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Identification and characterization of *srp1*, a gene of fission yeast encoding a RNA binding domain and a RS domain typical of SR splicing factors

Thomas Gross, Kathrin Richert, Claudia Mierke, Martin Lützelberger and Norbert F. Käufer*

Institut für Genetik-Biozentrum, Technische Universität Braunschweig, 38106 Braunschweig, Germany

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ABSTRACT

The SR protein family is involved in constitutive and regulated pre-mRNA splicing and has been found to be evolutionarily conserved in metazoan organisms. In contrast, the genome of the unicellular yeast *Saccharomyces cerevisiae* does not contain genes encoding typical SR proteins. The mammalian SR proteins consist of one or two characteristic RNA binding domains (RBD), containing the signature sequences RDAEDA and SWQDLKD respectively, and a RS (arginine/serine-rich) domain which gave the family its name. We have now cloned from the fission yeast *Schizosaccharomyces pombe* the gene *srp1*. This gene is the first yeast gene encoding a protein with typical features of mammalian SR protein family members. The gene is not essential for growth. We show that overexpression of the RNA binding domain inhibits pre-mRNA splicing and that the highly conserved sequence RDAEDA in the RBD is involved. Overexpression of Srp1 containing mutations in the RS domain also inhibits pre-mRNA splicing activity. Furthermore, we show that overexpression of Srp1 and overexpression of the mammalian SR splicing factor ASF/SF2 suppress the pre-mRNA splicing defect of the temperature-sensitive *prp4-73* allele. *prp4* encodes a protein kinase involved in pre-mRNA splicing. These findings are consistent with the notion that Srp1 plays a role in the splicing process.

INTRODUCTION

The main features of the eight mammalian SR family members are one or two RNA binding domains (RBD) at the N-terminus. The first RBD displays the conserved submotifs RNP-2 and RNP-1 which are typical of this type of RBD (Fig. 1C). These motifs have been shown to be involved in RNA interaction. In addition, all eight family members contain in the first RBD (RBD1) the highly conserved sequence RDAEDA. The SR family members which contain two RBDs invariably display the sequence SWQDLKD in the second RBD (RBD2), however, this domain does not contain typical RNP submotifs. The region at the C-terminus consists of alternating RS dipeptides and has in each family member a different length and arrangement of the dipeptides (1–5). *In vitro* studies showed that SR proteins function in the basic splicing reaction and in alternative splicing (6,7). SR proteins can bind to so-called exonic enhancer sequences, subsequently activating the splicing of weak upstream introns (8,9). Extensive work, mostly *in vitro*, has been done with the SR family members SC35 and ASF/SF2 (1,2). The latter protein contains two RBDs. It has been shown that ASF/SF2 binds to RNA containing 5′ splice sites and that the RNP-1 submotif of the first RBD is involved in RNA binding. ASF/SF2 proteins with mutations in RNP-1 cannot activate *in vitro* splicing (7,10,11). The RS domain becomes phosphorylated *in vitro* by two protein kinases, Clk/Sty and SRPK1 (12,13). The RS domain is essential for splicing to occur and there is evidence that phosphorylation of the domain is necessary for function (14). ASF/SF2 and SC35, for example, interact through the RS domain with U1 70K, a protein of the ribonucleoprotein particle U1 (snRNP U1). Based on the results of these biochemical studies with ASF/SF2, SC35 and other SR family members, it has been suggested that these proteins may mediate interactions between spliceosomal components and thereby facilitate proper assembly of a catalytic spliceosome (2,15,16).

In mammalian cells the SR proteins are found in speckles, which are subnuclear structures. It has been suggested that the SR proteins are moved from the speckles to the location of spliceosome assembly, and phosphorylation of the RS domain may play a role in this translocation (1,12,13,17).

We have isolated the *prp4* gene encoding a protein kinase of *Schizosaccharomyces pombe*. The protein kinase is involved in pre-mRNA splicing. The temperature-sensitive allele *prp4-73* causes accumulation of all pre-mRNAs tested at the restrictive temperature. We have also isolated a putative mammalian homolog of Prp4. Both the fission yeast and the mammalian kinase phosphorylate the mammalian SR protein ASF/SF2 in its RS domain *in vitro* (18–20).

In this study we show that overexpression of the RBD of *srp1* causes accumulation of pre-mRNA, which correlates with cessation of growth. Mutations in the RDAEDA motif of the RBD abrogate this effect, cells grow and produce mRNA. Using this *in vivo* approach we demonstrate that the entire RS domain and an

*To whom correspondence should be addressed. Tel: + 49 531 391 5774; Fax: + 49 531 391 5765; Email: n.kaeufer@tu-bs.de

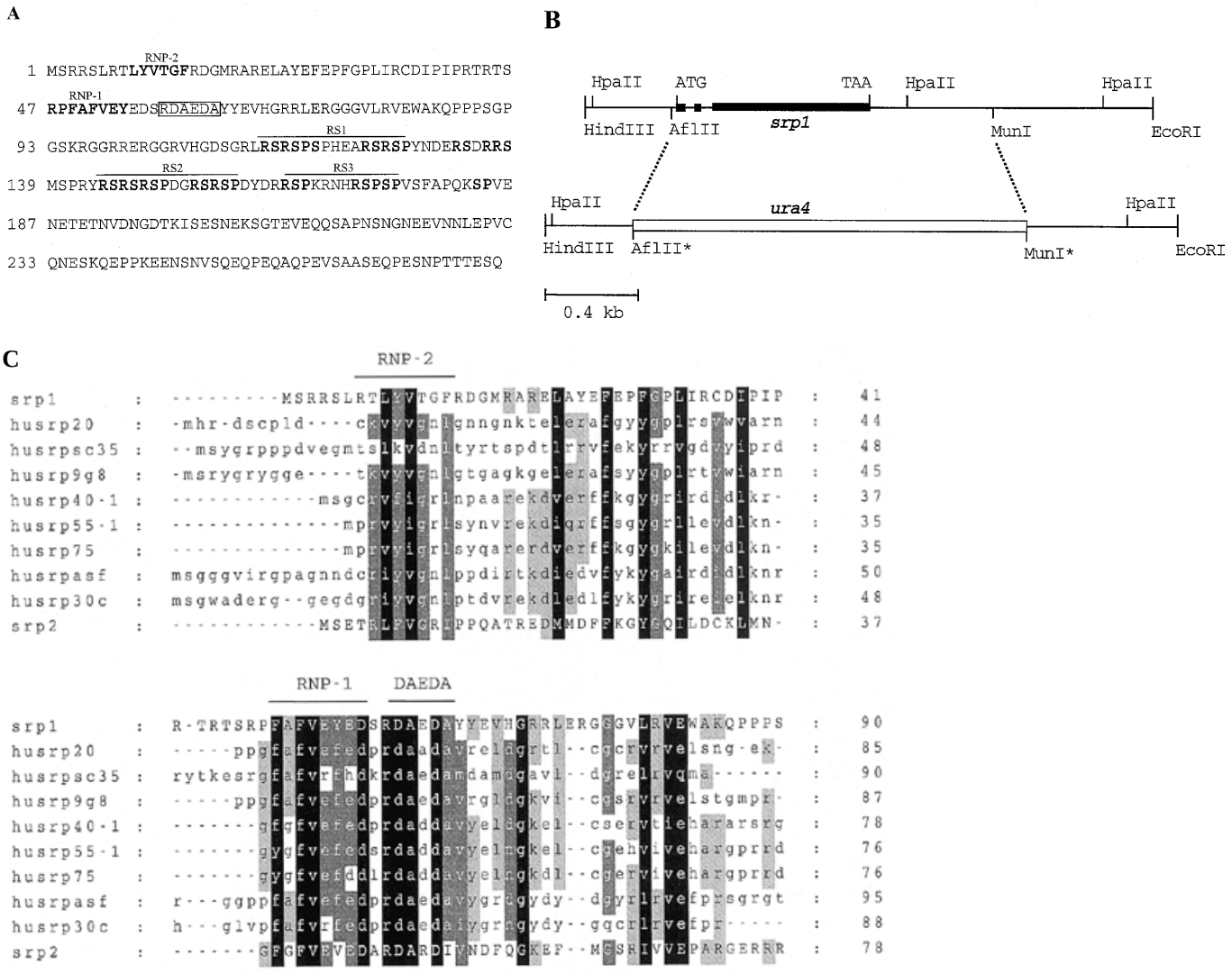

Figure 1. (**A**) Deduced amino acid sequence of Srp1. The RNP-2, RNP-1 and DAEDA submotifs in the RBD are in bold and boxed respectively. The three RS subdomains are designated RS1, RS2 and RS3. The RS motifs are highlighted in bold. (**B**) A schematic representation of the chromosomal *srp1* gene and of the strategy to replace the genomic copy with the *ura4* gene. ATG and TAA indicate the positions of the start and stop codons of *srp1* respectively; the black boxes represent exons, the thin lines between are introns. Restriction sites are indicated. The restriction sites *Hin*dIII and *Eco*RI are from the MCS of the cloning vector. Asterisks at the restriction sites indicate that these sites have been destroyed through cloning. The white box represents promoter, coding region and 3′-regulatory region of the *ura4* gene. (**C**) Alignment of RBD1 sequences of Srp1 and Srp2, encoded by the newly isolated genes from fission yeast, with the RBD1 of eight human SR proteins. The positions of RNP-2, RNP-1 and DAEDA are indicated. The positions of conserved residues are highlighted by vertical shading. GenBank accession nos U66833 (Srp1) and AF012278 (Srp2).

additional C-terminal region of Srp1 are functional domains of the protein. Furthermore, *srp1* in a genetic background containing the temperature-sensitive *prp4-73* allele suppresses the splicing defect of these cells. Overexpression of the mammalian splicing factor ASF/SF2 also suppresses the splicing defect of this strain. The findings using an *in vivo* approach reported in this paper are consistent with models that propose that in constitutive pre-mRNA splicing SR proteins play a role in spliceosome assembly.

MATERIALS AND METHODS

Plasmid and strain construction

Oligonucleotide-directed site-specific mutagenesis was used to create the mutations in the RBD and RS domains. We used the method developed by Kunkel as described previously (34), targeting single-stranded *srp1* cDNA with the following primers: VAVVA, 5′-GCATTCGTTGAGTACGCAGTTTCCGGAGTTGCTGTAG-TTGCG TATTAT-3′; RS1, 5′-CGGCTACGTTTAAGGTAC-CCTATTCCGCACGAAGCCCGATTTCGTGCCCCT-3′; RS2, 5′-GCCTCGTTATCGTTTGCGTATTCGTATTCCTGATGGAC-GTATACGGTTTCCCGAT-3′; RS3, 5′-TATGATCGTCG-TGCTCCTAAACGGAACCATCGTGCACCTTGGCCAGTCT-CT-3′. Isolation of phage DNA and further characterization of the mutated constructs was performed as described (20). The cDNA of wild-type *srp1* and the mutated versions were fused to the *nmt1* promoter in the pRIP2 vector via *Bam*HI linkers. The vector contains the *ura4* gene as a marker (21). The plasmid was linearized by cutting with *Stu*I, which has a unique restriction site in *ura4*, and integrated into the *ura4* locus of strains containing

the *ura4-294* mutant allele as described (20). Standard classical and molecular genetic procedures and media for growth of the *S.pombe* strains have been described by Gutz *et al*. (22) and Moreno *et al*. (23).

A cDNA library from HeLa S3 cells was used as template in a PCR reaction to isolate the cDNA of ASF/SF2. The product was subcloned and sequenced. The cDNA was fused to the *nmt1* promoter in the pREP1 vector using *Bam*HI adapters. pREP1 contains the *ars1* sequence for replication and the *ura4* gene as a marker (21).

RNA analysis

Isolation of RNA and Northern analysis was performed as described previously (19). Total RNA was fractionated on a 1.2% agarose gel, subsequently transferred onto nitrocellulose and probed with the complete *tfIId* gene encoding the TATA binding protein (TBP; 24). The probe was isolated in a PCR reaction using a genomic library as template and spans the complete gene containing three introns. Probes were labeled with a random priming kit using [α-^{32}P]dCTP (Amersham).

RESULTS

Isolation of *srp1*

Based on the finding that Prp4 protein kinase of *S.pombe* has a putative sequence homolog in mammalian cells and that the fission yeast and mammalian kinases were capable of phoshorylating the RS domain of human SR protein ASF/SF2 *in vitro* (18), we reasoned that fission yeast might contain SR proteins. Therefore, we used several approaches to identify genes displaying features of SR proteins. In one approach we searched genome sequences of *S.pombe* stored at the Sanger Centre (http://www.sanger.ac.uk/Projects/S_pombe/) for RBDs using structure-based criteria as suggested by Birney *et al*. (4). With this approach we found several putative RBDs, one of which contains the sequence FAFVEYEDSRDAEDA. The first eight amino acids fit the consensus of the RNP-1 submotif in a RBD (4). When this sequence was used in a search against GenBank it matched with all eight mammalian SR protein family members, showing between 58 and 86% identical amino acids (Fig. 1C). The *S.pombe* sequence for this putative open reading frame (ORF) resides on cosmid c13F4. This cosmid contains a partial gene comprising a RBD (accession no. Z69379). Based on this sequence information we used PCR and produced probes to clone the complete gene from a genomic and a cDNA library. Figure 1A shows the complete amino acid sequence (accession no. U66833), which contains one RBD, a glycine hinge region and a RS domain. The fission yeast sequence contains an additional C-terminal end of ~100 amino acids for which no related sequence was found in GenBank. Sequence analysis of the genomic clone revealed that the gene contains two introns of 49 and 45 bp (Fig. 1B). Both introns are found in the RBD and display the typical architecture of *S.pombe* introns discussed previously (25,26).

srp1 is not essential for growth

In order to test whether *srp1* is essential for growth we replaced the complete coding region of *srp1*$^+$ with the *ura4*$^+$ gene. As shown in Figure 1B, we replaced a 1.2 kb *Afl*II–*Mun*I fragment comprising the complete ORF of *srp1*$^+$ with a 1.75 kb *Hin*dIII fragment containing the *ura4* gene. The resulting 2.7 kb *Hpa*II–*Hpa*II fragment was transformed into diploid cells lacking the *ura4* gene and screened for colonies prototrophic for uracil. Growing colonies were sporulated and tetrad analysis was performed. All four spores of a tetrad developed as colonies, suggesting that *srp1* is not essential for growth (results not shown). However, comparing growth behavior of the *srp1* null strain with a *srp1* wild-type strain at temperatures of 17, 25, 30 and 36°C respectively revealed that strains in which *srp1* was deleted show a mild cold-sensitive phenotype at 17°C.

Overexpression of the RBD inhibits pre-mRNA splicing

We dissected the *srp1* gene by cloning individual domains into plasmid pRIP2, thereby fusing the constructs to the *nmt1* promoter (Fig. 2). The *nmt1* promoter is repressible with thiamine (27). The constructs were integrated into the *ura4* locus. All strains grew when the *nmt1* promoter was repressed (Fig. 2, +Thi). However, when they were incubated under derepressing conditions (–Thi) the strain overexpressing a truncated protein consisting of the RBD (RBD-D) and the strain overexpressing the RBD and the RS domain (RBD-RS) did not grow. In contrast, overexpressing the wild-type *srp1* gene and genes without the RBD, containing only the RS domain and the C-terminus (RS-C) or the RS domain alone (RS), had no effect on growth (Fig. 2). These results indicate that overexpression of the RBD causes the growth defect. It is possible that this domain, by binding randomly to RNA, disturbs RNA metabolism. However, it is also conceivable that growth inhibition is caused by a specific effect.

To investigate these possibilities the strain containing the RBD construct was grown under repressing and derepressing conditions. It takes ~12 h until the *nmt1* promoter is fully derepressed (27). Therefore, we inoculated the strains in medium with and without thiamine and monitored growth. As shown in Figure 3A, the strain overexpressing the RBD ceased to grow after 11 h. This was also true for the strain containing the truncated construct RBD-RS (results not shown and Fig. 2). We isolated RNA from the strains expressing the RBD over the indicated time period and performed a Northern analysis (Fig. 3B). The *tfIId* gene, containing three introns, was used as probe (24). Cessation of growth correlated with accumulation of pre-mRNA (Fig. 3B). When this strain was incubated under repressed conditions it grew normally and mRNA was produced (Fig. 3B). This suggests that overexpression of the RBD of Srp1 inhibits pre-mRNA splicing *in vivo*.

It is conceivable that this dominant negative effect might be due to binding of the RBD to pre-mRNA, thereby blocking further processing. It has been shown that ASF/SF2 proteins with mutations in the submotif RNP-1 of the first RBD cannot activate *in vitro* splicing (7,10,11). The eight amino acid RNP-1 submotif is followed by the RDAEDA sequence. This region of ASF/SF2 shares 86% identical amino acids with that of Srp1. Both proteins display the sequence RDAEDA (Fig. 1B). Based on these observations we hypothesized that if this sequence is involved in recognizing pre-mRNA, we should expect growing cells and spliced message when we overexpress the RBD containing a mutated version of RDAEDA. Therefore, we changed the sequence in the RBD from EDSRDAEDA to AVSGVAVVA. Overexpression of the RBD containing the mutated sequence indeed had no effect on growth and the cells produced mRNA (Fig. 3A and B). We also changed this region in the complete *srp1* gene. Overexpression of

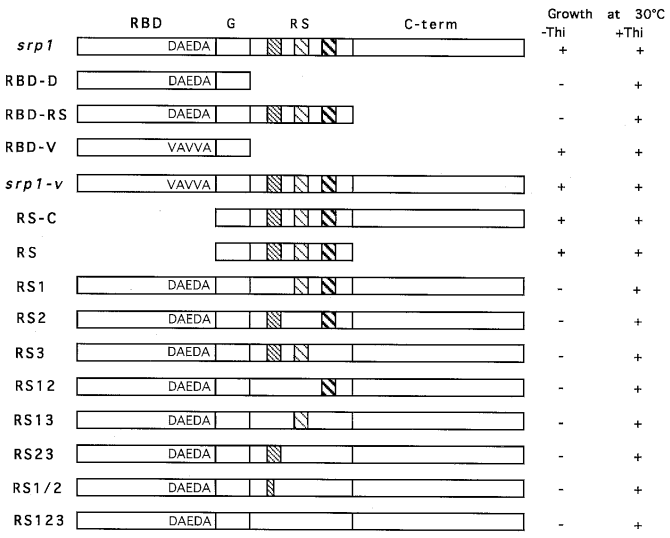

Figure 2. Summary of the effects of Srp1 mutations on growth. The structure of the wild-type and mutant genes are shown. The genes were fused to the repressible *nmt1* promoter and integrated in the *ura4* locus using the pRIP system (21). The effect on growth, indicated on the right, was monitored on plates under repressing (+thiamine, Thi) and derepressing conditions (–thiamine); +, growth; –, no growth. The domain structure of *srp1* is indicated by: RBD, RNA binding domain; G, glycine hinge; RS, RS domain; C-term, C-terminal domain. In the RBD the DAEDA motif is highlighted. In some mutants the DAEDA motif was changed to VAVVA as indicated. The three hatched boxes in the RS domain represent subdomains RS1, RS2 and RS3 respectively. The mutated RS1 reads RLRYPIPHEARFRAP; mutated RS2, RLRIRIPDGRIRFP; mutated RS3, RAPKRNHRAPWP.

this construct had, as expected, no effect on growth (*srp1-v*, Fig. 2). These results suggest that overexpression of the RBD inhibits pre-mRNA splicing and that the sequence EDSRDAEDA is involved in this inhibition. We also used other intron-containing genes as probes and observed the same pattern (results not shown). It is, therefore, possible that the RBD of Srp1 binds to pre-mRNA and blocks further processing since it lacks essential functional domains. This notion is consistent with the results discussed above. For example, the strain overexpressing the construct containing the RBD and the RS domain (RBD-RS) but lacking the C-terminal region does not grow (Fig. 2) and accumulates pre-mRNA (results not shown).

Mutations in the RS domain of Srp1 have dominant negative effects when overexpressed

The results of our approach discussed above encouraged us to use this *in vivo* system to investigate a possible role of the RS domain. The RS domain can be subdivided into three regions. We mutated each of the indicated subdomains by replacing the serines with other amino acid residues (RS1, RS2 and RS3, Figs 1A and 2). In RS1 we changed the five serines from left to right to leucine, tyrosine, isoleucine, phenylalanine and alanine; in RS2 the five serines were replaced with leucine followed by three isoleucines and phenylalanine; finally, in RS3 of the three serines two were changed to alanine and the last one to tryptophan (Fig. 2, legend). We constructed all possible combinations of mutated and non-mutated subdomains. In one experiment we only replaced the first three of the five serines of RS1 (RS1/2, Figs 1A and 2). Strains RS1, RS2 and RS3, which overexpress the mutated constructs, do not grow (Fig. 4A). When these strains were inoculated into medium without thiamine (–Thi) and RNA isolated after the indicated time period, Northern analysis showed accumulation of pre-mRNA (Fig. 4B). Again, accumulation of pre-mRNA correlated with cessation of growth (results not shown). We conclude from the results of these experiments that the entire RS domain of Srp1 is functionally involved. The dominant negative effect of these mutations can be explained by proteins mutated in the RS domain still binding to pre-mRNA, thereby disrupting further processing, such as assembly of functional spliceosomes.

Overexpression of *srp1*$^+$ and *ASF/SF2* suppresses the splicing defect of the *prp4-73* allele

We tested whether *srp1* can suppress the temperature-sensitive phenotype of *prp4-73*. We transformed the *srp1* gene under the control of the *nmt1* promoter into a strain which contained the temperature-sensitive *prp4-73* allele. A strain containing *prp4-73* grows well at the permissive temperature (25°C) and produces mRNA efficiently. However, when the culture is shifted to the restrictive temperature (36°C) pre-mRNA accumulates and cells stop growing (Fig. 5, lanes *prp4-73*). In addition, we determined by flow cytometry the DNA content of the cell population after 3 h at the restrictive temperature. In this cell population individual cells arrested with a 1C or 2C DNA content (results not shown). Since ~50% of the *S.pombe* genes, including genes regulating the cell cycle, contain introns (26), it is conceivable that without splicing activity cells arrest in different cell cycle stages.

The *prp4-73* strain containing the plasmid-borne *srp1*$^+$ gene shows a different pattern; when shifted for 3 h to the restrictive temperature the cells still showed splicing activity. This pattern was observed only when Srp1 was expressed from a high copy plasmid, independent of whether the *nmt1* promoter was repressed or derepressed (Fig. 5, lanes *srp1*).

The growth defect of this strain at 36°C was only partially suppressed, revealing minicolonies on plates. However, most of the cells of this culture arrested with a 2C content when shifted to 36°C for 3 h (results not shown). These results suggest that overexpression of Srp1 can suppress the splicing defect caused by *prp4-73*, however, suppression is not complete. This could be due to the generally lower splicing efficiency in this strain. Second, it is also possible that splicing of certain genes is not suppressed, leading to arrest of the cells in a specific cell cycle stage. Third, we have some indication that Prp4 might be pleiotropic (20); overexpression of Srp1 might suppress the splicing defect of *prp4-73*, but not other functional defects caused by this allele. We do not have experimental evidence for this scenario. Interestingly, overexpression of the mammalian SR protein ASF/SF2 also leads to splice activity in the *prp4-73* strain at the restrictive temperature. The cells produce mRNA, although splicing efficiency in these cells is significantly decreased (Fig. 5, lanes *ASF/SF2*).

DISCUSSION

We isolated *srp1*, which encodes a protein that is closely related to the SR protein family found in metazoa (3), from the unicellular yeast *S.pombe*. Since Srp1 is not essential for growth, it is conceivable that, as in metazoan organisms, other Srp or Srp-like proteins exist which might compensate for Srp1. In fact,

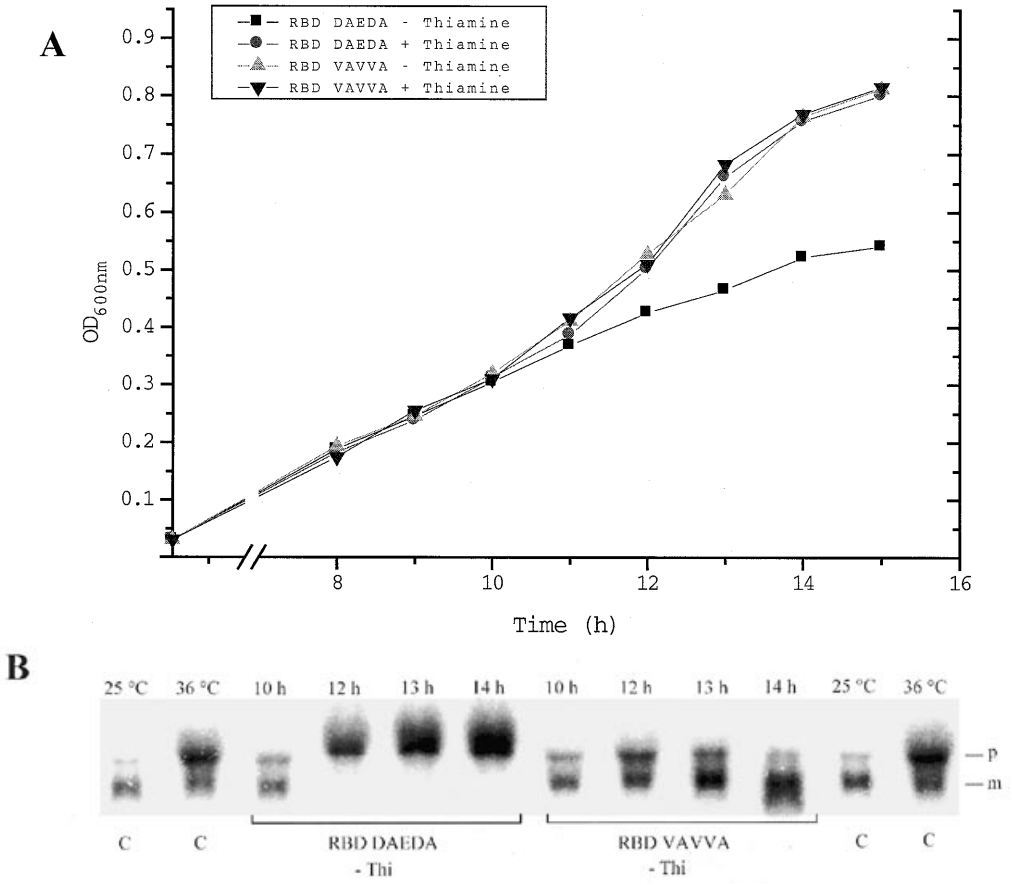

Figure 3. (**A**) Growth behavior of strains expressing the RBDs RBD-DAEDA and RBD-VAVVA respectively. The strains were inoculated (2×10^5 cells/ml) in medium +thiamine and –thiamine, as indicated in the inset, and grown at 30°C. (**B**) Northern analysis of total RNA isolated from cells expressing RBD-DAEDA and RBD-VAVVA under derepressed conditions (–Thi). Total RNA was isolated at the indicated time points (h) during growth. Aliquots of 15 µg RNA were fractionated on agarose, transferred to nitrocellulose and hybridized to the radiolabeled *tfIId* gene (see Materials and Methods). Lanes C contain total RNA of a strain containing *prp4-73* in the genetic background grown at 25°C and shifted for 3 h to 36°C as indicated. p, pre-mRNA; m, mRNA.

we have identified a gene, called *srp2* (accession no. AF012278), which contains two RBDs closely related to the mammalian SRp55 and SRp40 proteins (1). *srp2* is essential for growth (results not shown). Overlapping functions for the mammalian SR proteins have been demonstrated *in vitro*, however, there is growing evidence that different SR proteins might also have the capability to commit specific pre-mRNAs to splicing (1,28).

Our *in vivo* results presented here are consistent with results obtained *in vitro* showing that SR proteins can act in spliceosome assembly (1–3). The observed growth inhibition and accumulation of pre-mRNA in cells overexpressing the RBD with an intact RSDAEDA motif, as compared with cell growth and splicing activity of cells expressing a protein in which this motif was completely changed, suggest that this motif is involved in *in vivo* binding to pre-mRNA. This is the first experimental demonstration that this motif is most likely essential for function of the protein. All metazoan SR protein sequences available in GenBank contain this motif. Based on the highly conserved character of this sequence, which is, as yet, found only in typical SR proteins, it is proposed as a signature sequence of SR proteins involved in pre-mRNA splicing (37). Computer modeling with the first RBD of the SR proteins shows the α-helical RSDAEDA motif exposed on the surface of the modeled structure (results not shown).

The results obtained with mutation analysis of the RS domain of Srp1 indicate that the entire RS region is important for function of the protein. It has been shown *in vivo* that a mammalian ASF/SF2 protein lacking the RS domain could not complement a chicken cell line which was depleted of wild-type ASF/SF2 (29). The results reported here are consistent with the notion that RS domains can mediate interactions with other spliceosomal components during spliceosome assembly (15,16). The arrangement of the RS domain in Srp1 differs somewhat from that found in mammalian RS domains. It is remarkable that the SR dipeptides are often changed to SP dipeptides (Fig. 1A). Interestingly, we also find sequence motifs such as SRSPSP in the amino acid sequence of the above-mentioned *srp2* gene.

In addition to the RBD and RS domain, Srp1 has a C-terminus for which no related sequence was found in the data bank. This region also appears important for function of Srp1 in our system, however, the specific role it plays is not known.

We found that at the restrictive temperature (36°C) in a *prp4-73* strain all pre-mRNAs which we tested accumulated. This included pre-mRNAs from genes in which we had inserted randomly artificial introns (18,30). Based on these findings we suggested that *prp4-73* causes a general splicing defect and proposed that Prp4 kinase activity is required for pre-mRNA

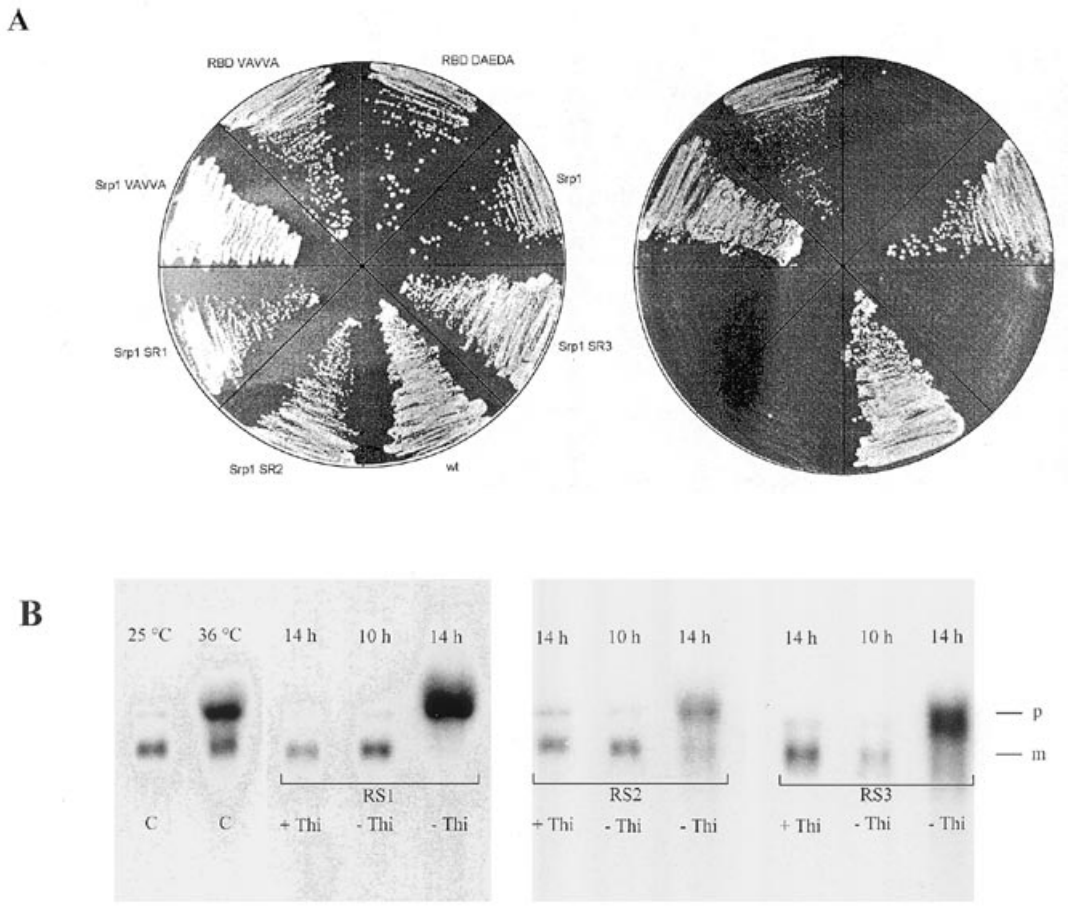

Figure 4. (**A**) Growth of strains expressing mutated *srp1* genes as indicated (see Fig. 2) under repressed (+thiamine) and derepressed conditions (–thiamine). *srp1* RS1, *srp1* RS2 and *srp1* RS3 express the mutated submotifs RS1, RS2 and RS3 respectively (see Fig. 2). (**B**) Northern analysis of total RNA isolated from strains expressing the mutated RS submotifs. RNA was isolated at the indicated time (h) after inoculation in medium +thiamine (+Thi) and –thiamine (–Thi) and hybridized to the *tfIId* gene as described in Figure 3. Lanes C, total RNA of a strain containing *prp4-73* grown at 25°C and shifted for 3 h to 36°C as indicated. p, pre-mRNA; m, mRNA.

splicing to occur (19,20). When Srp1 or the mammalian SR protein ASF/SF2 is overexpressed at the restrictive temperature mRNA is produced, suggesting that both proteins can, at least partially, suppress the pre-mRNA splicing defect of *prp4-73*. Suppression of this defect by Srp1 and ASF/SF2 indicates that these proteins somehow interact with the product of *prp4*. We have shown that the Prp4 protein kinase can phosphorylate ASF/SF2 *in vitro* (20). Preliminary results indicate that Prp4 can also phosphorylate Srp1 *in vitro*. This makes Srp1 a potential *in vivo* substrate of Prp4. On the other hand, overexpression of the SR proteins in a *prp4-73* background could also compensate for other splicing components which might be *in vivo* substrates of Prp4. It is noteworthy here that adding excess SR proteins to *in vitro* splicing systems could compensate for a lack of snRNP U1 particles, which are essential for assembly of commitment complexes and for recruitment of later splicing factors (31,32).

Taking these results into consideration, we suggest that Srp1 can act in pre-mRNA splicing in *S.pombe*. Moreover, we have evidence that Srp1 is not the only SR protein involved in pre-mRNA splicing. The approach presented here and the investigations of other groups demonstrate that fission yeast is a suitable system to investigate *in vivo* pre-mRNA processing (20,25,33,34). Since *S.pombe* is genetically amenable and easy to handle, studies with this system will be most helpful and complement studies in other systems in order to elucidate the complex functions of SR proteins and other splicing factors (35–37).

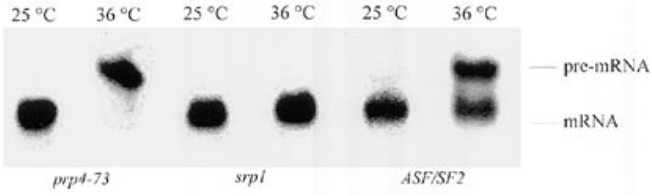

Figure 5. Northern analysis of a *prp4-73* strain transformed with wild-type *srp1* and cDNA coding for the human ASF/SF2 SR protein respectively. Total RNA was isolated from strains expressing *srp1* and *ASF/SF2* under control of the *nmt1* promoter. The strains were grown under derepressed conditions (–thiamine) at 25°C and then shifted for 3 h to 36°C as indicated. The *tfIId* gene was used as probe as described before. p, pre-mRNA; m, mRNA.

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