The fission yeast prp4+ gene involved in pre-mRNA splicing codes for a predicted serine/threonine kinase and is essential for growth

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ABSTRACT

Only four prp (pre-mRNA processing) genes of the fission yeast Schizosaccharomyces pombe have been reported. We exploited yeast genetics and identified and isolated the prp4 gene. Sequence analysis revealed that the splicing factor encoded by this gene contains the signature sequences that define the serine/threonine protein kinase family. This is the first kinase gene identified whose product is involved in pre-mRNA splicing. The prp4 gene contains one intron in the kinase domain. Gene replacement studies provided evidence that this gene is essential for growth and is located on chromosome III.

INTRODUCTION

Splicing of pre-mRNA takes place in a multi-component complex which has been termed the spliceosome. Intron recognition, spliceosome assembly and the splicing reaction are dependent on many factors that interact with each other and with the pre-mRNA. Major components of this dynamic process are the small nuclear ribonucleoprotein particles (snRNP U1, U2, U4/U6, U5) which consist of RNA and associated proteins. The U4/U6 snRNA molecule may play an important catalytic role in removal of the intron sequences (1, 2, 3, 5).

The pre-mRNA splicing process requires ATP (1, 4). One set of proteins consuming ATP during pre-mRNA splicing belong to the DEAD/DEAH family, which contain signature sequences assigned to RNA-dependent helicases with ATPase activity (4, 11, 35). DEAD/DEAH signature sequences have been found in several PRP (pre-mRNA processing) genes isolated from the budding yeast Saccharomyces cerevisiae (6, 8, 11). In mammalian splicing systems, the activity of other ATP consuming enzymes has been detected. In vitro experiments it has been shown that the spliceosome and isolated parts of the spliceosome display specific kinase activity (5, 9, 10, 39). Also, phosphoproteins have been identified as spliceosomal components (7, 36). Little, however, is known about protein kinases involved in the pre-mRNA splicing process.

Four temperature sensitive (ts) prp (pre-mRNA processing) mutants from the fission yeast S. pombe have been reported (12, 13). We have described the identification and characterization of the prp4 (61) mutation (13). This mutant strain does not grow at the restrictive temperature (36°C) and accumulates pre-mRNA of intron containing genes, while spliced mRNA is rapidly degraded. At the permissive temperature (25°C), however, no pre-mRNA is detected and growth behavior is normal.

Here we describe the isolation and characterization of the gene and its predicted product complementing the prp4 mutation. The gene complementing the prp4 mutation is an essential gene and contains the signature sequences predictive for a serine/threonine protein kinase. This is the first report of a gene whose product is involved in pre-mRNA splicing that is also predicted to be a protein kinase.

MATERIALS AND METHODS

Schizosaccharomyces pombe strains and manipulations

Strains used in this study were h−S (972), h+N (975) (21). The mutant strain h−S, ura4-D18, leu1-32, prp4 (61) strain has been described by Rosenberg et al. (13). Standard classical and molecular genetics procedures and media for growth of the S. pombe strains used in this study have been described by Gutz et al. (21) and Moreno et al. (19). The S. pombe genomic library cloned into the pUR shuttle vector containing the Amp+ gene as the bacterial marker and the ura4 gene as the yeast marker was a gift from A. Carr (25). The cDNA library was from Becker et al. (26). Transformation of S. pombe with shuttle-plasmids and the S. pombe genomic library was performed as previously described by Gatermann et al. (15).

Plasmid recovery from S. pombe and double stranded sequencing

Shuttle-plasmids from S. pombe were isolated via transformation of isolated S. pombe DNA into Escherichia coli using a protocol described by A.C. Ward (37). Genomic inserts were mapped by restriction analysis and subsequently subcloned into E. coli vectors.

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(23). Sequencing was performed as previously described (22). The predicted amino acid sequence of the PRP4 protein, derived from the nucleotide sequence was compared with sequences in the GenBank data base using BLAST server (40).

Nucleic acid isolation and analysis
Standard methods of cloning, subcloning and manipulating DNA in vitro were used (22). Procedures of DNA and RNA isolation from S. pombe, Southern and Northern blotting, and PCR analyses were performed exactly as described previously (13, 14, 15, 24).

RESULTS
Complementation of the prp4 mutation
To suppress the prp4 mutation, we transformed the prp4 strain with a partial genomic library constructed from a wild type S. pombe strain. After transformation the cells were incubated for two days at the permissive temperature (25°C) and then screened for growth at the restrictive temperature (36°C). We found several growing colonies. Plasmids were isolated from these colonies and retransformed into the prp4 strain. Those inducing growth at the restrictive temperature were further characterized.

A 2.5 kb genomic fragment suppresses the prp4 mutation
The shuttle vector pUR which was used to construct the genomic library contains a PstI and a KpnI restriction site in the multiple cloning site (25). Restriction analysis of the isolated shuttle plasmids revealed that we had recovered two different plasmids complementing the prp4 mutation. One plasmid contained a 4.3 kb PstI/KpnI fragment; the second plasmid released a 2.5 kb PstI/KpnI genomic fragment. A more detailed restriction map of both inserts indicated that the 4.3 kb PstI/KpnI fragment included the 2.5 kb fragment. Therefore, the smaller fragment was further characterized. The shuttle plasmid containing this 2.5 kb DNA fragment was called pSAK.

The prp4 strain containing pSAK splices the dis2 gene
To demonstrate that the plasmid pSAK suppresses the splicing deficiency caused by the mutation in the prp4 locus, we performed Northern analysis. Strain prp4 (a) transformed with the plasmid pSAK was grown at 25°C to mid log phase. RNA was isolated from parts of the culture. The remaining culture was shifted to 36°C for three hours. Then RNA was isolated.

Figure 1. Northern analysis to demonstrate that the splicing defect of the prp4 mutation is suppressed by a 2.5 kb genomic fragment. Total RNA isolated from the prp4 strain grown at 25°C (lane 1) and then shifted and incubated for 3 h at 36°C (lane 2) and total RNA isolated from cells containing pSAK, which rescues the prp4 mutation (lane 3, RNA from cells grown at 25°C and lane 4, RNA from cells grown at 36°C) was separated on a 1.2% agarose gel and transferred to nitrocellulose. The blot was hybridized to a 600 bp radio labeled HindIII/XbaI strain. Those colonies and retransformed into the prp4 strain. Those inducing growth at the restrictive temperature were further characterized.

Figure 2. Cloning and sequence determination. A. Restriction map of the 2.5 kb clone complementing the prp4 mutation. Restriction sites in parenthesis (PstI) and (KpnI) are sites in the multiple cloning site of the vector (25). A, AccI, B, BglII, Bc, BclI, E, EcoRI, F, SalI. ATG and TAA indicate start and stop codon of the ORF (open box); closed box shows the intron; dotted boxes represent 5' and 3' flanking areas of the S. pombe prp4 gene.

The predicted amino acid sequence of the PRP4 protein, derived from the nucleotide sequence was compared with sequences in the GenBank data base using BLAST server (40).
shows that the \textit{prp}^{4\text{(a)}} strain which has been transformed with the plasmid pSAK does not accumulate pre-mRNA of the \textit{dis2} gene at the restrictive temperature, only mature mRNA was detected (Fig.1, lane 4). As expected, the mutant strain \textit{prp}^{4\text{(a)}} accumulates at the restrictive temperature pre-mRNA of the \textit{dis2} gene (Fig.1, lane 2). 

DNA sequence analysis predicts that the gene suppressing \textit{prp}^{4} encodes a serine/threonine kinase

The complete nucleotide sequence of the 2.5 kb genomic fragment (Fig.2A) contained in pSAK was determined. The sequence is shown in Fig.2B. We find an open reading frame (ORF) comprising 375 amino acids which appears to be interrupted by a 109 bp intron (Fig.2B). The putative intron displays an architecture described for small 5'S/3'S S.\textit{pombe} introns and has a 5' splice site and a branch-sequence conforming with the consensus sequences (16). The deduced amino acid sequence predicts a protein with a molecular mass of 42 742 Dalton and a pI of 7.1.

The predicted polypeptide derived from this nucleotide sequence was compared to GenBank data base. The search revealed that the deduced amino acid sequence contains conserved features which are predictive of the catalytic domains of a serine/threonine protein kinase. The amino acids in Fig.2B depicted in bold letters indicate protein kinase signature sequences (27, 28, 29). The first conserved sequence LGXGXFSXV beginning at position 165 (Fig.2B in bold letters) conforms to a consensus sequence for nucleotide binding proteins (27, 28, 29); at position 188 we find a lysine (K) an amino acid residue which appears to be invariant in all serine/threonine kinases; the following sequences contain all the other conserved amino acid residues identified in proteins belonging to the protein kinase family (Fig.2B, conserved residues in bold letters, 27, 28, 29).

Within the kinase domain starting at position 161 (Fig.2B) in the region where the nucleotide binding site is located, we find stretches of high sequence similarities to two \textit{S.\textit{cerevisiae}} protein kinases and a \textit{Dictyostelium discoidum} kinase. The SPK1 kinase from \textit{S.\textit{cerevisiae}} (31) shows 67% similarity over 32 amino acids (Fig.3). While the YAK1 kinase (38) shows 57% similarity over 47 amino acids (Fig.3). DDK2, kinase-2 from \textit{D.\textit{discoideum}} shows 65% similarity to the \textit{S.\textit{pombe}} kinase in this region (Fig.3, signature sequences are underlined). In addition, starting at position 228 in Fig.2B we find a stretch of 18 amino acids with 78% identity to a stretch of amino acids in the YAK1 protein kinase from \textit{S.\textit{cerevisiae}} (Fig.3). Outside the kinase domain, in the N-terminus of the predicted protein a stretch of 64 amino acids display 25% identical and 51% similar amino acid residues with residues found in the N-terminus and also outside the kinase domain of protein kinase-2 from the slime mold \textit{D.\textit{discoideum}} (Fig.3).

To prove that the gene encoding the putative kinase contains an intron, we used oligonucleotides complementary to the 5' end and to the 3' end of the open reading frame (Fig.2B) and performed PCR (polymerase chain reactions) using a cDNA library made from a wild type \textit{S.\textit{pombe}} strain (26). With this approach we revealed a product of approximately 1 kb. This fragment was subcloned and sequenced (results not shown). The sequence was identical with the sequence shown in Fig.2B except that it was missing the predicted intron sequence.

Steady state transcripts of the \textit{prp}^{4} suppressing gene

We performed a Northern analysis using RNA isolated from wild type cells, from the \textit{prp}^{4\text{(a)}} strain and from the \textit{prp}^{4\text{dis1}} strain transformed with the plasmid pSAK complementing the \textit{prp}^{4} mutation. As probe we used the radiolabeled 1.1 kb \textit{Sall/BglII} fragment which contains most part of the open reading frame (Fig.2A). In the autoradiography we detected in all lanes a somewhat broad band of about 1200 nt in size (Fig.4). It is possible that the bands include pre-mRNA and spliced mRNA of the gene. It is also possible that the gene has more than one transcriptional start and transcriptional stop site. In the \textit{prp}^{4\text{dis1}} mutant strain the somewhat longer transcripts accumulate at the

Figure 3. Alignment of deduced amino acid stretches of the putative protein kinase (PRP) from \textit{S.\textit{pombe}} with deduced amino acids from kinases SPK1 (38) and YAK1 (31) from \textit{S.\textit{cerevisiae}} and with the protein kinase-2 (DDK2) from \textit{D.\textit{discoideum}} (32). Prot. protein, %Id, % Identity, %Si, % Similarity. When the amino acid residues between two sequences are identical it is indicated by the letter code, when the amino acid residues have similar biochemical features it is indicated by a + sign. Amino acid residues underlined are part of the protein kinase signature sequences indicating a nucleotide binding site (27, 28, 29).

![Figure 3](image-url)
type strain. The dissection of 42 asci yielded for each tetrad at
Therefore, we used one of these strains and crossed it to a wild
S.cerevisiae.
+ LEU2
prp4
and a close linkage between
strains tetrad analysis indicated
plasmid was integrated in chromosome HI. When these strains
locus which has been mapped to chromosome EH (20).
that this gene is essential for growth.
that the product
gene is involved in pre-
putative serine/threonine protein kinase. In this paper we clearly
isolation of a gene involved in pre-mRNA splicing encoding a
protein kinase gene potentially involved in pre-mRNA splicing
36°C four growing colonies. These data clearly demonstrate that
the plasmid suppressing the prp4 mutation was integrated at the
prp4 locus.

The putative protein kinase gene is essential for growth and
is located at the prp4 locus
To test whether the gene encoding this putative protein kinase
is essential for growth, we constructed a diploid strain h+/-h-
and homozygous for the leu1-32 and ura4-D18 alleles. We used
the Sall/BgllII fragment which had the functional ura4 gene
inserted into the HindIII site residing in the open reading frame
of the gene (Fig.5B). This construction was transformed into the
cells in its linearized form to target the genomic sequences and
to replace the wild type gene with the ura4 disrupted gene by
homologous recombination (19, 20). From six independently
isolated transformants tetrads were analyzed. In all tetrads no
more than two spores grew up at 30°C. The viable spores were
auxotrophic for uracil. When DNA isolated from these haploid
growing colonies was digested with Sall and BgllII and then
hybridized with the 1.1 kb Sall/BgllII fragment (Fig.5B) we
detected a fragment of about 1 kb representing the uninterrupted
genic copy of the gene (lanes 1 and 2). DNA isolated from
diploid cells contain one 2.7 kb ura4 interrupted allele and one
wild type allele (Fig.5A, lanes 3 and 4). This clearly demonstrates that
this gene is essential for growth.

Early observations indicated that the prp4 locus is linked to the
ura4 locus which has been mapped to chromosome III (20).
For the complementation experiments reported above we also
used a genomic library cloned in the vector pDB248. This vector
contains the S.cerevisiae LEU2 gene as a marker which
complements the leu1 gene of S.pombe (30) The leu1 gene is
located on chromosome II. Transformation of this clone bank
into the prp4(62) strain also revealed suppressors of the prp4
mutation and plasmids isolated contained the identical sequence
reported above. We found that in some cases the suppressing
plasmid was integrated in chromosome III. When these strains
were crossed to leu1-prp4 - strains tetrad analysis indicated a
close linkage between prp4 + and LEU2 from S.cerevisiae.
Therefore, we used one of these strains and crossed it to a wild
type strain. The dissection of 42 asci yielded for each tetrad at
restrictive temperature (Fig.4 lane 2 and 3). This is consistent
with the presence of the 109 bp intron in this gene.

DISCUSSION
We are investigating pre-mRNA splicing in the fission yeast
S.pombe. The fission yeast is only distantly related to the budding
yeast S.cerevisiae but is also genetically tractable. The number
and size of introns found in these two yeasts differ significantly.
The majority of the S.pombe introns are smaller than 150 bp and
about 40—50 percent of the genes contain introns. In the yeast
S.cerevisiae only about 5% of the genes contain introns. These
introns are on average significantly longer than S.pombe introns
(16, 18). Recently it has been demonstrated with a small typical
S.pombe intron that during the assembly of spliceosomes the 5'-
end of U1 SnRNA may not only interact by complementary base-
pairing with the 5' splice site but also may interact by
complementary base pairing with the 3' AG of the intron (17).
The fission yeast S.pombe splices efficiently the small t intron
(66 bp) in the early region of the SV40 virus (14). Interestingly,
the small t SV40 intron displays the features of a typical S.pombe
intron. Therefore, we suggested that the small S.pombe introns
might reflect the primitive, the archetype of pre-mRNA introns
(15).

To the best of our knowledge this is the first report of the
isolation of a gene involved in pre-mRNA splicing encoding a
putative serine/threonine protein kinase. In this paper we clearly
demonstrate that the product of the prp4 gene is involved in pre-
mRNA splicing and that it is essential for growth. Neither from
the budding yeast S.cerevisiae nor from any other organism a
protein kinase gene potentially involved in pre-mRNA splicing
has been isolated. We do not have an indication yet in which
step of the splicing process this kinase is involved. We also do
not know anything about the substrate(s) of this protein kinase
encoded by the prp4 gene.

We are currently trying to isolate the protein to further
investigate the biochemical features of this putative kinase. In
a mammalian system a snRNP U1 associated kinase activity

Figure 5. Southern analysis to confirm gene replacement. A. DNA was double digested with the restriction enzymes Sall and BgllII and separated on an 0.8% agarose
gel. Lane 1 and lane 2 contains DNA isolated from growing colonies after tetrad analysis, lane 3 and lane 4 contain DNA from uracil prototrophic diploid cells
after transformation with the ura4 interrupted 2.7 kb fragment shown in B. The DNA was transferred to nitrocellulose and hybridized to the radiolabeled 1.1 kb
Sall/BgllII fragment shown also under B. B. Construction of an interrupted prp4: ura4 allele. The 1.8 kb HindIII ura4 fragment was cloned into the HindIII site
of the 2.5 kb prp4 fragment. The HindIII site is in the ORF (Fig. 2). The 2.7 kb Sall/BgllII fragment was isolated and transformed as described in the text. H, HindIII, St, Sstl, EV, EcoRV, E1, EcoRl.
phosphorylates in vitro the serine/arginine rich domains of the U1-70K protein (9, 39). It has been argued that kinases may be involved in the proper assembly or disassembly of the spliceosome. The ordered association or dissociation of splicing components during the splicing process might be brought about by the phosphorylation and de-phosphorylation of specific substrates at each step of the splicing process.

In any case, the prp4 gene of S.pombe encodes a protein which is predicted to be a serine/threonine kinase (27, 28, 29). We have taken advantage of the genetic amenability of S.pombe and isolated several suppressors of the temperature sensitive prp4 mutation. This approach will allow us to genetically determine the protein(s) which may interact with this kinase and will hopefully help to shed more light on the specific functional involvement of kinase(s) in the pre-mRNA splicing process. Experiments to complement the prp4 mutation with a mouse cDNA library are currently in progress. It will be interesting to find out whether S.pombe has features in the pre-mRNA splicing process which can be studied across organismal borders like cell cycle regulation.

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