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Functional analysis of the fission yeast Prp4 protein kinase involved in pre-mRNA splicing and isolation of a putative mammalian homologue

Thomas Groβ, Martin Lützelberger, Holger Wiegmann, Andreas Klingenhoff, Suresh Shenoy+ and Norbert F. Käufer*

Institut für Genetik–Biozentrum, Technische Universität Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, Germany

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ABSTRACT

The *prp4* gene of *Schizosaccharomyces pombe* encodes a protein kinase. A physiological substrate is not yet known. A mutational analysis of *prp4* revealed that the protein consists of a short N-terminal domain, containing several essential motifs, which is followed by the kinase catalytic domain comprising the C-terminus of the protein. Overexpression of N-terminal mutations disturbs mitosis and produces elongated cells. Using a PCR approach, we isolated a putative homologue of Prp4 from human and mouse cells. The mammalian kinase domain is 53% identical to the kinase domain of Prp4. The short N-terminal domains share <20% identical amino acids, but contain conserved motifs. A fusion protein consisting of the N-terminal region from *S.pombe* followed by the mammalian kinase domain complements a temperature-sensitive *prp4* mutation of *S.pombe.* Prp4 and the recombinant yeast/mouse protein kinase phosphorylate the human SR splicing factor ASF/SF2 *in vitro* in its RS domain.

INTRODUCTION

The *prp4* gene of *Schizosaccharomyces pombe* was identified in a pool of temperature-sensitive (ts) *prp* (pre-mRNA processing) mutants whose molecular phenotype is the accumulation of pre-mRNA at the restrictive temperature (1). The gene is located on chromosome III and is essential for growth. The amino acid sequence of *prp4* predicts a serine/threonine protein kinase catalytic domain at the C-terminus of the protein. In addition to the kinase domain the protein consists of an N-terminus comprising 157 amino acids. The predicted M_r of Prp4 is 55 000 (2).

According to the protein kinase classification system of Hanks and Hunter (3), which is based on similarity in the amino acid sequence of the kinase domains, Prp4 belongs to the Clk (Cdc-like kinase) family. This family includes the mammalian SRPK1 and Clk/Sty protein kinases (4,5). The mammalian protein kinase Clk/Sty and Prp4 of fission yeast show the same domain arrangement: a short N-terminal region is followed by the catalytic kinase domain.

Both mammalian kinases have been shown to phosphorylate the RS (arginine/serine-rich) domains of pre-mRNA splicing factors, called SR proteins, *in vitro*. The SR proteins are involved in constitutive and alternative splicing (4,6–9). It has been suggested that these two kinases do not act directly at the spliceosome, but co-localize with the SR splicing factors in subnuclear structures, called speckles (9,10). Although the specific function of these kinases is still unknown, there is some evidence that they play an important role in regulating the traffic of SR splicing factors between speckles and the location of spliceosome assembly (4,9,11).

Five snRNPs (U1, U2, U4/U6 and U5) are required for pre-mRNA splicing (12,13). In mammalian cells a protein kinase activity co-purifying with the U1 snRNP has been detected. This kinase activity specifically phosphorylates the U1 70K protein in its RS domain. When the human SR splicing factor ASF/SF2 is added to an *in vitro* assay, the U1 snRNP-associated kinase activity also phosphorylates the RS domain of this protein (14). The gene for this kinase activity has not been identified.

As yet we do not know a physiological substrate of Prp4. The notion that Prp4 is involved in pre-mRNA splicing is based on the observation that intron-containing genes accumulate pre-mRNA at the restrictive temperature (36°C) when the *prp4-73* allele is in the genetic background. When the culture is shifted back to the permissive temperature (26°C) mature message appears again after 30 min. This observation gives no hint of whether Prp4 is directly or indirectly involved in the splicing process. In many reports it has been demonstrated that phosphorylation and dephosphorylation of spliceosomal components play a crucial role in spliceosome assembly and disassembly. The specific functions of the protein kinase(s) and phosphatase(s) involved are, however, still elusive (15–19).

In this report we describe a mutational analysis of the N-terminal and kinase domains of Prp4 protein kinase of *S.pombe*. This analysis revealed short motifs in the N-terminus which are essential for function *in vivo*. Overproduction of Prp4 containing

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

+Present address: CuraGen Corporation, 322 East Main Street, Branford, CT 06405, USA

§U488736, U488737, U66833 and L10739

the mutated motifs revealed phenotypes which indicate that the cells are impaired in mitosis.

We also describe the isolation of a mammalian kinase which is composed of an N-terminal region of ~170 amino acids followed by a catalytic protein kinase domain. The mammalian kinase domain shares 53% identical amino acids with Prp4sp of fission yeast. The N-terminal domain of the mammalian kinase, however, appears to be different, sharing <20% identical amino acids with the yeast N-terminus. The essential motifs found in the N-terminus of Prp4sp are highly conserved in the mouse N-terminus. When the complete mouse cDNA is expressed in *S.pombe* it does not complement the ts allele *prp4-73*, however a fusion of the yeast N-terminus with the mammalian kinase domain was complementary.

We show that Prp4sp of *S.pombe* and the recombinant yeast/mouse kinase phosphorylate predominantly the RS domain of the human splicing factor ASF/SF2 *in vitro* (20–22).

MATERIALS AND METHODS

Schizosaccharomyces pombe strains and general methods

The strains used in this study were L972, L975, h^{-s} *ura4-294 prp4-73*, h^{-s} *ura4-D18 leu1-32 prp4-73*. The prp4 ts mutant strain has been described by Rosenberg *et al.* (1). Standard classical and molecular genetic procedures and media for growth of the *S.pombe* strains used have been described by Gutz *et al.* (23) and Moreno *et al.* (24) Transformation of *S.pombe* with shuttle plasmids and linearized fragments for integration was performed as previously described by Gatermann *et al.* (25).

Site-specific mutagenesis

The system used for site-specific mutagenesis was based on the method developed by Kunkel (26). The procedure was performed as described previously (25). The following synthetic oligonucleotides were phosphorylated and annealed to the appropriate single-stranded uracil-containing phage DNA:

ΔNLS, 5′-GATGAAATTATACAGCAATTGTACCAACAGACTGGCAAT-3′;
ΔΔNLS, 5′-GAAATTATACAGCAATTCGAAGATGTCGACCAAGTCTCT-3′;
SX1, 5′-AGTACTACTGGTGATTTGCCCGCTATCAAATCTTCTGT-3′;
SX2, 5′-ATGTTTGCAGATATCCCTTTGCCTGCTGTTAAGCGGCA-3′;
EGY1, 5′-TGCAGGTTAACTCGGTCGTTTTTGCAGATAATAATACGGAAGTTCTTATGG-3′;
EGY2, 5′-TAATTGGGACGTTAACGCAGATAATAATACGGAAGTTCTTATGGAGG-3′;
mut1, 5′-CTTACTGATTTCTGCAGCACGATAAAATCG-3′;
mut2, 5′-CTCACGAGGTACCGTGTTATTTCATTTTCAG-3′;
mut3, 5′-CTCACGAGGTACCGTGTTATTTCATTTTCAG-3′;
mut4, 5′-CTCACGAGGTAAGCTGTTATT TCATTTTCAG-3′;
mut5, 5′-CGAGGTACGGTGCTATTTCATTTTCAGAGGC-3′;
mut6, 5′-CTCACGAGGAACGGTGTTATTTCATTTTCAG-3′;
mut7, 5′-CTCACGAGGAACGGTGCTATTTCATTTTCAG-3′.

All mutation constructs were sequenced and subsequently subcloned into the apropriate vectors for integration into the *ura4* locus.

Cloning of mammalian cDNA

The human cDNA was isolated using a UNI-Zap XR HeLa S3 cDNA library (Stratagene), the sense primer comprising part of the T-loop of the human sequence HsPK 27 (5′-CTGCTAGGATCCTCGGCTTCACATGTTGCGGA-3′; EMBL accession no. Z25435) and the T7 primer (5′-GTAATACGACTCACTATAGGGC-3′). A 1.7 kb PCR fragment produced in this reaction was isolated from the agarose gel and radiolabelled with [α-^{32}P]dCTP as described previously (2). This labelled fragment was used to screen the HeLa S3 cDNA. The filter hybridization conditions used were according to the protocol from Stratagene. The inserts of the hybridizing plaques were subcloned into pBluescript SK(–) by *in vivo* excision following the protocol from Stratagene. The mouse *prp4* cDNA was isolated using a λgt10 library from embryonic stem cells (Clontech). A 600 bp *Mun*I–*Eco*RV fragment comprising part of the ORF of the human *PRP4* cDNA was radiolabelled and hybridized to the filters. Recombinant phage DNA was isolated from positive plaques. Since the inserts contained an *Eco*RI site, the DNA was digested partially with this enzyme and subsequently cloned into pUC18.

Sequencing of the mammalian clones

The subcloned inserts from the human and mouse libraries were sequenced with [^{35}S]ATP using the universal and the reverse primer. Based on the first sequences we synthesized oligonucleotides which were used as primers in further sequence reactions. Databank accession numbers for the human and mouse sequences are U488736 and U488737 respectively.

prp4 S.pombe/Mus musculus swap constructs

cDNA of *S.pombe prp4* was isolated using the primers 5′-GAGCTCGGATCCGACGATAGATTTGCAGAAGAT-3′ and 5′-ATATGGATCCATGAACCCGCAGTTTATT-3′ and a *S.pombe* cDNA library. The 1.4 kb PCR product was inserted into the *Bam*HI site of a pREP1 vector in which the *Sal*I site in the multiple cloning site was destroyed by filling in the protruding ends (27). For the deletion construct *prp4ΔXI* the second primer was 5′-TCTAGAGGATCCTCAGGCGGTACGTTTCTCTG-3′. The same approach was used to insert a complete mouse cDNA into pREP1. The primers used were 5′-ATATGGATCCATGAAAGTTGAGCAAGAGTCT-3′ and 5′-CCGGGGATCCTTAAATTTTTTCCTGGATGAATGC-3′. The PCR reaction was performed with one of the isolated mouse clones and revealed a 1.4 kb product. This fragment was cloned into the *Bam*HI site of pREP1. Both constructs were sequenced to confirm the sequence and the proper ORF. The first swap leading to the construct Sp/Mm1 (Fig. 2) was a simple exchange of fragments from the pREP1 constructs using the *Eco*RV restriction site which both cDNAs share at the same position in the ORF. For the swap construct Sp/Mm2 (Fig. 4) we introduced an *Eco*RI restriction site 614 bp downstream of the start codon ATG of the *S.pombe* cDNA, performing site-specific mutagenesis as described previously (25). This manipulation allowed us then to exchange fragments from the pREP1 constructs using this *Eco*RI site, since the mouse cDNA contains an *Eco*RI site at the same position in the ORF. Sequence analysis of the constructs confirmed the proper ORF. The swap constructs Sp/Mm3 and Mm/Sp (Fig. 4) were constructed in a two step PCR reaction, called the 'Megaprimer' method, developed by Sarkar and Sommer (28). In the first reaction each chosen N- and C-terminal fragment was independently amplified using primers which create an overlap between the N- and C-terminal fragments of yeast and mouse. We used primers which had a *Mun*I site in this overlap. The *Mun*I site is only present in the mouse cDNA, not

in the *S.pombe* cDNA. In the second PCR reaction we combined the N- and C-terminal PCR products and used a primer sequence of the N-terminal fragment containing a *Bam*HI site and a primer of the C-terminal fragment also equipped with a *Bam*HI site. This PCR reaction revealed products of 1.4 kb which were isolated and cloned into the *Bam*HI site of pREP1. The primers used for the *S.pombe* N-terminal fragment were 5′-GAGCTCGGATCCGACG-ATAGATTTGCAGAAGATG-3′ and 5′-TGCATCTGTCCAATT-GTCCTGCATATCTG-3′; for the C-terminal fragment 5′-ACCTC-AGAGACAATTGGGACGATATTGAAG-3′ and 5′-ATATGGAT-CCATGAACCCGCAGTTTATT-3′. The primers used for the mouse N-terminal fragment were 5′-ATATGGATCCATGAAAGT-TGAGCAAGAGTCT-3′ and 5′-AATATCGTCCCAATTGTCTC-TGAGGTTGG-3′; for the C-terminal fragment 5′-CCGGGGATC-CTTAAATTTTTTCCTGGATGAATGC-3′. The constructs were sequenced to confirm the proper ORF.

Expression of recombinant *S.pombe* Prp4 and antibody preparation

A 1 kb *Bam*HI fragment of the *S.pombe* cDNA was ligated into plasmid pGEX2T (Pharmacia KB). The recombinant protein contains at the N-terminus gluthatione S-transferase (GST) followed by the N-terminal sequence of Prp4 and two thirds of the kinase domain. After transformation in *Escherichia coli* protein production was induced with 1 mM IPTG. Bacterial extract was prepared as described by Krämer *et al.* (29). Most of the recombinant protein appeared in the inclusion bodies. This material was separated by SDS–PAGE and the 69 kDa recombinant GST–Prp4 protein was electroeluted. The electroeluted protein was used as antigen. Antibodies were raised in rabbits by several injections with 150–250 µg recombinant GST–Prp4 (Pogonos Rabbit Farm, PA). We purified the antibodies using protein A–Sepharose.

For Western blot analysis proteins were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose. For antibody detection we used the ECL Western blotting analysis system from Amersham following the manufacturer's instructions.

Preparation of *S.pombe* protein extracts and immunoprecipitation

To prepare native and denatured protein extract we exactly followed the procedure described by Moreno *et al.* (24). For the preparation of native extract exponentially growing cells were used. Samples of 2×10^8 cells were resuspended in 200 ml extraction buffer HB (24) and broken with glass beads by vortexing. For immunoprecipitation 400 µl protein extract were used and incubated with anti-GST–Prp4 overnight at 4°C, then 25 µl protein A–Sepharose were added and incubated for a further 2 h at the same temperature. The precipitate was washed three times in HE buffer (50 mM Tris, pH 8, 150 mM NaCl, 50 mM β-glycerolphosphate, 50 mM NaF, 10 mM EDTA, 5 mM EGTA, 0.1 mM sodium vanadate, 1 mM DTT, 20 µg/ml leupeptin, 40 µg/ml aprotinin, 30 µg/ml pepstatin, 50 µg/ml Pefubloc SC) followed by three washes with kinase buffer I (20 mM HEPES, pH 7.9, 50 mM KCl, 3 mM $MgCl_2$, 5% glycerol).

In vitro kinase assay

The immunocomplex of Prp4 bound to anti-Prp4–protein A–Sepharose beads was used in the kinase assay. The kinase assay was routinely performed in a 20 µl volume containing kinase buffer II (20 mM HEPES, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DDT), 5 µCi [γ-^{32}P]ATP, 10 mM ATP and 5 µl immunoprecipitate. As possible substrates we added, in general, 36 pmol bacterially produced ASF/SF2, ASF/SF2ΔRS (provided by R.Lührmann, Marburg; 21,22), histone H1 (Boehringer Mannheim), myelin basic protein (MBP; Sigma) or β-casein (Sigma). This is 1 µg for protein ASF/SF2. The samples were incubated at 37°C for 30 min. The reaction was stopped by adding 20 µl 2× SDS sample buffer and boiling for 2 min. Samples were run on a 12.5% SDS–PAGE gel, transferred to nitrocellulose and exposed to X-ray film.

RESULTS

Experimental system to measure *in vivo* activity of Prp4

To learn about the function of the two domains of Prp4 we undertook a mutational analysis and tested the effect of the mutations *in vivo* by measuring the capability to complement the ts *prp4-73* allele. The mutation causing the temperature sensitivity of Prp4 is in kinase subdomain IV, changing the cysteine residue at position 235 to a tyrosine (Fig. 3). Haploid cells containing *prp4-73* grow normally at 26°C, but do not grow at all at 36°C (1). The mutation constructs were integrated into the genome via homologous recombination using the *ura4*$^+$ gene to target the *ura4-294* allele in a strain containing the *prp4-73* allele. This manipulation leads to two *prp4* alleles on chromosom III. As controls we constructed strains containing the *prp4-73* allele and in the *ura4* locus either the wild-type or the *prp4-73* allele. The wild-type gene complements *prp4-73* at 36°C, whereas the strain containing two *prp4-73* alleles shows no growth at 36°C (Fig. 1, WT and TS). The effects of the mutations were checked by spotting cells, which were grown to mid log phase at 26°C, on plates and monitoring growth at 36°C.

Mutational analysis of the N-terminus of Prp4

We made mutations in the N-terminus using a cDNA of *prp4*. The constructs were inserted into a vector which places them under the control of the *nmt1* promoter. The *nmt1* promoter allows down-regulation of expression in medium containing thiamine and leads to strong expression in thiamine-free medium (27).

The N-terminus of Prp4 of *S.pombe* consists of 157 amino acids. At position 15 in Prp4 we detected a putative nuclear localization signal (NLS) of the SV40 type, consisting of the five basic amino acid residues RRKRR (Fig. 1A). Five basic amino acids in the N-terminus of polymerase α of *S.pombe* have been shown to be solely responsible for moving the protein into the nucleus (30). We deleted 11 amino acids including the putative NLS and also made a more extensive deletion of 30 amino acids (Fig. 1, ΔNLS and ΔΔNLS). Neither the deletion of 11 amino acids nor the second deletion had an effect on growth at 36°C. Both deletion constructs complemented the *prp4-73* allele under repressed (Fig. 1A) and derepressed conditions (results not shown).

In positions 90–95 and 112–117 we find two elements, SDSPSI and SPSPSV, which we call serine elements (Fig. 1A, SX1 and SX2). We replaced the serines with other amino acids as shown in Figure 1A. Changing the serines in one of the elements had no effect. These mutations still complemented the *prp4-73* allele. However, the mutation construct in which the serines of both elements had been replaced by other residues did not complement

Figure 1. (**A**) Mutations in the N-terminus of Prp4sp. WT shows the wild-type amino acid sequence. A putative NLS (nuclear localization sequence), SX (serine dipeptide) elements and the EGY sequence are indicated in the diagram and described in the text. The open boxes in the ΔNLS and ΔΔNLS mutants indicate amino acid residues that were deleted. Amino acid changes are displayed under the corresponding wild-type sequence. The dashes indicate wild-type residues. Numbers indicate position of amino acid residues relative to the N-terminal methionine. For the complementation test the mutations were integrated into the *ura4* locus of a strain containing the temperature-sensitive *prp4-73* allele, grown at 26°C to log phase, then spotted on plates and growth was monitored at 26 and 36°C. All constructs were under the control of the repressible *nmt1* promoter. Growth of cells at 36°C was monitored under repressed conditions (+ thiamine). WT contains two wild-type *prp4* alleles; TS contains two *prp4-73* alleles. (**B**) Integrant strains containing (a) prp4 WT, (b) SX1+SX2, (c) EGY1 and (d) EGY2 grown for 20 h at 26°C under derepressed conditions (– thiamine). Lower panel cells stained with DAPI (4′,6-diamidino-2-phenylindole) and photographed with a Zeiss Axiophot microscope.

the *prp4-73* allele (Fig. 1A, SX1+SX2), indicating that these two motifs together play a crucial role in the proper functioning of Prp4.

The amino acid sequence DNWDDIEGYYKV starting at position 138 is highly conserved in sequence and position in Prp4sp of *S.pombe* and a mammalian protein kinase which we have isolated (see below, Fig. 3). Therefore, we changed this sequence in Prp4sp of *S.pombe* to VNSVVFADNNTE (Fig. 1, EGY1). In a second construct DIEGYYKV was changed to VNADNTE (Fig. 1, EGY2). Neither mutation construct complemented the *prp4-73* allele (Fig. 1A). These results suggest that this motif including EGYYK is essential for function.

Overexpression of the non-complementing mutations impairs mitosis

When we overexpress the mutation constructs at 26°C by growing the cells in medium without thiamine, the strains containing the mutations which do not complement the *prp4-73* allele (SX1+SX2, EGY1 and EGY2) exhibit significantly elongated cells after 20 h (Fig. 1B). The DAPI staining pattern of these cells suggests that overexpression of the mutated Prp4 proteins impairs mitosis. Unequal and puffy staining patterns of DNA in one cell can be observed, particularly in panels (c) and (d) of Figure 1B.

Mutational analysis of the kinase catalytic domain

The kinase catalytic domain of Prp4 consists of 321 amino acids and shows the 12 subdomains defined by the signature sequences indicative of the serine/threonine protein kinase family (Fig. 3; 3). We searched the databases using the sequence between the signatures DFG and APE of Prp4 as query sequence (Fig. 2A). This region of a protein kinase is called the T-loop and serves in some kinases as a switch for up- and down-regulating activity (31). The search revealed a partial human sequence that was isolated in a screen for Cdc2-like kinases (32). Between the signature sequence DFG and APE the human sequence HsPK 27 and Prp4sp share 79% identical amino acid residues (Fig. 2A). We also found in this search the T-loops of the MAP kinases Erk1

A

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ERK2     155  DFGLARVADPDHDHTGFLTEYVATRWYRAPE

SAPK     169      DFGLARTACTNFMMTPYVVTRYYRAPE

Prp4sp   302    KICDLGSASDASENEITPYLVSRFYRAPEIILGFPYSCPIDTWSVG
                KI D GSAS  ++N+ITPYLVSRFYRAPEII+G +Y++ ID WS+G
HsPK27     1    KIGDFGSASHVADNDITPYLVSRFYRAPEIIIGKSYDYGIDMWSIG
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B

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                                                                complementation
                                                                  of prp4-73
Mutation                                                          phenotype

                                                                 26 °C  36 °C

        D L G S A S D A S E N E I T P Y L V S R F Y R A P E
mut1    - - - - - - - - - - - - - - - - - - - - - - - - A -    +      -
mut2    - - - - - - - - - A - A - - - - - - - - - - - - - -    +      +
mut3    - - - - - - - - - - - - - - R - - - - - - - - - - -    +      +
mut4    - - - - - - - - - - - - - - A - - - - - - - - - - -    +      +
mut5    - - - - - - - - - - - - - A - - - - - - - - - - - -    +      +
mut6    - - - - - - - - - - - - - - - F - - - - - - - - - -    +      +
mut7    - - - - - - - - - - - - - A - F - - - - - - - - - -    +      +
```

Figure 2. (**A**) Databank search using the T-loop (see text) of the kinase catalytic domain of Prp4 of *S.pombe* as query sequence (Prp4sp). The human sequence HsPK27 is stored as a partial sequence under PIR S37427 and EMBL Z25435. ERK2 (extracellular signal-regulated protein kinase, PIR S23426); SAPK (stress-activated protein kinase, SP S23426). Numbers indicate amino acid residue position relative to the total amino acid sequence stored in the databank. Signature sequences DF/LG and APE of serine/threonine protein kinases (3) are underlined. (**B**) Mutations in the T-loop of the kinase catalytic domain of Prp4sp. The wild-type sequence of the T-loop is displayed. Amino acid changes are indicated naming the changed residues under the corresponding wild-type residue. The complementation test was performed as described in Figure 1 except that all mutated *prp4* genes were under the control of their own promoter. +, normal growth; –, no growth.

and Erk2 and of the stress-activated kinases (SAPKs). The T-loops of these kinases contain the sequences TEY and TPY (Fig. 2A). Activation of these kinases requires both threonine and tyrosine phosphorylation in these sites (31,33). Interestingly, these phosphorylation sites resemble the sequence found in the Prp4 and HsPK T-loops (Fig. 2A).

To test whether the T-loop sequence plays a regulatory role in the function of Prp4, we replaced threonine with alanine and tyrosine with phenylalanine. In one construct we made a double mutation replacing threonine and tyrosine. We also changed other amino acid residues in the T-loop region (Fig. 2B). As a control we mutated the signature sequence APE to AAE (Fig. 2B, mut1). The constructs were integrated into the *ura4* locus as described before, however, in this series of experiments the *prp4* gene was under the control of its own promoter. As expected, the mutation mut1 in the signature sequence APE does not complement the *prp4-73* allele (Fig. 2B). However, all the other mutations rescued the ts phenotype (Fig. 2B) and none, including the double mutation mut7, had any influence on growth. Thus, under these growth conditions the T-loop appears not to play a regulatory role for Prp4.

Isolation of a mammalian cDNA using the human T-loop sequence as primer

We produced PCR fragments using a HeLa cDNA library and a sequence of the human T-loop HsPK 27 and the T7 sequence in the λ phage arm as primers (Fig. 2A). The PCR products were used as a probe to screen the HeLa library. Out of this screen we isolated and sequenced several cDNAs. A defined fragment of the ORF of the human cDNA was then used to screen the mouse cDNA library. With this approach we isolated cDNA from human and mouse encoding a protein kinase which shares 98% identical amino acids. Overall the mammalian sequence shares 44% identical amino acids with the yeast sequence. Throughout the kinase domains, however, we find 53% identical amino acids. This high conservation changes apruptly in the N-terminal domains. The N-termini share <20% identical amino acids. However, the EGY motif mentioned above appears to be conserved in the mammalian and yeast N-termini (Fig. 3). We also detected in the mammalian N-terminus a serine element, SRSPSP, which resembles the serine elements found in Prp4sp (Fig. 3).

Complementation of the *S.pombe prp4-73* mutation

To test whether the mammalian gene complements the ts mutation we inserted the mouse cDNA behind the *nmt1* promoter into the expression vector pREP1 and transformed it into a strain containing the *prp4-73* allele. The pREP1 vector containing the complete *S.pombe prp4* cDNA complements the ts mutation under thiamine repressing conditions as well as under derepressed conditions (Fig. 4, Prp4sp). The vector containing the complete mouse cDNA was not complementary (Fig. 4, Prp4m). Therefore, we designed swap constructs switching mouse with yeast sequences as shown in Figure 4. The construct Sp/Mm1, containing 55% of the mammalian kinase domain, complements *prp4-73* under repressed and derepressed conditions, whereas the construct Sp/Mm2, spanning 75% of the mammalian kinase domain, complements only under derepressed conditions (Fig. 4). Neither construct Sp/Mm3 nor Mm/Sp complemented the *prp4-73* allele. As a negative control we used a *prp4* cDNA of *S.pombe* which has a 13 amino acid deletion in kinase subdomain XI. This construct does not rescue the ts mutation (Fig. 4, Prp4spΔXI). In addition, a *prp4* cDNA of *S.pombe* in which the N-terminal region was deleted did not rescue the ts mutation (results not shown).

The swap construct Sp/Mm2 complemented only when the recombinant protein was highly expressed (Fig. 4). This indicates

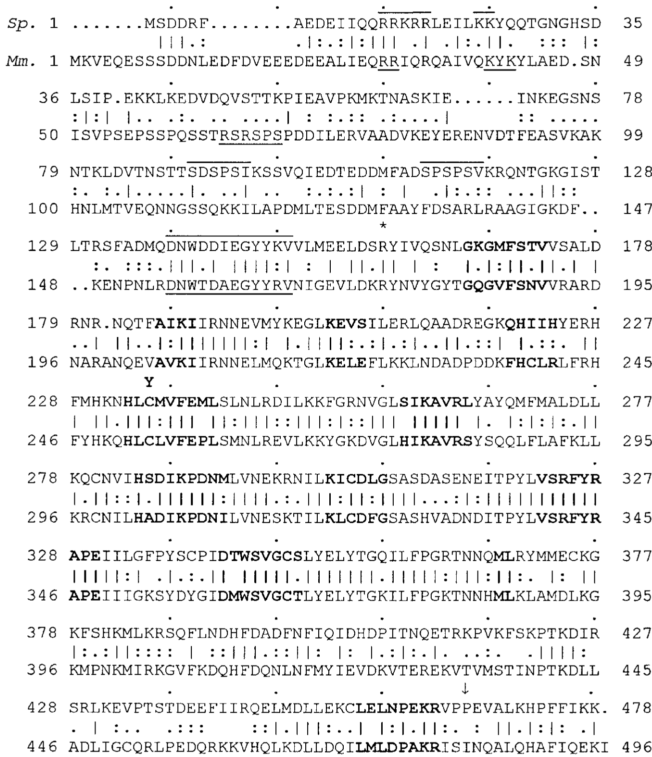

Figure 3. Amino acid alignment of Prp4 of *S.pombe* (*Sp*) with Prp4 of *M.musculus* (*Mm*). Over- and underlined, beginning at the N-terminus: putative nuclear localization signal (NLS), serine dipeptide elements (SX) and EGY sequence. Asteriks indicate the beginning of the kinase catalytic domain (3). Y (tyrosine) at position 235 of the *S.pombe* sequence indicates the ts mutation in the *prp4-73* allele. The arrow indicates the extent of the deletion of the C-terminus in construct Prp4spΔXI (Fig. 4). Signature sequences of serine/threonine protein kinases (3) are in bold.

some differences in kinase subdomains I–II. The simplest explanation for this result would be that the fusion products of Sp/Mm2 and Sp/Mm3 induce some structural changes in that region which causes the decrease and loss of activity *in vivo* respectively. The results also indicate that differences in the primary sequence of the N-termini of the proteins might account for the failure of the mouse N-terminus to complement.

In vitro kinase activity of Prp4

As yet we have no *in vitro* data on the kinase activity of Prp4sp, since we do not know a physiological substrate. All kinase activities in mammalian cells which have been implicated as involved directly or indirectly in pre-mRNA splicing phosphorylate SR proteins *in vitro*. It has been shown that these kinases and kinase activities preferably phosphorylate the RS domains of SR proteins (4,9,14,34). Therefore, we decided to test the mammalian SR protein ASF/SF2 and a mutated version of ASF/SF2 as *in vitro* substrates. The mutated protein ASF/SF2ΔRS lacks the RS dipeptides in the RS domain at the C-terminus (21,22).

A polyclonal antiserum raised against a glutathione S-transferase–Prp4sp fusion (GST–Prp4, see Materials and Methods) was used to examine the proteins expressed with pREP1. Protein extracts of *S.pombe* cells containing the Prp4sp cDNA, the mouse cDNA and the chimeric constructs (shown in Fig. 4) were isolated and probed in a Western analysis with polyclonal antibodies. The antibodies recognized the product of the Prp4sp cDNA, but failed to detect a product of the mouse cDNA (Fig. 5A, lanes 1 and 2); recombinant proteins, however, that contained the N-terminus of the *S.pombe* protein were recognized (Fig. 5A, lane 4). This suggests that the antibody population consists mostly of antibodies against epitopes in the N-terminus of the *S.pombe* protein. Based on this result and the fact that *prp4sp* cDNA and the chimeric yeast/mouse gene (Sp/Mm2, Fig. 4) complemented the *prp4-73* allele, we immunoprecipitated Prp4sp and the Sp/Mm2 proteins

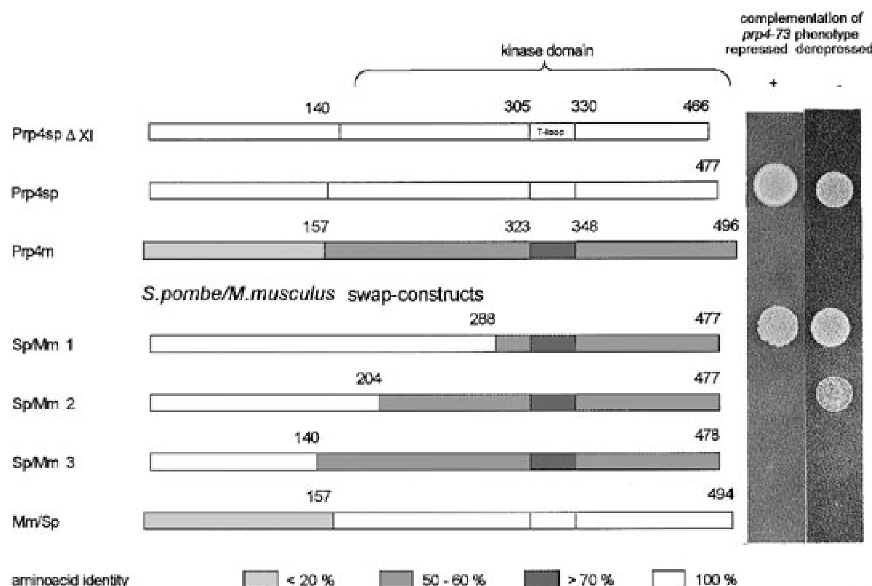

Figure 4. Complementation studies with cDNA constructs of fission yeast (Prp4sp, white boxes), mouse (PRP4m, stipled boxes) and swap constructs as indicated. Prp4spΔXI has a 39 bp deletion in kinase subdomain XI. The constructs were inserted into the expression vector pREP1 containing the repressible *nmt1* promoter and transformed into a *S.pombe prp4-73* strain. The constructs were tested for complementation at 36°C under repressed (+thiamine) and derepressed (–thiamine) conditions.

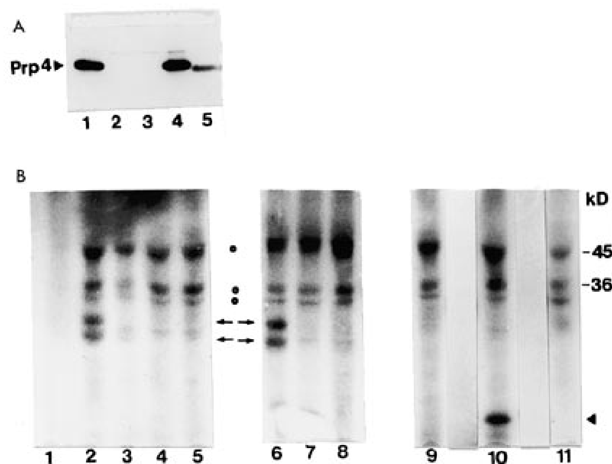

Figure 5. (**A**) Western blot analysis. Protein extracts of *S.pombe* strains transformed with the constructs shown in Figure 4 were separated by SDS–PAGE, transferred to nitrocellulose and probed with the antibodies raised against GST–Prp4 of fission yeast. Lane 1, Prp4sp; lane 2, Prp4m; lane 3, Mm/Sp; lane 4, Sp/Mm2; lane 5, Prp4spΔXI. (**B**) Kinase assays. Protein extracts of an *S.pombe* strain transformed with the constructs shown in Figure 4 were immunoprecipitated with antibodies against GST–Prp4, incubated for 30 min at 37°C in the presence of [γ-^{32}P]ATP, separated by SDS–PAGE and autoradiographed. Arrows show the position of ASF/SF2, which runs as a doublet. Lane 1, Prp4spΔXI + ASF/SF2; lane 2, Prp4sp + ASF/SF2; lane 3, Prp4sp; lane 4, Prp4sp; lane 5, Prp4sp + ASF/SF2ΔRS; lane 6, Sp/Mm2 + ASF/SF2; lane 7, Sp/Mm2 + ASF/SF2ΔRS; lane 8, Sp/Mm2; lane 9, Prp4sp + histone H1; lane 10, Prp4sp + myelin basic protein (arrowhead); lane 11, Prp4sp + β-casein.

with GST–Prp4 antibodies and performed *in vitro* kinase assays using [γ-^{32}P]ATP. Prp4 and the recombinant yeast/mouse protein phosphorylated human ASF/SF2 protein *in vitro* (Fig. 5B, lanes 2 and 6, arrows). ASF/SF2ΔRS protein was hardly phosphorylated in this assay (Fig. 5B, lanes 5 and 7). These results suggest that *in vitro* the kinase activity phosphorylates ASF/SF2 protein at the RS domain. We conclude that this *in vitro* kinase activity is due to Prp4 and the recombinant yeast/mouse protein, since the immunoprecipitate containing the Prp4ΔXI deletion protein did not phosphorylate ASF/SF2 (Fig. 5B, lane 1). This is consistent with the observation that *in vivo* the deletion construct Prp4spΔXI does not complement the ts mutation (Fig. 4). Furthermore, in the autoradiographs we see additional distinct bands in the range 30–45 kDa (Fig. 5B circles, all lanes except lane 1). This suggests that proteins which were phosphorylated by Prp4 co-precipitated.

We also tested the capability of the Prp4 kinase to phosphorylate *in vitro* kinase substrates like histone H1, myelin basic protein (MBP) and β-casein, which have been used as substrates for the kinases Clk/Sty, SRPK1 and the U1 snRNP-associated kinase activity. Prp4 phosphorylated MBP (Fig. 5B, lane 10), but did not phosphorylate histone H1 and β-casein (Fig. 5B, lanes 9 and 11). The recombinant Sp/Mm2 protein kinase revealed the same pattern (results not shown).

DISCUSSION

This is the first report of a protein kinase of the fission yeast *S.pombe* involved in pre-mRNA splicing showing *in vitro* kinase activity. Prp4sp and the recombinant yeast/mouse kinase are capable of phosphorylating the the RS domain of the mammalian splicing factor ASF/SF2. Splicing factor ASF/SF2 is a member of the SR family of phosphoproteins which appears to be highly conserved throughout metazoan organisms (11,35). SR proteins, including ASF/SF2, which have been studied in a mammalian *in vitro* pre-mRNA splicing system play a role in constitutive and alternative splicing (6,8,11,36,37). In particular, ASF/SF2 has been shown to be an important component in determining 5′-splice sites of alternatively spliced genes (21,22). Typical SR proteins involved in pre-mRNA splicing have not been reported from the yeasts *Saccharomyces cerevisiae* and *S.pombe*. However, very recently we isolated a gene *srp1* of *S.pombe* which encodes a 30 kDa protein containing a RNA recognition motif (RRM) and a domain which resembles metazoan RS domains (T.Groß, C.Mierke and N.F.Käufer, unpublished results, EMBL accession no. U66833). Whether Srp1 is a potential substrate of Prp4 is currently under investigation.

The complementation studies with the swap constructs indicate a difference in kinase subdomains I and II of the yeast and mammalian proteins. It is conceivable that for optimal *in vivo* activity of the protein kinase other factors, including the substrate(s), might need to interact with the protein kinase through the N-terminus and the early kinase domain. Taking the differences in the N-terminus of these proteins into consideration, it is, therefore, possible that *in vivo* the fission yeast factors cannot interact properly with the recombinant product of *Sp/Mm3* (Fig. 4).

We know from experiments with fusions of Prp4 and green fluorescent protein (GFP) that Prp4 accumulates in the nucleus (results not shown). Transport into the nucleus appears not to be solely dependent on commonly known NLS signals (Fig. 1; 38). However, the information for targetting the nucleus appears to be in the N-terminus, since the chimeric yeast/mouse construct Sp/Mm2, which contains the *S.pombe* N-terminus and the mouse kinase domain, is detected in the nucleus, whereas a fusion protein of GFP with the kinase domain of Prp4sp is detected in the cytoplasm (results not shown). The mouse Prp4–GFP fusion protein does not reach the nucleus when expressed in *S.pombe* (results not shown). The N-terminus of the mouse protein contains a putative NLS which resembles a bipartite NLS of the nucleoplasmin type, but it does not fit the consensus well (Fig. 3; 38).

It has been proposed that Clk/Sty and SRPKI regulate ASF/SF2 function *in vivo* by phosphorylating the protein in the RS domain to induce release from the speckles (9). The repeats of RS/SR dipeptides in RS domains have been implicated in protein–protein interaction (39,40,41). The N-terminus of the mammalian protein kinase Clk/Sty does not contain a typical RS domain, but scattered throughout it contains 10 SR/RS dipeptides and one RSRS motif. The N-terminus of Clk/Sty has been shown to interact with SR proteins (9). With this in mind we have substituted the serines in the serine elements of Prp4sp (Fig. 1). When the serines are replaced in both elements, the mutated protein does not compelement the *prp4-73* allele. This indicates that the two serine elements are part of the protein architecture which might be involved in interaction with other components.

The results of the mutational analysis of the N-terminus of Prp4sp are consistent with the idea that the N-terminus is involved in interaction with other components. These components might be substrates, but interaction partners with other functions, such as inhibitor or docking functions, are also conceivable. We still do not know whether Prp4 is associated with spliceosomes or, perhaps, as demonstrated for the mammalian kinases Clk/Sty and SRPKI, located in subnuclear structures such as speckles (4,9).

The highly elongated phenotype of cells caused by overexpression of mutations in the N-terminus of Prp4sp warrants some comment. Overproduced mutated proteins do not impair cell growth. The cells appear to be disrupted in mitosis. It has been shown that overexpression of the kinases Clk/Sty and SRPKI in mammalian cells causes disruption and rearrangement of speckles containing splicing components (4,9). Speckles are subnuclear structures embedded in the nuclear scaffold (10,42). It is conceivable that overproduced mutated Prp4 protein effects nuclear segregation by disrupting orderly rearrangement of the nuclear scaffold. This notion, however, needs further investigation. In any case, a functional connection of pre-mRNA splicing and cell cycle events has been observed in *S.cerevisiae* and *S.pombe* (43,44). The fission yeast gene *cdc28* encodes an RNA-dependent ATPase/helicase and is allelic with *prp8.* The temperature-sensitive alleles *cdc28-P8* and *prp8-1* accumulate pre-mRNA at the restrictive temperature and show a cell cycle phenotype (44).

Interestingly, a homologue of the mammalian SRPKI kinase in *S.pombe* appears to be Dsk1 (4). The *dsk1* gene has been detected as a multicopy suppressor of *dis1* mutants. These mutants are blocked in mitosis due to non-disjunction of sister chromatids (34,42). Whether Dsk1 kinase in *S.pombe* is involved in splicing is not known. We first detected the role of Prp4 protein kinase in pre-mRNA splicing in fission yeast with a genetic approach, isolating *prp* mutants which accumulate pre-mRNA at the restrictive temperature (1,2). The data presented in this paper indicate that the function of Prp4 might be pleiotropic. Further studies in the fission yeast and mammalian systems will help to elucidate the biological role of Prp4 protein kinase.

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