on growth. However, the double mutation SR1+SR2 did not rescue function (Fig. 3B). Furthermore, we replaced the tyrosine with phenylalanine in RRDs in all 11 YRR repeat motifs (Fig. 1C). Much to our surprise, these extensive mutations had no visible effect on function: the cells grew well even when the *srp2* allele was repressed.

We wanted to know whether the function of Srp2 and Srp1 is redundant. Therefore, we tested whether Srp1 can provide the function of Srp2. Srp1 cannot substitute for Srp2 (Fig. 3B). Since both proteins have an RBD1 in common, we constructed an *srp2* gene in which we had replaced the RBD1 of *srp2* with the RBD1 of *srp1* (Fig. 3B). This chimeric gene complemented the switched-off *srp2* allele (Fig. 3B). The results of these experiments indicate that the function of Srp2 and Srp1 is not redundant, and demonstrate the domain character of these proteins: both RBD1 modules appear to serve the same function. In addition, we also tested in this system the mammalian SR protein ASF/SF2. The mammalian protein could not rescue the function of Srp2 (Fig. 3B).

Role of the different domains of Srp2 in cellular distribution

Next we investigated the cellular distribution of Srp2 and of its domains using GFP. We fused portions of Srp2 with GFP by cloning them in pTG plasmids and transformed them into an *srp2*$^+$ background (Materials and Methods). The complete Srp2 localized in the nucleus (Fig. 4). Note here that nuclear regions were identified by DAPI staining (results not shown). RBD1 and RBD1-RBD2 appear distributed in the cytoplasm and the nucleus. The RRD alone localized in the nucleus (Fig. 4). It is obvious from these experiments that whenever RRD is combined with any of the RBDs, the protein appears in the nucleus (Fig. 4). This suggests that the RRD contains a signal which is necessary and sufficient for nuclear targeting. However, neither RBD1 nor RBD2 nor the sequence of RRD contain a canonical nuclear localization signal (NLS; 54). For some mammalian SR and SR-like proteins, it has been shown that parts of the sequences of the RS domain are involved in guiding SR proteins into the nucleus and to sub-nuclear structures, called speckles (17,24,28,55). Based on these findings,

we tested whether the SR elements found in Srp2 might serve a similar function. Therefore, we fused the *srp2* constructs containing the mutated SR elements to *GFP* and analyzed the cellular distribution.

The molecules containing the mutated SR1 or SR2 element, respectively, are still targeted to the nucleus (results not shown). However, the expression of an Srp2 molecule containing both mutated SR elements revealed a drastically different picture. When we monitored a cell culture, which expressed this construct, we observed the appearance of structures distributed in the cytoplasm (Fig. 4). These results indicate that the SR1/SR2 double mutation causes the protein to accumulate in the cytoplasm.

Overexpression of the modular domains of Srp2

Recently, we have shown that overexpression of modular domains of Srp1 causes in an *srp1*$^+$ background a dominant negative phenotype resulting in growth arrest which correlates with the accumulation of pre-mRNA. In order to observe accumulation of pre-mRNA, the overexpressed constructs need to contain RBD1 of Srp1. This and other observations led us to suggest that RBD1 of Srp1 may be involved in the recognition of pre-mRNA (34). It is noteworthy here that the complete Srp1 protein and its modular RBD1 localized exclusively in the nucleus (our unpublished results). This is in contrast to the localization of RBD1 of Srp2 as shown above.

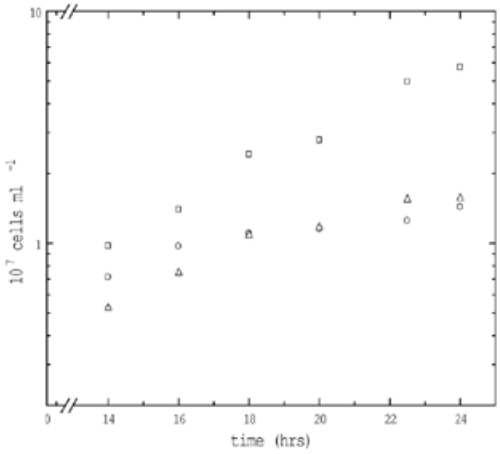
To explore the effect of Srp2 overexpression on growth, we placed the complete cDNA, the modular domains and some mutation constructs behind the strong *nmt1* promoter and transformed the plasmid borne constructs under repressing conditions (+Thi) into an *srp2*$^+$ strain. Transformed colonies were monitored for growth on plates and in liquid culture at 30°C under derepressing conditions (–Thi). A summary of the results of these experiments is shown in Figure 5A. We observed a clear dominant negative effect on growth when the *srp2* construct, RBD1-RRD, which lacks RBD2, was overexpressed (Fig. 5A and B). We also observed a negative effect on growth when we overexpressed the *srp2* construct in which the signature sequence SWQDLKD in RBD2 was mutated (Fig. 5A and B). All the other constructs, including *srp2*$^+$, do not have an effect on growth when overexpressed (Fig. 5A). Interestingly, overexpression of the construct containing both mutated signatures in RBD1 and RBD2 has no negative effect on growth (Fig. 5A). The mutation in the signature sequence of RBD1 apparently alleviates the dominant negative effect caused by the mutated signature sequence in RBD2. Taken together with the results from the complementation studies, these observations are consistent with the notion that RBD1 and RBD2 are functionally interrelated in Srp2.

We showed previously that in cells overexpressing RBD1 of Srp1, the negative effect on growth correlated with the accumulation of pre-mRNA, whereas the mRNA is rapidly turned over (34). Therefore, we tested in a northern analysis whether the overexpression of the construct RBD1-RRD of Srp2, which causes a negative growth effect, also correlates with the accumulation of pre-mRNA. As a probe we used the gene coding for the TATA binding protein (*tfIId*). This gene contains three introns and is expressed constitutively (56). No accumulation of *tfIId* pre-mRNA was observed (Fig. 5, panel RBD1-RRD).

A

Domain(s)	+Thi	–Thi
RBD1–RBD2–RRD (srp2)	+	+
RBD1–RBD2	+	+
RBD1	+	+
RBD1–RRD	+	–
RBD2–RRD	+	+
RRD	+	+
RBD1*	+	+
RBD2*	+	–
RBD1*RBD2*	+	+

B



C

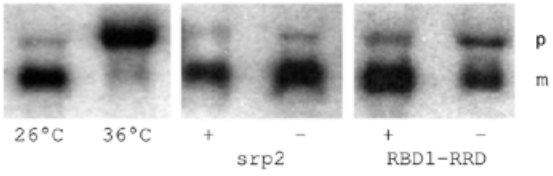


Figure 5. (**A**) Overexpression of Srp2 and the indicated modular domains. RBD1-RBD2-RRD represents the complete *srp2* gene. RBD1*, complete gene with mutated RBD1 signature; RBD2*, complete gene with mutated RBD2 signature; RBD1*RBD2*, complete gene with both mutated signature sequences. +Thi, thiamine added; –Thi, without thiamine; growth behavior on plates was observed at 30°C; +, growth; –, no growth. (**B**) Growth behavior of RBD1-RBD2-RRD (squares), RBD1-RRD (circles) and RBD2* (triangles) in liquid culture. Each culture was inoculated with 5×10^5 cells/ml in medium without thiamine (–Thi) and incubated at 30°C for the indicated time. (**C**) Northern analysis of total RNA isolated from cells expressing RBD1-RBD2-RRD (panel srp2) and the construct which lacks RBD2 (panel RBD1-RRD). +, cultures were supplemented with thiamine; –, cultures without thiamine. RNA was isolated from cultures incubated at 30°C, 16 h after inoculation. The radiolabeled *tfIId* gene was used as a probe. As a control, lanes 26°C and 36°C contain total RNA isolated from a strain containing the temparature-sensitive *prp4-73* allele which causes accumulation of pre-mRNA at 36°C (27). m, mRNA; p, pre-mRNA.

The replacement of RBD1 in Srp2 with RBD1 of Srp1 revealed a proper functioning Srp2 molecule (Fig. 3A). When we overexpressed this chimera, cells grew well (Fig. 6A,

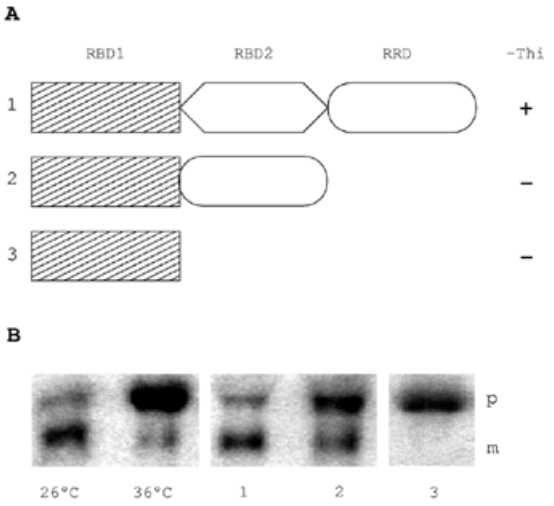


Figure 6. (**A**) Overexpression of chimeric constructs of Srp1 and Srp2. Hatched square, RBD1 of Srp1. The hexagon represents RBD2 and the oval the RRD region of Srp2, respectively. Column –Thi, growth behavior of the strain when the indicated construct is overexpressed. +, growth; –, growth arrest. (**B**) Northern analysis of total RNA isolated from cells overexpressing the chimeric constructs shown in (A). Radiolabeled *tfIId* fragment was used as the probe. Lanes 1, 2 and 3 contain RNA from strains overexpressing the constructs shown under 1, 2 and 3 in (A). RNA was isolated from cultures incubated at 30°C, 16 h after inoculation. Lanes 26°C and 36°C serve as control to roughly estimate pre-mRNA accumulation as described in the legend of Figure 5. m, mRNA; p, pre-mRNA.

panel 1) and as expected *tfIId* pre-mRNA did not accumulate (Fig. 6B, lane 1). However, when we overexpressed the second chimera containing RBD1 of Srp1 and RRD of Srp2, growth was impaired (Fig. 6A, panel 2). This growth arrest correlated with the accumulation of *tfIId* pre-mRNA as shown in Figure 6B (lane 2). These results suggest that the chimeric product inhibits splicing of the *tfIId* pre-mRNA and, particularly, that it is RBD1 of Srp1, which mediates this effect (Fig. 6B, lane 3). It seems that when RBD1 of Srp1 is placed in the sequence context of Srp2, it is affected by RBD2 and assumes the specificity of the authentic RBD1 in Srp2 (Fig. 3A).

DISCUSSION

We have characterized *in vivo* Srp2 by using three approaches: (i) testing the complementation capability of specific mutations of Srp2 in a conditional *srp2* strain; (ii) measuring the effects of overexpression of individual domains on cell growth and pre-mRNA splicing of the *tfIId* transcript; and (iii) investigating the role of individual domains of Srp2 in cellular distribution.

In vivo RBD2 of Srp2 determines the specificity of Srp2 function

RBD1 and RBD2 of Srp2 contain motifs, highly conserved in sequence and position, which have been found in the RBD domains of typical SR splicing factors (Fig. 1A) (2,4). A mutation in the signature sequence of RBD2 leads to loss of function of Srp2 in the complementation test, whereas the mutation in the signature sequence of RBD1 is still able to rescue the conditional *srp2* allele. In addition, RBD1 of Srp2 can be replaced by RBD1 of Srp1 without an observable negative effect for the cells in the complementation test. Taken together, these results are consistent with the idea that, in the context of Srp2, it is RBD2 which plays an important role in determining functional specificity of the molecule.

Overexpression studies confirm that RBD2 determines specificity in Srp2

Overexpression of the RBD1 of Srp1 inhibited growth. The growth arrest correlated with the accumulation of pre-mRNA of the *tfIId* gene (Fig. 6B, lane 3) (34). We suggested previously that Srp1 may be a general splicing factor and that its RBD1 may be involved in recognizing pre-mRNA (34). The overexpression of RBD1 and RBD1-RBD2 of Srp2 does not cause a dominant negative effect on growth. Since both molecules are distributed in nucleus and cytoplasm (Fig. 4), it is conceivable that the concentration in the nucleus is too low to exert a dominant negative effect. In contrast, overexpression of RBD1-RRD, which is located exclusively in the nucleus (Fig. 4), causes a dominant negative effect on growth, but does not lead to the accumulation of *tfIId* pre-mRNA (Fig. 5, panel RBD1-RRD). On the other hand, the replacement of RBD1 in this construct with RBD1 of Srp1 leads to the accumulation of *tfIId* pre-mRNA (Fig. 6B, lane 2). This result confirms that the inhibition of splicing of *tfIId* pre-mRNA is caused by the RBD1 of Srp1. This specificity for *tfIId* pre-mRNA can be changed when RBD1 of Srp1 is combined with RBD2 of Srp2. Overexpression of RBD1(Srp1)-RRD does not lead to the accumulation of pre-mRNA of *tfIId*, but still inhibits growth (Fig. 6B, lane 1). In addition, the chimera RBD1 (Srp1)-RBD2(Srp2)-RRD behaved as the authentic Srp2, judged by the perfect complementation of the strain in which *srp2* was switched off (Fig. 3A). These results obtained *in vivo* are consistent with results obtained with mammalian SR proteins *in vitro*. It has been shown that swaps of RBD1 of the SR protein SC35 with RBD1 of the SR protein ASF/SF2, which contains two RBDs, can change specificity for pre-mRNAs and that RBD2 of ASF/SF2 can affect other RBDs negatively and positively (8–13).

The SR elements are involved in nuclear localization of Srp2

The two RBDs of Srp2 are not sufficient to move the molecule into the nucleus. It is the RRD, particularly the two SR elements, which are required to target Srp2 to the nucleus. The two SR elements in this sequence context are signals for nuclear localization of Srp2 (Fig. 4). This is in marked contrast to the RBD1 of Srp1. The RBD1 domain lacking the RS domain still localized exclusively in the nucleus (results not shown). Taken together, these observations are reminiscent of findings reported in an investigation of the distribution pattern of modular domains of mammalian SR proteins (17). In the case of ASF/SF2, the RS domain is required for targeting the nucleus, but is not sufficient for the sub-nuclear localization in the speckles. For SC35 and Srp20, the RS domain is necessary and sufficient to reach the nucleus and the sub-nuclear localization (17,55). Srp2 does not contain a typical RS domain. However, it is conceivable that the SR elements are the basic units of an RS domain (Fig. 1A). That is, the variable length of RS domains in SR proteins could have arisen by multiplication of basic units which assumed different functions, such as nuclear localization signals or speckle signals, thus providing

regions for dynamic interaction with other nuclear components (6,15).

The results presented here and in a recent study (34) demonstrate that fission yeast contains proteins closely related to the SR protein family. Our results suggest that the RBDs of Srp1 and Srp2 are closely related in structure and function to the RBDs found in SR proteins. However, we have no direct evidence that either Srp1 or Srp2 is a bona fide splicing factor. However, based on circumstantial evidence it seems likely that Srp1 plays a role in this process, whereby the involvement of Srp2 in pre-mRNA splicing remains to be shown.

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