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Isolation of novel pre-mRNA splicing mutants of *Schizosaccharomyces pombe*

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Abstract New *prp* (pre-mRNA processing) mutants of the fission yeast *Schizosaccharomyces pombe* were isolated from a bank of 700 mutants that were either temperature sensitive (ts^-) or cold sensitive (cs^-) for growth. The bank was screened by Northern blot analysis with probes complementary to *S. pombe* U6 small nuclear RNA (sn RNA), the gene for which has a splicesomal (mRNA-type) intron. We identified 12 *prp* mutants that accumulated the U6 snRNA precursor at the nonpermissive temperature. All such mutants were also found to have defects in an early step of TFIID pre-mRNA splicing at the nonpermissive temperature. Complementation analyses showed that seven of the mutants belong to six new complementation groups designated as *prp8* and *prp10-prp14*, whereas the five other mutants were classified into the known complementation groups *prp1*, *prp2* and *prp3*. Interestingly, some of the isolated *prp* mutants produced elongated cells at the nonpermissive temperature, which is a phenotype typical of cell division cycle (*cdc*) mutants. Based on these findings, we propose that some of the wild-type products from these prp^+ genes play important roles in the cellular processes of pre-mRNA splicing and cell cycle progression.

Key words *Schizosaccharomyces pombe* · Pre-mRNA splicing · *prp* mutant · Cell cycle

Introduction

The accurate removal of introns from a nuclear pre-mRNA is an essential step in eukaryotic gene expression. The splicing reaction proceeds via two transesterification reactions. In the first step, the 5′ splice site is cleaved, and a lariat intermediate is formed. Then, cleavage at the 3′ splice site and ligation of two exons occur in the second step. These reactions take place in a large complex called the spliceosome, which consists of the U1, U2, U4/U6 and U5 small nuclear ribonucleoprotein particles (snRNPs) and numerous non-snRNP protein factors (for reviews, see Green 1991; Guthrie 1991; Moore et al. 1993). The identification and characterization of these factors are necessary to elucidate the mechanism of pre-mRNA splicing. The *trans*-acting protein factors involved in pre-mRNA splicing have been identified by biochemical approaches using a mammalian in vitro splicing system or genetic approaches using yeast mutants defective in pre-mRNA splicing. The basic mechanism of pre-mRNA splicing has been evolutionarily conserved among the eukaryotes, although there are some differences. Yeasts are considered to be advantageous for studying the splicing mechanism, because classical genetic, biochemical and molecular approaches can be readily used in these organisms. In the budding yeast *Saccharomyces cerevisiae*, over 40 pre-mRNA processing (*prp*) mutants have been isolated (for reviews, see Guthrie 1991; Ruby and Abelson 1991; Moore et al. 1993), and more than 20 *PRP* gene products have been characterized by in vitro splicing analyses. PRP4p, PRP6p, PRP24p and PRP38p were found to associate with the U6 or U4/U6 snRNP (Banroques and Abelson 1989; Petersen-B et al. 1989; Abovich et al. 1990; Shannon and Guthrie 1991; Blanton et al. 1992). PRP8p and PRP18p are protein components of the U5 snRNP (Lossky et al. 1987; Whittaker et al. 1990; Horowitz and Abelson 1993). PRP28p interacts with PRP24p, and perhaps also associates with U5 snRNP (Strauss and Guthrie 1991). Genetic and biochemical assays have shown that PRP5p, PRP9p, PRP11p and PRP21p interact with U2 snRNP (Ruby and Abelson 1993). PRP19p is an integral component of the spliceosome

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(Cheng et al. 1993; Tarn et al. 1993). *PRP2* and *PRP16* encode RNA-dependent ATPases (Schwer and Guthrie 1991; Kim et al. 1992). PRP5p and PRP28p are putative members of the DEAD box helicase-like protein family, while PRP2p, PRP16p and PRP22p are members of the DEAH box helicase-like protein subfamily (for reviews, see Wassarman and Steitz 1991; Schmid and Linder 1992).

The fission yeast *Schizosaccharomyces pombe* is believed to be a good model organism for analysis of pre-mRNA splicing for the following reasons. First, genetic manipulation is as effective in this organism as in *S. cerevisiae*. Secondly, the gene structure in *S. pombe* is more similar to that of vertebrate genes than that in *S. cerevisiae*. About 40% of *S. pombe* genes have introns and about half of them have multiple introns, whereas *S. cerevisiae* genes rarely have introns. Thirdly, the similarity of splicing signals between *S. pombe* and mammals is greater than that between *S. cerevisiae* and mammals (Mertins and Gallwitz 1987a; Prabhala et al. 1992). Actually, there has also been a previous example of mammalian pre-mRNA splicing in *S. pombe* (Käufer et al. 1985). In *S. pombe*, however, only four temperature-sensitive (ts) *prp* mutants have been reported so far: *prp1*, *prp2*, and *prp3* by Potashkin et al. (1989), and *prp4* by Rosenberg et al. (1991). Of these, only two prp^+ genes have been cloned. The $prp2^+$ gene encodes spU2AF^{59}, which is a yeast homolog of the mammalian splicing factor U2AF large subunit, U2AF^{65} (Potashkin et al. 1993). The $prp4^+$ gene product is predicted to be a serine/threonine kinase (Alahari et al. 1993).

We describe herein the isolation of *prp* mutants from ts^- and cold-sensitive (cs^-) growth mutant banks, and characterize their phenotypes. Twelve mutants that accumulate precursors for U6 snRNA and TFIID mRNA at the nonpermissive temperature were identified by Northern blot analysis. Interestingly, some of the isolated *prp* mutants exhibited phenotypes characteristic of *cdc* (cell division cycle) mutants at nonpermissive temperatures. The products of these *prp* genes may be involved in both pre-mRNA splicing and cell cycle progression either directly or indirectly.

Materials and methods

Yeast strains and genetic analyses

The *S. pombe* strains used in this study are listed in Table 1. The standard genetic procedures for *S. pombe* were followed as described in Gutz et al. (1974), Sherman et al. (1986), Moreno et al. (1991) and Alfa et al. (1993).

Media and culture conditions

Yeast was grown in YPD or YE complete medium (Sherman et al. 1986; Gutz et al. 1974) or PM minimal medium (Moreno et al. 1991). Growth supplements were added to PM to attain a final concentration of 75 mg/l (adenine and histidine) or 250 mg/l (leucine) (Alfa et al. 1993). YEP (YE containing 2.5 mg/l of phloxin B) was used to stain dead cells (Moreno et al. 1991). Media containing 2% agar were used for plating. SPA was used for mating and sporulation (Gutz et al. 1974).

The ts^- mutants were grown at 26° C (permissive temperature) or at 36° C (nonpermissive temperature), while the cs^- mutants were grown at 32° C (permissive temperature) or at 22° C (nonpermissive temperature). Wild-type strains were usually grown at 30° C. Mating and sporulation were done at either 26° or 30° C.

Table 1 Strains of *Schizosaccharomyces pombe*

Strain	Genotype	Source
L972	h^-	U. Leupold
L975	h^+	U. Leupold
HM123	h^- *leu1-32*	M. Yanagida
SU23-4A	h^- *ade6-M216*	This study
SU24-20B	h^+ *ade6-M210*	This study
JP21	h^- *prp2-1*, *ade6-M216*	D. Frendewey
UR107	h^- *prp1-4*	This study
SU50-5B	h^- *prp2-2*	This study
SU13-11B	h^- *prp3-3*	This study
UR130	h^+ *prp3-4*, *ade6-M216*	This study
UR142	h^- *prp3-5*, *ade6-M210*	This study
SU26-9B	h^- *prp8-1*	This study
UR230	h^- *prp10-1*	This study
SU93-2D	h^- *prp11-1*	This study
SU100-2A	h^- *prp12-1*	This study
SU101-9C	h^- *prp13-1*	This study
UR402	h^- *prp14-1*	This study
UR412	h^- *prp14-2*	This study

Mutagenesis and mutant isolation

The wild-type strain L792 was mutagenized with ethylmethane sulfonate (EMS) as described by Sherman et al. (1986) or with 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) according to the method of Uemura and Yanagida (1984). After mutagenesis, the cells were plated on YPD at a density of about 200 survivors per plate and incubated at 26° C to isolate ts^- mutants or at 32° C to isolate cs^- mutants. The colonies grown on plates were replica-plated on to YEP plates and incubated at 36° C overnight for ts^- mutant isolation or at 22° C for 2 days for cs^- mutant isolation. Strains that grew poorly or died only at the nonpermissive temperature were identified and included in a mutant bank.

Preparation of RNA

Each ts^- or cs^- mutant was grown in 10 ml YPD broth to mid-log phase at the permissive temperature, and then shifted to the nonpermissive temperature for either 2 h (at 36° C) or for 5 h (at 22° C). The cells were harvested, washed twice with sterile water and frozen at − 80° C. Subsequently, total RNA was prepared from them by glass bead disruption as described by Nischt et al. (1986). The yield of total RNA was usually 50-300 μg by this method.

Preparation of probes for Northern blot analysis

The following oligonucleotides were used as probes for Northern blot analysis. U6-IN1 (20-mer), 5′-TCGAACCTTGGTAAA-TATTG-3′, is complementary to nucleotides 55-74 in the intron of *S. pombe* pre-U6 snRNA and hybridizes with the U6-snRNA

precursor, lariat intermediate and excised intron. U6-EX2 (20-mer), 5′-CAGTGTCATCCTTGTGCAGG-3′, is complementary to positions 106-125 of *S. pombe* pre-U6 snRNA and can detect the mature U6 snRNA, pre-U6 snRNA and lariat intermediate (Tani and Ohshima 1989). TFII-IN1 (20-mer), 5′-GAAATCTCGTGACATGGTAG-3′, is complementary to the first intron of *S. pombe* TFIID pre-mRNA. TFII-EX3 (20-mer), 5′-GAGCTTGGAGTCATCCTCGG-3′, is complementary to the third exon of *S. pombe* TFIID pre-mRNA (Hoffmann et al. 1990). ACT1 (20-mer), 5′-ACAAGCAAGGGTGCTCCTCA-3′, is complementary to *S. pombe* actin mRNA, the gene for which has no intron (Mertins and Gallwitz 1987b). These oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 380A and labeled at the 5′ ends using [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Sambrook et al. (1989).

Northern blot analysis

To analyze pre-U6 snRNA splicing, total RNA was mixed with formamide dye (80% formamide, 1 × TBE, 0.1% bromophenol blue and 0.1% xylene cyanol), heated at 65° C for 2 min and then fractionated on 8% polyacrylamide (acrylamide : bis-acrylamide = 19 : 1), 8.3 M urea gels. After removal of the urea from the gel by soaking in 7% formaldehyde and 20 mM TRIS-HCl, pH 7.5 for 30 min, for RNAs were transferred onto nylon membranes (Biodyne A, PALL) in 20 × SSC by capillary blotting. For the analysis of pre-mRNA, 30 μg of total RNA from each mutant was electrophoresed on a 1% formaldehyde agarose gel and blotted onto a nylon membrane (Gene Screen, NEN) by capillary blotting (Sambrook et al. 1989). The RNA was crosslinked to the membrane by exposure to a low dose of ultraviolet irradiation (Sambrook et al. 1989).

The membranes were prehybridized in 6 × SSC, 50 mM sodium phosphate, pH 7.5, 2 mM EDTA, 0.1% SDS, 5 × Denhardt's solution and 0.2 mg/ml boiled salmon sperm DNA at 42° C. Hybridization was performed in the same solution with more than 5×10^5 cpm/ml of the oligonucleotide probes at 42° C overnight. The membranes were washed three times in 6 × SSC at room temperature, and then soaked in 6 × SSC, 0.1% SDS at 50° C for 5 min. The filters were then exposed to Kodak X-ray film with an intensifying screen at − 70° C for 2 or 3 days.

Removal of the TFIID probes from the membranes for rehybridization with the actin probe was done according to the method of Potashkin et al. (1989).

DNA staining and in situ hybridization

Nuclei of *S. pombe* were stained as follows: the cells were fixed with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate, pH 6.0 for 1 h at room temperature. The cells were washed with 0.1 M sodium phosphate, pH 6.0 and 0.3 M glycine twice and resuspended at 1×10^7 cells/ml in PBS (Nissui-seiyaku). Subsequently, the cells were placed onto a multiwell slide (Polyscience.) coated with 1 mg/ml poly(L-lysine) and stained with 0.1 μg/ml 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min. The samples were washed with PBS once, mounted with a drop of mounting medium (Moreno et al. 1991) and observed by fluorescence microscopy. In situ hybridization using an oligo(dT) probe was performed as described by Kadowaki et al. (1992).

Results

Generation of a bank of ts$^-$ or cs$^-$ mutants

We mutagenized a wild-type strain of *S. pombe* with EMS or MNNG, and isolated about 600 ts$^-$ mutants that were able to form colonies at 26° C but were not able to do so at 36° C. We also isolated about 100 cs$^-$ mutants that were able to grow at 32° C but not at 22° C. In contrast, the wild-type parental strain could form colonies at all four temperatures. The doses of EMS and MNNG were selected to give a 20–30% and 5–10% survival rate, respectively. Under these conditions, ts$^-$ or cs$^-$ mutants represented 0.1–0.3% and about 1% of the survivors after EMS treatment and MNNG treatment, respectively (data not shown). These ratios are similar to those reported by Potashkin et al. (1989) and by Nurse et al. (1976).

Screening for mutants defective in pre-U6 snRNA splicing

To isolate pre-mRNA splicing mutants of *S. pombe*, we initially screened the ts$^-$ and cs$^-$ mutant banks for mutants defective in the splicing of the U6 snRNA precursor by Northern blot analysis. The *S. pombe* U6 snRNA gene is interrupted by an intron that is 50 bp in length and has a structure similar to those of introns in the nuclear mRNA genes (spliceosomal introns), despite the fact that it does not encode a protein (Tani and Ohshima 1989). We chose U6 snRNA for primary screening, since the detection of pre-U6 snRNA was easier than the detection of pre-mRNA for the following reasons. First, U6 snRNA is abundant in cells (for review, see Reddy and Busch 1988). Secondly, the splicing of pre-U6 snRNA was impaired in the three previously identified *prp* mutants of *S. pombe* (Potashkin and Frendewey 1989), which suggested that pre-U6 snRNA is spliced by the same mechanism as that for pre-mRNA splicing.

Among the 700 ts$^-$ or cs$^-$ mutants screened, 12 mutants (9 ts$^-$ and 3 cs$^-$) were identified as candidates that were defective in the splicing of pre-U6 snRNA. They accumulated the U6 snRNA precursor at the nonpermissive temperature, whereas the wild-type strain showed no accumulation (Fig. 1). The mature U6 snRNA was also detected at the nonpermissive temperature in each candidate. As U6 snRNA is highly stable in the cells, the detected mature U6 snRNAs are likely to have been produced prior to the inhibition of splicing by the temperature shift.

Most of these mutants showed an accumulation of pre-U6 snRNA only at nonpermissive temperatures, though *ts2*, *ts18*, *ts40* and *cs89* accumulate a small amount of U6 snRNA precursor at the permissive temperature as well. These three strains are likely to have a slight defect in pre-U6 snRNA splicing even at the permissive temperature, although their growth was normal at this temperature. In the case of the three *ts332*, *cs66* and *cs72*, the amounts of accumulated pre-U6 snRNA were similar at both temperatures. It is unlikely that the accumulation of pre-U6 RNA in these mutants at the permissive temperature is due to the

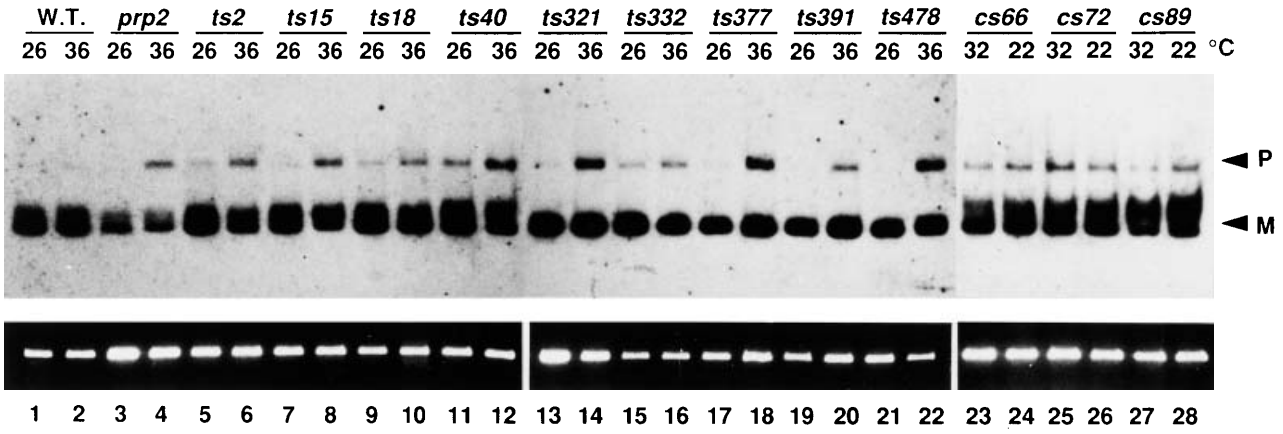


Fig. 1 Northern blot analysis of U6 and pre-U6 snRNA in the temperature-sensitive (ts) and cold-sensitive (cs) mutants (*upper panels*). Total RNA was isolated from the wild type and each of the 12 candidates that had been grown at the respective permissive temperatures (ts = 26° C, cs = 32° C) or shifted to nonpermissive temperatures (ts = 36° C, cs = 22° C) for 2 or 6 h. Each RNA (5 μg) was fractionated on a polyacrylamide gel and blotted to the membranes. The *prp2-1* strain (JP21) is known to accumulate pre-U6 snRNA at nonpermissive temperatures (Potashkin and Frendewey 1989). *P* and *M* on the right of the panels mark the positions of the precursor and mature U6 snRNAs, respectively. The results for the wild-type strain at 32° and 22° C (data not shown) are similar to the results at 26° and 32° C that are shown in the figure. The *lower panels* show 5S rRNA stained with ethidium bromide, and serve to indicate the quantity of total RNA used in each lane

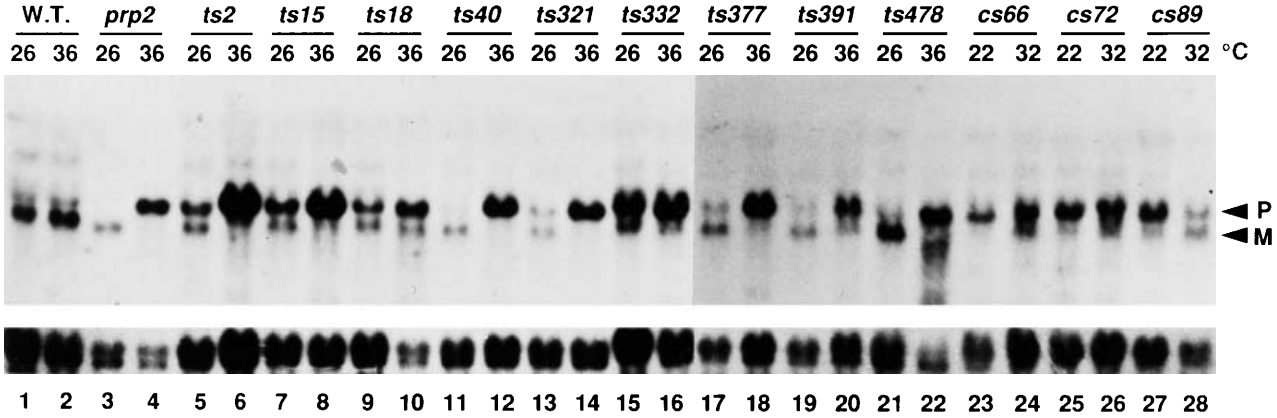
presence of two independent mutations, one affecting RNA processing and the other leading to ts or cs lethality, because the accumulation patterns did not change even after eight backcrossings with wild-type cells. Their growth was significantly slow even at permissive temperatures. Each of these three mutants is thus considered to have a severe mutation that affects growth and pre-U6 snRNA splicing even at permissive temperatures.

Analyses of TFIID pre-mRNA splicing

To examine whether or not the isolated mutants also have defects in pre-mRNA splicing, we performed a Northern blot analysis of TFIID mRNA. The TFIID gene encodes a TATA box-binding factor and contains three introns (Hoffmann et al. 1990). We chose TFIID mRNA since it is relatively abundant in *S. pombe* and intron-containing transcripts of the TFIID gene are barely detectable in wild-type cells. Figure 2 shows the results of a Northern blot analysis using a mixture of probes for the intron (TFII-IN1) and an exon (TFII-EX3) of the *S. pombe* TFIID gene. The patterns of accumulation of TFIID mRNA precursors in the mutants at both temperatures were similar to those of

Fig. 2 Northern blot analysis of TFIID mRNA and pre-mRNA in the pre-U6 snRNA splicing mutants (*upper panels*). Total RNA (30 μg) from each mutant or the wild-type strain was isolated under the same conditions as in Fig. 1, fractionated on a 1% formaldehyde agarose gel and blotted. The blot was probed with a mixture of the TFII-IN1 and TFII-EX3 probes. *P* and *M* on the right indicate the precursor and mature mRNAs of TFIID, respectively. The probes were subsequently removed from the membranes, and then the membranes were rehybridized with the ACT1 probe to indicate the quantity of RNA in each lane. The two bands in each lane in the *lower panels* indicate two of the three transcripts of the *act1*$^+$ gene

▼



pre-U6 snRNA. Only mature mRNA was detected in the wild-type strain at both temperatures (Fig. 2, lanes 1 and 2). The *prp2* mutant (Potashkin et al. 1989) was used as a positive control that accumulated TFIID pre-mRNA only at nonpermissive temperatures (Fig. 2, lanes 3 and 4). Each pre-U6 snRNA splicing mutant accumulated the TFIID mRNA precursor at the nonpermissive temperature as well as pre-U6 snRNA. In strains *ts2*, *ts15*, *ts18*, *ts321* and *cs89*, the TFIID mRNA precursor was detected in addition to mature mRNA even at permissive temperature (26° or 32° C), albeit at a reduced amount. Pre-mRNA splicing in these strains seems to be inhibited slightly at permissive temperatures. The mutants *ts332*, *cs66* and *cs72* showed similar levels of pre-mRNA at both temperatures, which is consistent with the results on pre-U6 snRNA. However, in all the mutants, the shift to the nonpermissive temperature reduced the levels of mature mRNA, suggesting that the splicing of TFIID pre-mRNA is more severely blocked at nonpermissive temperatures. In *ts2*, *ts15* and *ts18*, β-tubulin pre-mRNA splicing was also blocked at nonpermissive temperatures (data not shown). These results indicate that the 12 isolated mutants are deficient not only in pre-U6 snRNA splicing but also in pre-mRNA splicing at nonpermissive temperatures. We therefore designate these mutants as *prp* mutants of *S. pombe*.

Genetic analyses

The isolated *prp* mutants were crossed with a wild-type strain and a tetrad analysis was performed. Analyses of 20 tetrads for each mutant showed that ts$^-$ or cs$^-$ and wild-type phenotypes segregated 2:2 in all the observed cases, suggesting that a single nuclear mutation was present. Each mutant was backcrossed at least six times with a wild-type strain. Cosegregation of the splicing defect with the ts$^-$ or cs$^-$ phenotype was observed in all the *prp* mutants (data not shown). This suggests that the ts$^-$ or cs$^-$ growth phenotype is linked to a mutation that leads to the defects in pre-U6 snRNA and pre-mRNA splicing.

To examine whether the mutations in the isolated *prp* mutants are dominant or recessive, heterozygous diploids were obtained by crossing with an appropriate wild-type strain with the opposite mating type. All diploids grew at nonpermissive temperatures, indicating that all the ts$^-$ or cs$^-$ mutations are recessive.

Eight *prp* complementation groups have previously been isolated as ts$^-$ mutations in *S. pombe*: *prp1*, *prp2* and *prp3* (Potashkin et al. 1989), *prp4* (Rosenberg et al. 1991), and *prp5*, *prp6*, *prp7* and *prp9* (Frendewey et al. personal communication). Complementation analyses revealed that *ts15* and *ts40* are allelic with *prp1* and *prp2*, respectively, and three mutations, *ts2*, *ts321* and *ts377*, are allelic with *prp3*. However, four ts$^-$ mutations, *ts18*, *ts332*, *ts391* and *ts478*, were found to belong to new complementation groups. When mutants carrying one of these mutations were crossed with each other, all diploids were able to form colonies at 36° C, suggesting that these ts$^-$ mutations belong to different complementation groups. Because the *cs66*, *cs72* and *cs89* mutations had a cs$^-$ phenotype, we carried out recombination analyses between each cs$^-$ strain and

Table 2 The *S. pombe prp* mutations isolated in this study. (*EMS* ethylmethane sulfonate, *MNNG* 1-methyl-3-nitro-1-nitrosoguanidine)

Complementation group	Allele no.	Original mutant name	Mutagen	Phenotypes at the nonpermissive temperature			
				Growth defect	Splicing defect	Poly (A)$^+$ RNA transport defect	Cell morphologies
prp1	*prp1-4*	*ts15*	EMS	ts	+	+	Chromosome condensation Dumpy cell body
prp2[a]	*prp2-2*	*ts40*	EMS	ts	+	−	Dumpy cell body
prp3	*prp3-3*	*ts2*	EMS	ts	+	−	Dumpy cell body
	prp3-4	*ts321*	MNNG	ts	+	−	Dumpy cell body
	prp3-5	*ts377*	MNNG	ts	+	−	Dumpy cell body
prp8[b]	*prp8-1*	*ts18*	EMS	ts (leaky)	+	−	Elongation of cell body
prp10	*prp10-1*	*ts478*	MNNG	ts	+	−	Dumpy cell body
prp11	*prp11-1*	*cs89*	MNNG	cs	+	−	Elongation of cell body
prp12	*prp12-1*	*ts391*	MNNG	ts (leaky)	+	−	Elongation of cell body
prp13	*prp13-1*	*ts332*	MNNG	ts	+	−	Elongation of cell body Appearance of multinuclei, abnormal branching and knobs
prp14	*prp14-1*	*cs66*	MNNG	cs	+	−	Elongation of cell body Appearance of abnormal branching and knobs (*prp14-1*)
	prp14-2	*cs72*	MNNG	cs	+	−	

[a] Prp2p is the yeast homolog of human U2AF^{65} (Potashkin et al. 1993), and the *prp2*$^+$ gene is identical to *mis11*$^+$ (Takahashi et al. 1994)
[b] *prp8* is allelic to *cdc28* (Lundgren et al. 1996)

each ts$^-$ strain. In all cases, wild-type recombinants were observed at a high frequency, suggesting that each pair of mutations is unlinked. As for the three cs$^-$ mutations, complementation analyses showed that *cs66* and *cs72* were allelic with each other, while *cs89* belongs to a different complementation group. Therefore, we classified *ts18*, *ts478*, *cs89*, *ts391* and *ts332* as *prp8*, *prp10*, *prp11*, *prp12* and *prp13*, respectively, while *cs66* and *cs72* were identified as *prp14* (Table 2).

Characterization of the phenotypes of the new *prp* mutants

To characterize further these *prp* mutants, we examined the morphological changes at nonpermissive temperatures. The cell morphology and staining pattern of the nuclear DNA of the wild type and *prp10*, did not change at the nonpermissive temperature (Fig. 3C, D, K and L) except for the fact that some *prp10*, cells became shorter and fatter, or dumpy (Fig. 3K, shown by arrowheads). The *prp1*, *prp2* and *prp3* mutants also showed similar phenotypes, although some *prp1* cells showed condensed chromatin (data not shown). Interestingly, *prp8* and *prp11-prp14*, produced elongated cells at the nonpermissive temperature (Figs. 3G, O, S, 4G, K). Elongation of the cell at the nonpermissive temperature is characteristic of *cdc* mutants, which are defective in the progression of the cell cycle. The *prp8*, *prp11*, *prp12* and *prp14-2* mutants (Figs. 3G, O, 4G, K) showed twofold or threefold elongated cells compared with wild-type cells at the nonpermissive temperatures (Figs. 3C, 4C), and each of them carried a single nucleus. *prp13* and *prp14-1* cells also became elongated at permissive temperatures, albeit less than at nonpermissive temperatures (Figs. 3Q, S, 4I, K). In addition, some cells from these two *prp* strains showed multiple nuclei per cell (Fig. 3T), and often formed abnormal branches and knobs at nonpermissive temperatures (data not shown).

We then investigated the growth characteristics of these *prp* mutants after shifting to the restrictive temperature (Fig. 5). In *prp10*, *prp11*, *prp13* and *prp14*, the cell numbers increased two- to threefold at nonpermissive temperatures (Fig. 5A, C), suggesting that these cells had divided at least once before cell cycle arrest or death. In *prp8*, the cell number increased about fourfold at 36° C (Fig. 5A), which suggests that the cells had divided at least twice. In *prp12*, the cell number increased up to 4 h, like the wild type, and then continued slight growth at 36° C (Fig. 5A). These observations are consistent with leaky temperature sensitivity for growth in *prp8* and *prp12*. The cell viabilities of *prp10* and *prp13*, decreased to less than 10% and to 50% after 10 h at 36° C (Fig. 5C), respectively. These mutants were mostly dead after 24 h at 36° C (data not shown). Although there was a slight increase in viable cells of *prp8* and *prp12* at 36° C for 12 h (Fig. 5C), most of these

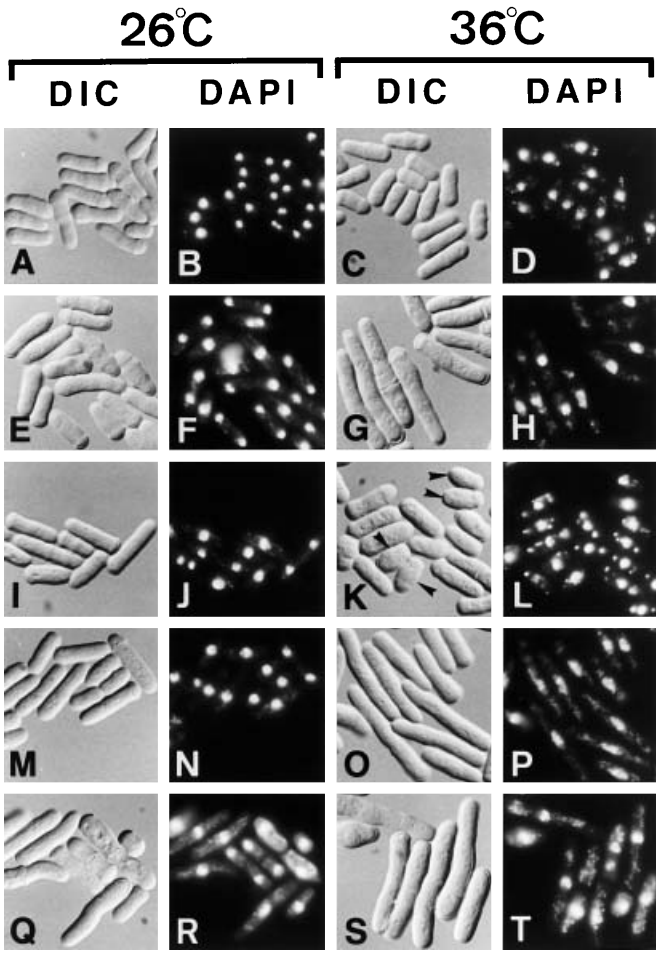


Fig. 3A–T Morphologies of the new ts$^-$ *prp* mutants. The wild type and the new ts$^-$ *prp* mutants were cultured at the permissive temperature (26° C, first and second columns), and then were transferred to the nonpermissive temperature for 8 h (36° C, third and fourth columns). **A–D** Wild type; **E–H** *prp8-1*; **I–L** *prp10-1*; **M–P** *prp12-1*; **Q–T** *prp13-1*. The *first* and third columns in each row show the differential interference contrast (DIC) pictures. The second and the fourth columns show the cells stained with 4′,6-diamidino-2-phenylindote (*DAPI*). The first and second columns, and the third and the fourth columns in each row show the same fields. *Arrowheads* in **K** indicate dumpy form cells (see text). The original magnification of the photographs in 1000

cells were dead after 24 h at 36° C (data not shown). In *prp11* and *prp14*, the cell viabilities decreased to approximately 60% after 20 h at 22° C (Fig. 5D), which was consistent with the observation that these cs$^-$ *prp* mutants can resume their growth after being shifted back to the permissive temperature.

We next examined by in situ hybridization whether or not 12 *prp* mutants have defects in transporting mRNA from the nucleus to the cytoplasm. All *prp* mutants were grown and shifted to the nonpermissive temperature for 4 h (ts$^-$) or 10 h (cs$^-$), fixed, and the cell walls digested with enzymes. The cells were incubated with a biotin-labeled oligo(dT) probe, which hybridizes with poly(A) tails of mRNAs, followed by

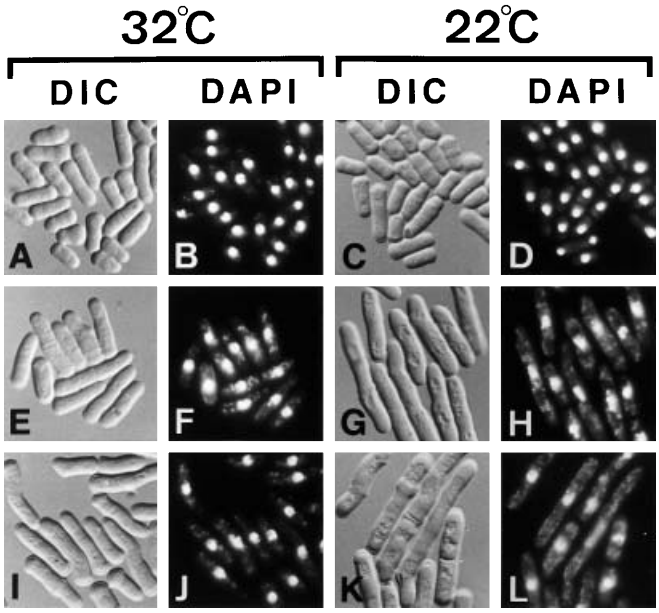


Fig. 4A–L Morphologies of the new cs$^-$ *prp* mutants. The wild type and the new cs$^-$ *prp* mutants were cultured at the permissive temperature (32° C, first and second columns), and then were transferred to the nonpermissive temperatures for 24 h (22° C, third and fourth columns). **A–D** wild type; **E–H** *prp11-1*; **I–L** *prp14-2*

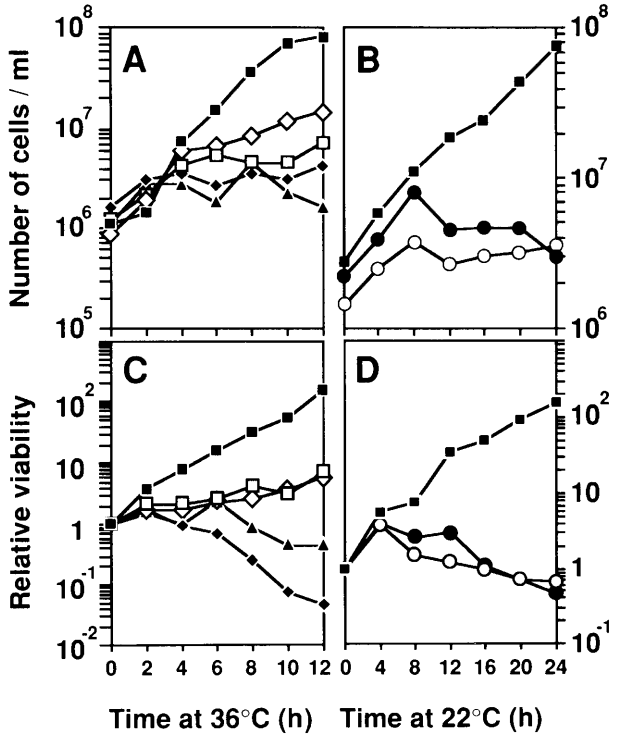


Fig. 5A–D Growth characteristics of the *prp* mutants. The wild-type strain and the *prp* mutants were cultured in YPD broth at the permissive temperature until mid-log phase and then were transferred to the nonpermissive temperature (either 36° or 22° C). The total cell number was measured with a hemacytometer at the indicated time (**A**, **B**). The relative viabilities of cells were obtained from the number of the colonies grown at the permissive temperature (**C**, **D**). The *symbols* indicate the strains as follows: ■ wild type, □, *prp8*, ◆ *prp10*, ◇ *prp12*, ▲ *prp13*, ○ *prp11*, ● *prp14*

treatment with fluorescein isothiocynate-avidin and DAPI. *prp1* showed hybridizing signals in the whole nucleus only at the nonpermissive temperature (unpublished result), suggesting that nucleocytoplasmic export of poly(A)$^+$ RNA is inhibited in this mutant. However, other *prp* mutants gave signals both in the nucleus and in the cytoplasm at both temperatures as did the wild-type strain (data not shown), which suggested that limited or no defects exist in poly(A)$^+$ RNA export in these mutants.

Discussion

Splicing defects of the mutants

As a first step in isolating mutants defective in pre-mRNA splicing, we screened ts$^-$ or cs$^-$ banks for mutants defective in pre-U6 snRNA splicing. This screening strategy is based on the hypothesis that the machinery for pre-U6 snRNA splicing in *S. pombe* is identical to, or shares components with, that for pre-mRNA splicing (Potashkin and Frendewey 1989). In fact, all the pre-U6 snRNA splicing mutants isolated in the primary screening also had defects in TFIID pre-mRNA splicing. In addition, the mutants, which were either slightly or severely blocked in pre-U6 snRNA splicing at permissive temperatures, showed similar levels of blocking in pre-mRNA splicing. These results thus support the hypothesis described above. We cannot rule out the possibility that the block in pre-U6 snRNA splicing in some *prp* mutants takes place as a secondary effect of a specific defect in pre-mRNA splicing. As suggested by Potashkin and Frendewey (1989), some factors specific for pre-U6 snRNA splicing might exist and be encoded by a gene with an intron. However, this possibility is less likely, since we could not isolate mutants specific for pre-U6 snRNA splicing. If there is any factor specific for pre-mRNA splicing and not for pre-U6 snRNA splicing, mutants defective in such a factor would not be isolated by the primary screening used in our study. Before we can definitively conclude that pre-mRNA and pre-U6 snRNA are spliced by the same machinery, more information on both splicing pathways will be necessary.

All isolated mutants accumulate only precursor RNAs at the nonpermissive temperature. No intermediates or excised introns were detected. As a result, these *prp* mutants seems to have defects in early steps of the splicing pathway, such as in (pre-)spliceosome assembly.

mRNA transport of *prp* mutants

In situ hybridization to poly(A)$^+$ RNA in the *prp* mutants showed stronger nuclear signals only in *prp1*, and

uniform signals in the other mutants. These results suggest that *prp1* has not only a pre-mRNA splicing defect, but also a defect in the nucleocytoplasmic export of poly(A)$^+$ RNA at the nonpermissive temperature, and that the others do not have an export defect. The *prp1*$^+$ gene product might function both in the pre-mRNA splicing and in the nucleocytoplasmic export of poly(A)$^+$ RNA.

Block of the cell cycle in *prp* mutants

At the nonpermissive temperatures, five new *prp* mutants, *prp8* and *prp11-prp14*, all produced abnormally elongated cells (Figs. 3, 4), which are commonly observed in *cdc* mutants (Nurse et al. 1976; Nasmyth and Nurse 1981). In other *prp* mutants, *prp1*, *prp2*, *prp3* and *prp10*, no such morphological change was observed, although some cells in these mutants changed to dumpy forms at nonpermissive temperatures. Recently, Frendewey et al. (personal communication) found that *prp5*, *prp6* and *prp7* also produced elongated cells at nonpermissive temperatures. Furthermore, *cdc28* and *prp8* of *S. pombe* were recently found to be allelic with each other (Lundgren et al. 1996). Namely, they found that a *cdc28* mutant accumulated U6 snRNA precursor at the nonpermissive temperature, and that the wild-type *cdc28*$^+$ gene complemented a *prp8* mutation. The *cdc28* mutant was previously isolated as a ts$^-$ mutant that was unable to complete the cell division cycle at the nonpermissive temperature. The *cdc28*$^+$ gene was found to be required for entry into mitosis (Nasmyth and Nurse 1981). The *cdc28*$^+$ gene encodes a putative RNA-dependent ATPase that is most similar to the members of the RNA helicase family with a DEAH box motif (Lundgren et al. 1996). A DEAH motif has also been found in PRP2p, PRP16p and PRP22p of *S. cerevisiae*. All these results suggest that these *prp*$^+$ gene products are not only involved in RNA splicing, but also in cell cycle progression. There might be a mechanism that links pre-mRNA splicing and cell cycle progression. Similar observations made in *S. cerevisiae* and mammalian cells have also suggested a relation between pre-mRNA splicing and cell cycle progression. *prp20* of *S. cerevisiae* was initially isolated as a pre-mRNA splicing mutant (Vijayraghavan et al. 1989). The *PRP20* protein was found to be an *S. cerevisiae* homolog of the mammalian RCC1 protein (Aebi et al. 1990), which is a guanine nucleotide exchange factor for the Ras-like guanosine triphosphatase (GTPase) Ran/TC4 (Bischoff and Ponstingl 1991). The RCC1 protein is known to be involved in controlling the initiation of mitosis in addition to RNA export, protein import and chromosome stability (for review, see Dasso 1993). Little is known as to how the *PRP20* protein affects the pre-mRNA splicing reaction. Recently, the *DBF* (Dumb-bell former) gene of *S. cerevisiae* was found to be identical to the *PRP8* gene, which encodes a protein component of U5 snRNP in *S. cerevisiae* (Shea et al. 1994). The *dbf3* mutant was originally isolated as a ts$^-$ mutant, which has a defect in the initiation of DNA synthesis in the cell cycle (Johnston and Thomas 1992a, b). *DBF3* was shown to play a role in the S phase of the cell cycle, possibly in the initiation of DNA synthesis (Shea et al. 1994). They thus proposed that one or more of the snRNP complexes may play a dual role in both pre-mRNA splicing and cell cycle progression. In addition, one of the *S. pombe* minichromosome loss mutations, *mis11*, was recently found to reside in the *prp2*$^+$ gene (Takahashi et al. 1994). In *mis11* mutants, the progression of the G1 and G2 phases is defective and the average cell size is significantly reduced at the nonpermissive temperature. The *prp2* gene encodes an *S. pombe* homolog of the large subunit of mammalian splicing factor U2AF (Potashkin et al. 1993), which is one of the non-snRNP protein factors identified in HeLa cell nuclear extracts and is required for U2 snRNP binding to a branch point of a pre-mRNA and for pre-spliceosome assembly (Ruskin et al. 1988; Zamore and Green 1989). Functional spliceosomes were thus suggested to be important for proper nuclear architecture, and their absence may lead to minichromosome instability (Takahashi et al. 1994). Furthermore, mammalian SRPK1, a kinase that is regulated by the cell cycle and specifically phosphorylates serine residues in SR proteins, has recently been identified. It was proposed that SRPK1 is one of the regulators for the organization of splicing factors in interphase cells, although its precise function remains to be clarified (Gui et al. 1994).

Taken together, the products of the *prp*$^+$ genes, whose mutations show *cdc*-like phenotypes, may not only regulate the activities of the splicing factors either directly or indirectly, but may also play a role in the cell cycle. Alternatively, if cell cycle genes have introns, an inhibition in the splicing of the primary transcripts at the nonpermissive temperature may result in arrest of the cell cycle. Furthermore, *prp1*, *prp3* and *prp10* mutants do not show cell elongation at nonpermissive temperatures, but their phenotypes, including reduced cell sizes, closely resemble those of the *prp2* mutants described above (data not shown). This finding suggests that these *prp* mutants without cell elongation may also have defects in their cell cycle progression. Further characterizations of the isolated *prp* mutants are still needed to elucidate the relationship between pre-mRNA splicing and cell cycle progression.

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