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Pre-mRNA splicing mutants of *Schizosaccharomyces pombe*

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A collection of temperature sensitive (ts^-) mutants was prepared by chemical mutagenesis of a wild type *Schizosaccharomyces pombe* strain. To screen the ts^- mutants for pre-mRNA splicing defects, an oligodeoxynucleotide that recognizes one of the introns of the β-tubulin pre-mRNA was used as a probe in a Northern blot assay to detect accumulation of intron sequences. This screening procedure identified three pre-mRNA splicing mutants from 100 ts^- strains. The three mutants are defective in an early step of the pre-mRNA splicing reaction; none accumulate intermediates. The precursors that accumulate at 37°C are polyadenylated. Analysis of the splicing of another pre-mRNA showed that the mutations are not specific for β-tubulin. The total RNA pattern in the three splicing mutants appears to be normal. In addition, the amounts of the spliceosomal snRNAs are not drastically changed compared to the wild type and splicing of pre-tRNAs is not blocked. Genetic analyses demonstrate that all three splicing mutations are tightly linked to the ts^- growth defects and are recessive. Crosses among the mutants place them in three complementation groups. The mutants have been named *prp1*, *prp2* and *prp3*.

Key words: pre-mRNA splicing/prp^- mutants/*Schizosaccharomyces pombe*

Introduction

The splicing of nuclear messenger RNA precursors (pre-mRNAs) is accomplished by two separate cleavage–ligation reactions that occur in a large ribonucleoprotein complex known as the spliceosome (for recent reviews see Padgett *et al.*, 1986; Green, 1986; Krainer and Maniatis, 1988). The chemical reactions that bring about the excision of introns and the joining of exons appear to be simple transesterifications. A similar mechanism has been described for the splicing of the group II introns found in yeast mitochondria (Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986; van der Veen *et al.*, 1986). In this case a specific and highly conserved intron structure is responsible for both catalysis and alignment of the cleavage-ligation events. The heterogeneous nature of nuclear pre-mRNA structure implies that a more complex biochemistry determines the precision of splicing for this class of RNA.

The expected complexity of pre-mRNA splicing has been abundantly confirmed by analysis of the structure of the spliceosome. Spliceosomal constituents include the RNA components of the U1, U2, U4/6 and U5 small nuclear ribonucleoproteins (snRNPs); (Konarska and Sharp, 1986, 1987; Grabowski and Sharp, 1986; Pikielney and Rosbash, 1986; Pikielney *et al.*, 1986; Bindereif and Green, 1987; Kretzner *et al.*, 1987; Cheng and Abelson, 1987) and at least some of the proteins associated with the individual snRNPs (Grabowski *et al.*, 1985; Bindereif and Green, 1986, 1987; Kaltwasser *et al.*, 1986; Konarska and Sharp, 1986; Chabot and Steitz, 1987; Lossky *et al.*, 1987). The hnRNP C protein has also been shown to be associated with the spliceosome (Choi *et al.*, 1986). Other non-snRNP proteins have been implicated in the pre-mRNA splicing process (Furneaux *et al.*, 1985; Krämer and Keller, 1985; Krainer and Maniatis, 1985; Cheng and Abelson, 1986; Perkins *et al.*, 1986; Krämer *et al.*, 1987; Lin *et al.*, 1987). These proteins are likely to be components of the spliceosome or be required for its assembly and activity. Thus, the splicing of nuclear pre-mRNA requires the action in *trans* of a diverse collection of RNAs and proteins that function in concert in a large structure. The identification and characterization of these components is necessary for a more complete understanding of the mechanism of pre-mRNA splicing.

Most of the information on pre-mRNA splicing factors obtained to date has come from biochemical studies on *in vitro* splicing systems for both HeLa cell nuclear extracts and *Saccharomyces cerevisiae* whole cell extracts. The yeast system has demonstrated an additional advantage. A set of mutants designated *RNA2–RNA10/11* have been described that exhibit an inability to splice pre-mRNAs at an elevated temperature (Hartwell *et al.*, 1970; Rosbash *et al.*, 1981). These mutants have been used in biochemical studies to try to elucidate the components required at various stages in the pre-mRNA splicing pathway (Lustig *et al.*, 1986; Lin *et al.*, 1987; Cheng and Abelson, 1987). The mutations also highlighted genes involved in pre-mRNA splicing. Some of these genes have been cloned (Last *et al.*, 1984; Lee *et al.*, 1984; Soltyk *et al.*, 1984; Jackson *et al.*, 1988) and have been shown to encode proteins (Last and Woolford, 1986; Lee *et al.*, 1986; Jackson *et al.*, 1988). The *RNA2* and *RNA3* gene products are found within the nucleus (Last and Woolford, 1986) and the *RNA11* protein has been localized to the periphery of the nucleus (Chang *et al.*, 1988). *RNA8* and *RNA11* encode proteins that are associated with the spliceosome (Lossky *et al.*, 1987; Chang *et al.*, 1988). This combined genetic and biochemical approach is certain to lead to further progress in identifying pre-mRNA splicing factors and revealing their function.

We have begun an investigation of pre-mRNA splicing in the fission yeast *S.pombe*. In addition to being genetically well defined and amenable to modern techniques of genetic manipulation, *S.pombe* exhibits a number of characteristics of interest to pre-mRNA splicing. Compared to *S.cerevisiae*, genes containing introns appear to be more prevalent in *S.pombe* (approximately half of the sequenced genes) and several interrupted genes possessing multiple introns have

been described (Hindley and Phear, 1984; Hiraoka *et al.*, 1984; Hindley *et al.*, 1987; Barker *et al.*, 1987; Hirano *et al.*, 1988). In addition to these features that are reminiscent of vertebrates, the *S.pombe* U-RNAs are similar in size to their vertebrate homologs (Tollervey, 1987; Tollervey and Mattaj, 1987; Brennwald *et al.*, 1988a,b). At least one mammalian intron, that for the simian virus 40 small t antigen, has been shown to be spliced correctly in *S.pombe* (Käufer *et al.*, 1985); although attempts to splice other mammalian introns in *S.pombe* have been unsuccessful (tropomyosin; J.Potashkin, S.Erster, D.Helfman and D.Frendewey; Adenovirus E1a; J.Harper and R.Roberts, unpublished results).

Encouraged by the genetic contribution to the understanding of pre-mRNA splicing in *S.cerevisiae*, we have initiated a search for mutants defective in pre-mRNA splicing in *S.pombe*. We have collected a bank of *S.pombe* mutants that are temperature sensitive for growth. The mutants were screened for accumulation of pre-mRNA introns by a Northern blot assay. We have identified three mutants that exhibit a general defect in the splicing of pre-mRNAs at the non-permissive temperature. Genetic analysis indicates that the three pre-mRNA splicing mutants are members of separate complementation groups.

Results

Isolation of temperature sensitive mutants

A wild type strain of *S.pombe* was mutated with ethylmethanesulfonate (EMS) and ~100 temperature sensitive (ts^-) mutants were isolated by a dual temperature replica plating technique. Isolates which formed colonies at 23°C but grew slowly or were dead at 37°C were included in the *ts* bank. The parental wild type strain, 972, formed colonies at both temperatures. In order to optimize the method of mutagenesis, the quantity of EMS was varied in a series of experiments. The percentage killing increased as expected with increasing doses of EMS (data not shown). The lethality observed in these experiments ranged from 40 to 95% (Table I). To obtain the maximum number of ts^- mutants at the lowest lethality, a dose of EMS that produced 80% killing was used. Under these conditions, the ts^- mutants represented 0.2% of the survivors (Table I). This frequency of appearance of ts^- mutants is 5-fold less than what has been reported for nitrosoguanidine mutagenesis of *S.pombe* (Nurse *et al.*, 1976).

Screening the ts⁻ mutants for splicing defects

Each of the ts^- mutants was grown to mid-log phase at 23°C and then shifted to 37°C for 2 h. The generation time at 23°C for the ts^- mutants is approximately 5 h and does not differ substantially from a wild type strain grown at the same temperature. Total RNA was isolated from each mutant, fractionated on formaldehyde agarose gels and transferred to nylon membranes. The blots were probed with a 31mer oligonucleotide, I4 (see Materials and methods and Figure 1A), the sequence of which is complementary to the coding strand of the fourth intron of the $nda3^+$ gene of *S.pombe* (Hiraoka *et al.*, 1984), which encodes β-tubulin. The $nda3^+$ gene is one of ~12 cloned genes in *S.pombe* that are known to contain introns. The coding region of $nda3^+$ is interrupted by five introns (Hiraoka *et al.*, 1984). We chose β-tubulin for analysis of accumulation of intervening sequences because its transcript is relatively abundant.

Table I. Yield of ts^- mutants from ethylmethanesulfonate (EMS) mutagenesis.

% Killing	% ts^- mutants
40	0.05
50	0.08
70	0.16
80	0.20
95	0.20

The left-hand column indicates the degree of cell death obtained from several independent experiments in which the dose of mutagen was varied. % killing = 100 × [1− (number of survivors of EMS treatment ÷ number of cells before mutagenesis)]. The right-hand column indicates the proportion of ts^- mutants among the survivors of the mutagenesis. % ts^- = 100 × (number of ts^- colonies ÷ number of survivors).

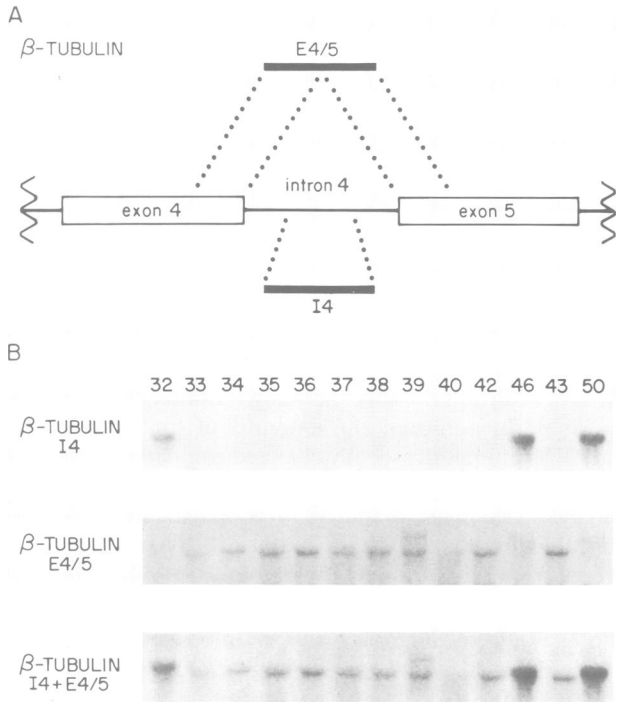

Fig. 1. Northern blot analysis of the ts^- mutants. **A**, oligonucleotides used as probes for the RNA blots. I4 is an intron specific probe which recognizes the fourth intron of *S.pombe* β-tubulin. E4/5 is an exon specific probe which recognizes 15 nt of exon 4 and 15 nucleotides of exon 5 of β-tubulin. **B**, Northern analyses of the ts^- mutants. Total RNA (20 μg/sample) was fractionated on a formaldehyde agarose gel and blotted onto nylon membranes. The blots were then probed with I4 to test for accumulation of pre-mRNA (**upper panel**). The probe was subsequently removed from the blots and the blots were rescreened with E4/5 (**center panel**). The **lower panel** is a composite of two autoradiographs, one showing the I4 signal and the other showing the E4/5 signal. Alignment for the composite was made using mol. wt markers (not shown). The I4 and E4/5 probed blots were autoradiographed for 3 days.

Wild type *S.pombe* cells showed no accumulation of pre-mRNA at the non-permissive temperature (data not shown). Northern analysis of total RNA prepared from the ts^- mutants grown at 37°C showed that *ts32*, *ts46* and *ts50* had accumulated the β-tubulin precursor (Figure 1B, upper panel). The I4 probe was removed from the RNA blots and the membranes were reprobed with an oligonucleotide, E4/5, that is complementary to 15 nucleotides (nt) of exon 4, and

15 nucleotides of exon 5 that are contiguous and span the splice junction between these two exons in the mRNA (Figure 1A). Preliminary experiments indicated that E4/5 was specific for spliced RNA under stringent hybridization conditions. E4/5 detected the mature β-tubulin transcript in all of the RNAs prepared from the *ts*$^-$ mutants except for *ts32*, *ts46* and *ts50* (Figure 1B, center panel). The E4/5 signal was confirmed with a β-tubulin antisense RNA probe (data not shown). The β-tubulin transcript has five small introns whose combined length is ~300 nt (Hiraoka *et al.*, 1984). This size difference was resolved on the formaldehyde agarose gels as shown in a composite exposure of the I4 and E4/5 films (Figure 1B, lower panel).

After the initial observation of pre-mRNA accumulation in *ts32*, *ts46* and *ts50*, each of these mutants was restreaked on YE plates and retested for temperature sensitive growth by replica plating at 37°C. Both *ts32* and *ts50* are dead at the non-permissive temperature; *ts46*, however, grows slowly at the elevated temperature, indicating a leaky mutation. Each of these three mutants was also examined by phase contrast microscopy after growth at both 23°C and 37°C. There are no apparent morphological changes in the mutants at either temperature compared to a wild type strain grown under the same conditions.

Pre-mRNA accumulation

To further characterize the temperature sensitivity of the three mutants with regard to their splicing defects, RNA was prepared from each mutant grown at 23°C and after growth at 37°C for 1 or 2 h and analyzed on Northern blots. When the intron specific probe I4 was used, there was no signal in the three splicing mutants at 23°C (Figure 2A). At 37°C, each of the mutants showed a rapid accumulation of β-tubulin pre-mRNA (Figure 2A). The kinetics of pre-mRNA accumulation were reproducible and differed slightly in each of the mutants. The amount of precursor appears to peak at 1 h in *ts32* and then decline; whereas, pre-mRNA levels remain the same in *ts46* and increase from 1−2 h in *ts50*. The upper band which appears in the *ts50* sample in the experiment shown in Figure 2A was not reproduced in several independent RNA preparations made from this mutant.

When the exon specific probe E4/5 was used to identify mature mRNA, a signal was observed at 23°C in each of the mutants (Figure 2B). The mRNA signal is completely absent by 1 h at 37°C in both *ts32* and *ts50*. For *ts46* there is a reduction in the amount of mature mRNA that is present at both 1 and 2 h after the shift to 37°C (Figure 2B). These results are consistent with the leaky *ts*$^-$ growth phenotype exhibited by *ts46*. The lower transcript detected with E4/5 in each of the mutants is background hybridization to rRNA (data not shown).

Because the above experiments were all done with total cellular RNA, it was unclear whether there may be a small amount of precursor RNA present at 23°C that was not detected. To enrich for mRNA, total RNA was prepared from cells grown at the permissive and non-permissive temperatures and a fraction of this RNA was selected on an oligo(dT)-cellulose column and analyzed by Northern blotting. The results of this experiment are shown in Figure 3. No pre-mRNA is present in *ts46* and *ts50* at 23°C. A very small amount of precursor was detected in *ts32* at the permissive temperature. Because the pre-mRNA from all

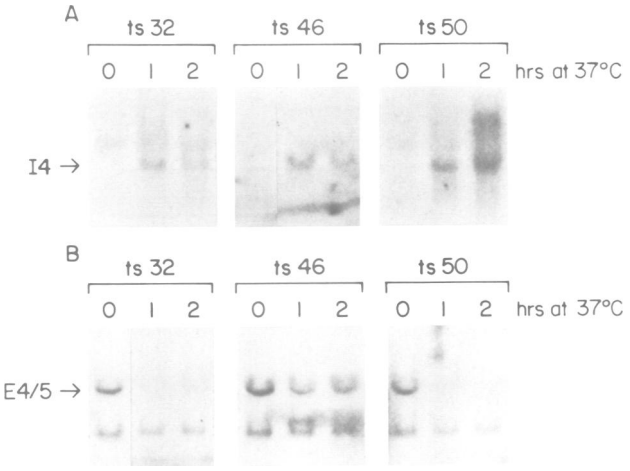

Fig. 2. Northern blot analysis of accumulation of β-tubulin pre-mRNA in *ts32*, *ts46* and *ts50*. Cells were grown at 23°C and shifted to 37°C for 0, 1 or 2 h. Total RNA (20 μg/sample) was analyzed by Northern blotting as in **Figure 1**. The blots were probed with I4, the intron specific probe (**panel A**) or E4/5, the exon specific probe (**panel B**). The I4 and E4/5 probed blots were autoradiographed for 3 days.

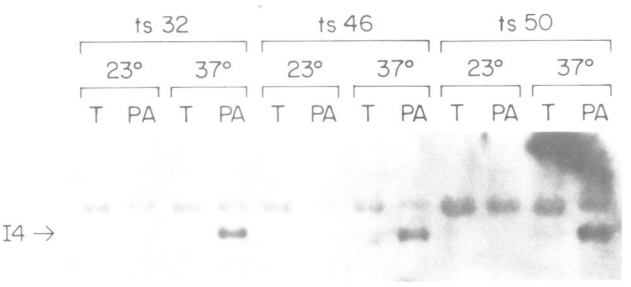

Fig. 3. Northern blot analysis of poly(A)$^+$ RNA from *ts32*, *ts46* and *ts50*. Total RNA was prepared from cells grown at 23°C or after shift to 37°C for 2 h. Part of each RNA preparation was selected on an oligo(dT)-cellulose column. Total RNA(T) or poly(A$^+$) selected RNA (PA); (5 μg/sample) was analyzed by Northern blotting as in **Figure 1**. The blot was probed with I4, the intron specific probe. The blot was autoradiographed for 3 days.

three mutants can be selected on an oligo(dT)-cellulose column, the block in splicing is not associated with, or the result of, a lack of polyadenylation. (The upper band on the blot is a background band which is present on some blots and not on others under stringent hybridization conditions). Therefore in *S.pombe*, as in mammals, polyadenylation can occur on an unspliced pre-mRNA (Nevins and Darnell, 1978). We have not tested in this experiment whether or not polyadenylation is occurring at the correct site. A similar analysis with a probe for the alcohol dehydrogenase mRNA, which lacks introns, showed that neither the size of the mRNA nor its ability to be selected on oligo(dT)-cellulose were affected by a temperature shift from 23°C to 37°C in the three mutants (data not shown).

Analysis of α-tubulin splicing

Since the results presented up to this point are based on the analysis of the splicing of the fourth intron of the β-tubulin pre-mRNA, the question remained whether the mutants were specific for this particular intron. On a statistical basis, it is highly unlikely that three mutations specific for a particular intron in a single copy gene would be isolated from a small

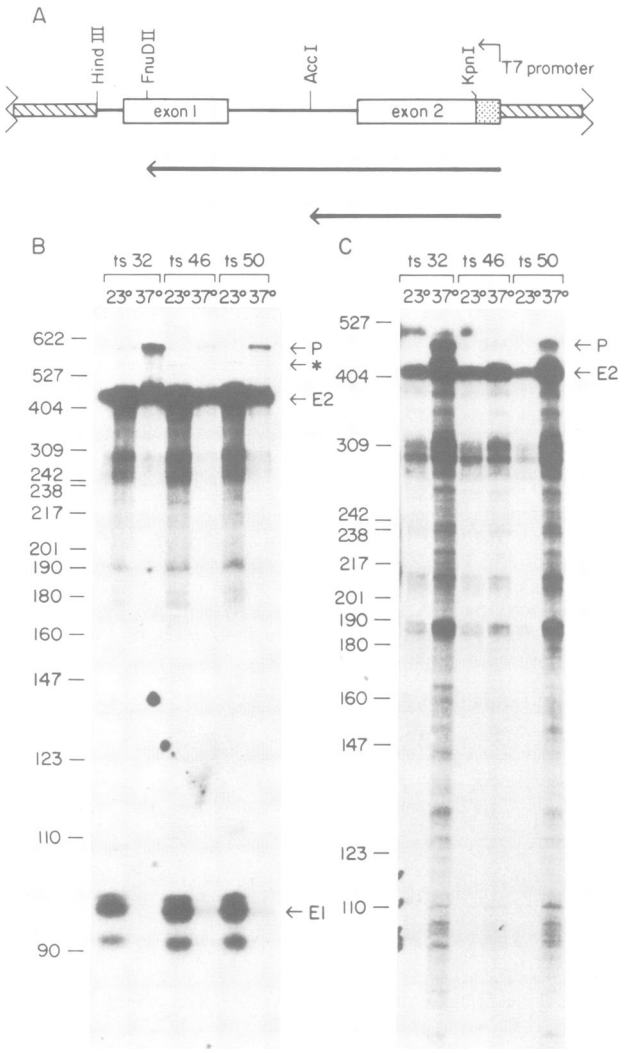

Fig. 4. Ribonuclease protection assays of α1-tubulin RNA in *ts32*, *ts46* and *ts50*. Two antisense RNAs which recognize the α1-tubulin transcript of *S.pombe* were prepared as described in Materials and methods. One antisense RNA prepared from a *Fnu*DII cut template recognizes exon 2, the intron and part of exon 1 as shown schematically in **panel A**. This probe was used in ribonuclease protection assays with total RNA prepared from *ts32*, *ts46*, and *ts50* grown at either 23°C or after shift to 37°C for 2 h (**panel B**). The other antisense RNA was prepared from an *Acc*I digested template and recognizes exon 2 and part of the intron as shown schematically in **panel A**. The results of the ribonuclease protection assays with this smaller antisense probe are shown in **panel C**. E1 is the exon 1 protected fragment, E2 is the exon 2 protected fragment, P is the pre-mRNA protected fragment and *indicates the fragment expected to be protected by the lariat intermediate. The gels were autoradiographed for 1 day.

collection of 100 ts^- mutants. In addition, the pre-mRNAs that accumulate are identical in size in the three mutants and the length is consistent with a pre-mRNA having all five introns. However, small differences in size may not have been resolved on the formaldehyde agarose gels. The recessive nature of the mutations (see below) suggests a lesion in a *trans*-acting factor as opposed to a *cis*-acting mutation (for example in a splice site) that would probably be co-dominant. Despite these lines of evidence, it was possible that one of the splicing mutants was specific for β-tubulin.

To more directly test the generality of the splicing defects in *ts32*, *ts46*, and *ts50* we investigated the processing of a second transcript, that from the *nda2*$^+$ gene. This gene encodes one of two α-tubulins in *S.pombe* (Toda *et al.*, 1984). The coding sequence of the α1-tubulin gene is interrupted by a 90 bp intervening sequence. For these analyses a ribonuclease protection assay was employed. A portion of the *nda2*$^+$ gene was subcloned into the vector Bluescribe M13 (Figure 4A) in an orientation such that an antisense RNA could be synthesized from the T7 promoter using T7 RNA polymerase. The plasmid contains the entire coding sequence of exon 1, the intron and part of exon 2. In one experiment, the antisense RNA was transcribed after digesting the DNA template with *Fnu*DII, which cleaves upstream of the 5′ splice site (Figure 4A). This RNA was used as a probe to detect RNase T1 and RNase A resistant fragments in total RNA prepared from each of the mutants when grown at either 23°C or after a shift at 37°C. If splicing occurs, two protected fragments were expected of 92 nt for exon 1 and 393 nt for exon 2. If splicing is blocked, a protected fragment of 574 nt complementary to exon 1, the intron and part of exon 2 was expected. RNA prepared from the mutants grown at 23°C showed two protected fragments indicative of spliced mRNA. In contrast, RNA prepared from cells that had been shifted to 37°C showed an accumulation of precursor RNA (Figure 4B). At 37°C there appears to be spliced product present. This may reflect the increased sensitivity of the RNase protection assay compared to the Northern procedure used to investigate the splicing of the β-tubulin pre-mRNA. Alternatively, the α1-tubulin mRNA may have a longer half-life at 37°C than the β-tubulin mRNA. In this case the spliced RNA that we detect at 37°C would be mRNA that was made before the temperature shift. In addition to the portion of the probe protected by pre-mRNA and exons 1 and 2, there is an RNA migrating below the pre-mRNA band at the expected position of an antisense RNA protected by the intermediate lariat. There appears to be no accumulation of this RNA at 37°C in the three mutants.

When the experiment was repeated with antisense RNA prepared from *Acc*I cleaved template, a fragment of 393 nt encoding exon 2 was expected if splicing occurs; a fragment of 448 nt was expected if splicing was blocked. The results (Figure 4C) are identical to those obtained with the *Fnu*DII probe. Splicing occurs in each of the mutants at 23°C and pre-mRNA accumulates at 37°C. These analyses confirm the results obtained with β-tubulin both qualitatively and quantitatively. For example, the block in splicing in the leaky mutant *ts46* is significantly less complete than in *ts32* or *ts50* for both β-tubulin (Figure 2) and α-tubulin (Figure 4) splicing. The splicing defect in all three mutants is therefore a general defect in pre-mRNA splicing that manifests its effect before the first cleavage−ligation reaction.

Analysis of other RNAs in ts32, ts46 and ts50

The results presented above concern effects on pre-mRNA splicing and mRNA levels in the ts^- mutants. It was possible that the synthesis or processing of other RNAs were defective in the three pre-mRNA splicing mutants. As an initial experiment we compared the whole cell RNA profiles for the wild type and the three splicing mutants. Total RNA was prepared from *ts32*, *ts46*, *ts50* and wild type cells that had been grown to mid-log at 23°C and then shifted to 37°C for 2 h. Equal amounts of each RNA (as determined by

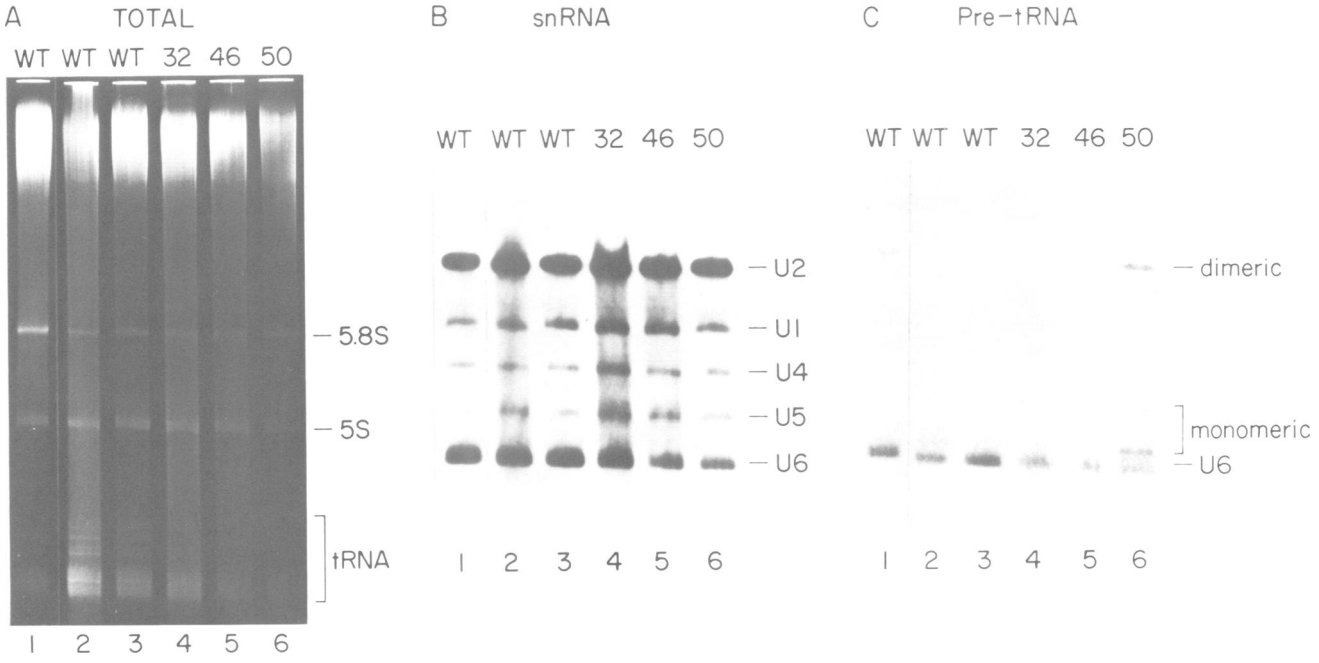

Fig. 5. Northern blot analysis of snRNAs and pre-tRNA in *ts32*, *ts46* and *ts50*. Total RNA (20 μg/lane) from wild type (WT) or mutant cells grown to mid-log at 23°C and then shifted to 37°C for 2 h was fractionated on a polyacrylamide/urea gel. **A**, ethidium bromide stained RNAs. The gel was stained with ethidium bromide and exposed to UV irradiation to visualize the RNAs. **B**, snRNAs. The gel shown in **A** was electroblotted and hybridized with probes specific for U1, U2, U4, U5 and U6 RNAs (see Materials and methods). The snRNAs were visualized by autoradiographing for 24 h. **C**, pre-tRNAs. The snRNA probes were removed and the blot was reprobed with an oligonucleotide specific for the intron of the $sup9^+$ pre-$tRNA^{Ser}$. The pre-tRNAs were visualized by autoradiographing for 3 days.

absorbance at 260 nm) were fractionated on a 10% polyacrylamide/8 M urea gel. The gel was stained with ethidium bromide and the RNAs were visualized by fluorescence under UV irradiation and photographed. Figure 5A shows that although there were small variations in the quantities of RNA loaded (compare the three wild type samples in lanes 1–3), the total RNA patterns for the wild type and the mutants are essentially indistinguishable. In particular, the amounts and positions of the 5.8S and 5S rRNAs and tRNA are the same in all samples. We have not examined the large rRNAs.

Because U1, U2, U4, U5 and U6 RNAs are required for pre-mRNA splicing, the mutants were also analyzed for alterations in the amounts of these snRNAs. The RNAs displayed in Figure 5A were electroblotted onto a nylon membrane. The Northern blot was hybridized with probes complementary to human U1, U2 and U6 RNAs, *S.pombe* U4 RNA and *S.cerevisiae* U5 RNA (see Materials and methods). These probes each recognize RNAs of the expected sizes (Tollervey, 1987; Brennwald *et al.*, 1988a,b; and compared with the human snRNAs) in RNA prepared from *S.pombe* snRNPs, which were purified by immunoaffinity chromatography with an antibody specific for the trimethylguanosine cap structure found on most snRNAs (A.Krainer and D.Frendewey, unpublished results). As heterologous probes were used in this analysis, the intensities of the hybridization signals may not reflect the true absolute or relative abundances of the *S.pombe* snRNAs. The snRNA pattern in the three ts^- mutants is essentially identical to that observed for the wild type (Figure 5B). However, the amount of U6 RNA appears to be slightly reduced in each of the mutants. We have observed this reduction with three independent RNA preparations. The pre-mRNA splicing defects exhibited by *ts32*, *ts46* and *ts50* are, therefore, not due to large changes in the concentrations of the spliceosomal snRNAs.

To investigate whether the defects in *ts32*, *ts46* and *ts50* were specific for pre-mRNA splicing, we also assayed the three mutants for defects in pre-tRNA splicing. The Northern blot that was used to obtain the results shown in Figure 5B was stripped and reprobed with an oligonucleotide that recognizes the intron of the $sup9^+$ $tRNA^{Ser}$ precursor. (Some of the U6 probe remained on the blot.) Mature $tRNA^{Ser}$ is produced from a dimeric precursor consisting of $tRNA^{Ser}$ sequences joined to $tRNA^{Met}$ (Willis *et al.*, 1984). The processing of the dimeric precursor includes cleavage to produce separate pre-$tRNA^{Ser}$ and pre-$tRNA^{Met}$, trimming of 5′ and 3′ flanking nucleotides, addition of CCA to the 3′ ends and removal of the intron from pre-$tRNA^{Ser}$ by splicing (Willis *et al.*, 1984, 1986). The $sup9^+$ probe detects two sets of RNAs (Figure 5C). The larger group represents the various forms of the dimeric precursor. The smaller RNAs identified by the probe are unspliced $tRNA^{Ser}$ precursors. Since splitting of the dimeric precursor and removal of the flanking sequences normally occurs before splicing (Deutscher, 1984), a mutant that is defective in pre-tRNA splicing would be expected to accumulate the smallest of the unspliced $tRNA^{Ser}$ precursors. As seen in Figure 5C, the steady state levels of $sup9^+$ precursor transcripts are low and the signal is variable in RNA from wild type cells (Figure 5C, lanes 1–3). The amounts and relative abundances of the various $sup9^+$ RNAs observed in *ts32*, *ts46* and *ts50* (Figure 5C, lanes 4, 5 and 6) are not significantly different from the wild type pattern. In *ts50* all of the pre-tRNAs detected by the $sup9^+$ probe appear to be slightly elevated compared to the wild type. However, the unspliced pre-$tRNA^{Ser}$ monomer (the smallest of the monomeric species) is not increased relative to the other pre-tRNA

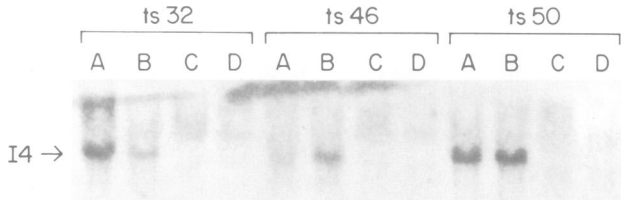

Fig. 6. Northern analysis of the progeny of each of four spores from a single tetrad of *ts32*, *ts46* and *ts50*. *ts32*, *ts46* and *ts50* were each mated to a wild type strain and allowed to undergo meiosis. Tetrads were dissected and total RNA was prepared from the progeny of each of the four spores (**A, B, C** and **D**) after shift to 37°C for 2 h. RNA was blotted and probed with I4, the intron specific probe as in **Figure 1**. The blot was autoradiographed for 3 days.

species. Therefore, pre-tRNA splicing is not specifically blocked in *ts50* and tRNA processing in general is not drastically affected in the three pre-mRNA splicing mutants.

Genetic analysis

Each of the three ts^- mutants was crossed with an h^+ *ade6-M210* strain of *S.pombe* and the resulting tetrads were dissected. The segregation of the ts^- and ade^- phenotypes was followed by replica plating onto minimal medium at 23°C and 37°C. Analysis of 12 tetrads for each mutant showed that they exhibited 2:2 segregation of the ts^- phenotype. Each mutant was backcrossed three times to a wild type strain carrying an ade^- marker and the tetrad analysis was repeated to confirm the initial observation. These results indicate that the ts^- mutations are within single genes.

After backcrossing, cells originating from each of the four spores in a single tetrad were grown at 23°C to mid-log phase and then shifted to 37°C for 2 h. Total RNA was prepared from each culture and analyzed on Northern blots for the accumulation of precursor RNA. Figure 6 shows that the ts^- phenotype and the splicing defect correlate in all three mutants and segregate 2:2. Therefore, for each of the mutants *ts32*, *ts46* and *ts50*, the ts^- phenotype is the result of a mutation in a single nuclear gene required for proper pre-mRNA splicing.

Each of the mutations was tested for dominance by crossing strains carrying complementing ade^- alleles of opposite mating type. For example, an h^+ ts^-*ade6-M210* strain was mated to an h^- *ade6-M216* strain. Diploids were selected on minimal medium at 23°C and then streaked to YE plates and grown at 37°C. Some diploids were also sporulated at 23°C and tetrads were dissected. The progeny were replica plated to minimal plates and grown at 23°C and 37°C to confirm the genotypes of each of the parental strains in the diploids. In all three crosses the diploids were not temperature sensitive when grown at 37°C. Whole cell RNA was prepared from diploids after they were grown at 37°C. (Cells were examined by phase contrast microscopy and compared to haploid parental cells grown under the same conditions to confirm that they were diploid). The RNA prepared from the diploids was examined by Northern analysis using both I4 and E4/5 as probes. No accumulation of β-tubulin pre-mRNA was detected with the I4 probe, while the E4/5 oligonucleotide confirmed the presence of β-tubulin mRNA in the diploids (data not shown). Both the ability of the diploids to grow at 37°C and the absence of accumulation of pre-mRNA at the elevated temperature indicate that all three mutations are recessive.

To determine whether or not *ts32*, *ts46* and *ts50* carried mutations in the same or separate genes, each strain carrying ts^- and *ade6-M210* mutations was mated pairwise to each of the other two ts^- mutants carrying the complementing *ade6-M216* mutation. The resultant diploids were streaked onto YE plates and incubated at 37°C in order to check for the ts^- growth defect. In each case, colonies were present when each of the three ts^- mutants was crossed with a different ts^- mutant. When each of the ts^- mutants was crossed with itself as a control, the diploid exhibited the ts^- growth defect. Overall, the results indicate that *ts32*, *ts46* and *ts50* complement each other and therefore represent three different genes. Following the yeast nomenclature for pre-RNA processing mutants agreed upon at the recent Cold Spring Harbor RNA processing meeting, and based on the chronology of the results obtained in our laboratory, we have named *ts50*, *ts32* and *ts46* respectively *prp1*, *prp2* and *prp3*.

Discussion

As part of our investigation of pre-mRNA splicing in the fission yeast *S.pombe*, we have initiated an effort to identify genes involved in pre-mRNA splicing by isolating mutants that are defective in this essential nuclear process. We have produced a collection of ~100 ts^- mutants by ethylmethanesulfonate mutagenesis of a wild type *S.pombe* strain. The ts^- mutants were selected for slow growth or death at 37°C. The ts^- bank was screened for mutants defective in pre-mRNA splicing by a Northern blot assay designed to detect accumulation of intron sequences. Three of the ts^- mutants, *ts32*, *ts46* and *ts50*, accumulated the precursor for the β-tubulin mRNA at the non-permissive temperature (Figure 1). These three mutants also had elevated levels of the α1-tubulin pre-mRNA (Figure 4). The mRNAs for the α1 and β-tubulins are abundant transcripts in *S.pombe*, which allowed for the detection of their pre-mRNAs in the splicing mutants. Attempts to assay for the accumulation of pre-mRNAs of less abundant mRNAs by the Northern blot procedure were unsuccessful. However, the fact that the splicing of the pre-mRNAs for both α1- and β-tubulin was impaired in *ts32*, *ts46* asnd *ts50* strongly indicates that the splicing defects are general. That is, the mutations are not specific for a particular pre-mRNA.

Each of the three pre-mRNA splicing mutants is blocked at an early step in the splicing pathway, before the first cleavage–ligation reaction, since extensive accumulation of splicing intermediates was not observed (Figure 4). This is also the case for the *S.cerevisiae* splicing mutants *RNA2–RNA10/11*. These mutants are all defective in spliceosome formation (Lin *et al.*, 1987). Most do not assemble splicing complexes *in vitro* at an elevated temperature. In one mutant, *RNA2*, an incomplete splicing complex is formed that is unable to cleave and ligate the pre-mRNA. A similar analysis of the *S.pombe* mutants will have to await the development of a homologous *in vitro* splicing system.

Slight differences in the molecular phenotypes of the *S.pombe* pre-mRNA splicing mutants were observed. Mutants *ts32* and *ts50* are completely blocked in pre-mRNA splicing at 37°C. Consistent with its weak ts^- growth phenotype, *ts46* allows some formation of mRNA (Figure 2). In

addition, the kinetics of precursor accumulation are slightly different for each of the three mutants (Figure 2).

The precursors that accumulate in *ts32*, *ts46* and *ts50* can be selected on an oligo(dT)-cellulose column (Figure 3), which indicates that they are polyadenylated. Although we do not know if polyadenylation occurs at the correct site and is the normal length, this result is a preliminary indication that polyadenylation is not defective in the three splicing mutants. Thus, the block in pre-mRNA splicing in the mutants is not the result of an inhibition of polyadenylation. In *S.pombe*, pre-mRNAs that are not spliced are apparently substrates for polyadenylation, as was previously observed in a mammalian system (Nevins and Darnell, 1978). It remains to be shown whether an independent block in polyadenylation will affect pre-mRNA splicing.

Since the U1, U2, U4, U5 and U6 RNAs have been shown to be required for pre-mRNA splicing, we investigated the possibility that *ts32*, *ts46* and *ts50* might have reduced levels of one or more of these RNAs. Northern blot analysis indicated that the amounts of the U1, U2, U4 and U5 RNAs present at 37°C in the three splicing mutants were normal (Figure 5A). A slight reduction in the concentration of U6 RNA was observed in each of the mutants. We do not know whether this reduction is a cause or an effect of the pre-mRNA splicing defects. However, results obtained with another mutant that we have isolated (to be described elsewhere) indicate that when the amount of U6 is the same or less than that seen in Figure 5B, pre-mRNAs do not accumulate to an extent detectable by Northern analysis. It is our conclusion, therefore, that the splicing defects are not explained by changes in the levels of the essential spliceosomal snRNAs, including the small decrease in the U6 levels shown in Figure 5B. We have not eliminated the possibility that the function of the snRNPs may be impaired in the mutants at the non-permissive temperature.

The results discussed above suggest that the defects in *ts32*, *ts46* and *ts50* are specific for the splicing of mRNA precursors. To further investigate the question of specificity, the three pre-mRNA splicing mutants were also assayed for accumulation of unspliced pre-tRNA precursors. In *S.pombe* the only RNAs known to have introns are pre-mRNAs and pre-tRNAs. The three ts^- mutants exhibited no obvious defects in the splicing or overall processing of their pre-tRNAs (Figure 5C). In addition, a comparison of the RNAs stained with ethidium bromide in the wild type and the three mutants indicates no substantial differences in the concentrations of the 5.8S and 5S rRNAs and tRNA (Figure 5A). To summarize the molecular analyses, *ts32*, *ts46* and *ts50* are blocked in the splicing of pre-mRNAs and these defects are not due to lack of polyadenylation or gross reductions in the steady state concentrations of the major snRNAs.

We have also analyzed the three splicing mutants genetically. After three backcrosses to the wild type strain, the defect in pre-mRNA splicing in each of the mutants segregates 2:2 and correlates with the ts^- growth defect (Figure 6). The mutations are recessive, indicating that they are probably in genes that encode factors that act in *trans* as opposed to point mutations in the splice sites or recognition sequences of individual introns. This genetic result confirms the molecular analysis that demonstrated that the mutations are general pre-mRNA splicing defects. Crosses among the three splicing mutants places them in separate complementation groups. We have therefore assigned *ts50*, *ts32* and *ts46* the names *prp1*, *prp2* and *prp3*, respectively, indicating mutations in pre-RNA processing. In summary, the mutations in *prp1*, *prp2* and *prp3* are most likely lesions in single nuclear genes that are required for both pre-mRNA splicing and growth.

It is our hope that *prp1*, *prp2* and *prp3* will be used in future studies to aid in the elucidation of the mechanism of pre-mRNA splicing. The cloning of the wild type genes that are defective in these strains will shed light on the nature of some of the factors essential for pre-mRNA splicing in *S.pombe*. Ultimately, a structural comparison between the factors required in the human, *S.cerevisiae* and *S.pombe* systems will inform us about the generality and conservation of the splicing components.

Materials and methods

Yeast strains and genetic analysis

All *S.pombe* strains used in this study were derived from strains 972 (h^-) or 975(h^+) introduced by U.Leupold. The following strains were used: h^- *ade6-M216* and h^+ *ade6-M210*. The standard genetic procedures for *S.pombe* described by Gutz *et al.* (1974) were used in this study.

Media and growth conditions

Yeast were grown in YE medium (0.5% yeast extract, 3% glucose), PM (*S.pombe* minimal) medium, which is a modification of EMM2 medium (Mitchison, 1970; Nurse, 1975) or PMA medium, which in addition contains 75 mg/l of adenine. To assist the identification of the ts^- lethals, phloxin B, which readily stains dead cells, was added to the PM and PMA medium.

Wild type strains of yeast were grown at 30°C. Temperature sensitive mutants were grown at 23°C for the permissive temperature and 37°C for the non-permissive temperature. These temperatures were chosen to create a wide difference in the optimal growth temperature of the mutants.

Isolation of temperature sensitive mutants

The wild type strain 972 was mutated with ehtylmethanesulfonate (EMS, Aldrich) by the method described for *S.cerevisiae* (Sherman *et al.*, 1986). After EMS treatment, cells were allowed to recover overnight in YE medium at 23°C with good aeration. The following day, cells were plated at a concentration of 100 to 200 cells per plate on YE plates and incubated at 23°C until colonies were 2–3 mm in diameter. Duplicate replicas were plated on YE plates containing phloxin B. One set was incubated at 23°C, while the other set was grown at the non-permissive temperature, 37°C. Colonies that grew well at 23°C but grew slowly or were dead at 37°C were included in the ts^- bank.

Preparation of RNA

To prepare total RNA from each of the mutants, cells were grown to mid-log phase at 23°C. An aliquot of 5 ml was shifted to 37°C for 2 h. Cells were harvested, washed once with distilled water and frozen at −20°C. RNA was extracted from the cells by disruption with glass beads followed by phenol/chloroform/isoamylalcohol extractions (Nischt *et al.*, 1986). 100–200 μg of RNA could readily be obtained by this method.

When larger quantities of RNA were required, 500 ml of mid-log phase cells were either grown at 23°C (for the control) or shifted to 37°C (the non-permissive temperature for the ts^- mutants) for 2 h. Cells were harvested and washed once in distilled water. The pellet was resuspended in 1 ml of cold breaking buffer (0.32 M sucrose, 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 0.5 mg/ml heparin) and an equal volume of sterile cold glass beads (Braun, 0.45–0.5 mm diameter) was added. The cells were broken by vortexing for 90 s. The volume was then brought to 20 ml by the addition of buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% SDS and 0.5 mg/ml heparin. An equal volume of phenol was added and the mixture was vortexed vigorously. The aqueous phase was extracted again with phenol at 65°C for 10 min during the second extraction. The second aqueous phase was chloroform extracted. RNA was precipitated by the addition of LiCl to a final concentration of 0.5 M and an equal volume of ethanol. 1–5 mg of RNA could be obtained using this procedure.

Polyadenylated RNA was separated from 1–5 mg of non-polyadenylated RNA by chromatography on an oligo(dT)-cellulose column as previously

described (Maniatis *et al.*, 1982) except that the 0.1 M NaCl wash was omitted after the RNA sample was applied to the column. Poly(A)$^+$ RNA was then eluted from the column with 10 mM Tris−HCl (pH 7.5), 1 mM EDTA.

Northern analysis

Total or polyadenylated RNA was fractionated on formaldehyde agarose gels as described (Maniatis *et al.*, 1982). RNA was then transferred to Gene Screen Plus (New England Nuclear) by capillary blotting as described by the manufacturer. For analysis of the snRNAs, total RNA was electrophoresed on a 0.15 cm × 20 cm × 20 cm 10% polyacrylamide (19:1, acrylamide:bisacrylamide)/8 M urea gel. The gel was stained in a Tris−acetate, EDTA buffer containing 0.5 μg/ml of ethidium bromide for 5 min. RNAs were visualized by fluorescence on a UV transilluminator and photographed with polaroid type 55 film for 90 s at f8. For Northern analysis the RNAs were electroblotted onto Gene Screen Plus in 10 mM Tris−acetate (pH 7.8), 5 mM sodium acetate, 0.5 mM EDTA for 16 h at 20 V. Blotted RNA was hybridized with oligonucleotide probes. Prehybridization was done for 2 h in 6 × SSPE (1 × SSPE contains 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, and 1 mM EDTA), 0.1% SDS, 50 μg/ml denatured salmon sperm DNA and 100 μg/ml of 25 mM homochromatography mix RNA, (Silberklang *et al.*, 1979). The prehybridization buffer was removed completely and the membranes were then hybridized overnight in 5 ml 6 × SSPE, 0.1% SDS containing 1 × 10^6 d.p.m. of the probe. The blots were washed in an excess of 6 × SSPE, 0.1% SDS at room temperature. In general, prehybridization and hybridization was done at Tm−10°C in a Bachofer rotisserie oven (Bachofer, Reutlingen, FRG). Blots were exposed to Kodak X-ray film at −70°C with an intensifying screen for the times indicated in the figure legends.

Probes were removed from blots by washing blots in four changes of 0.01 × SSC and 0.01% SDS at 70°C for a total of 2 h. Blots could be reprobed at least four times with little or no loss of RNA.

Preparation of probes for Northern analysis

The I4 and E4/5 oligonucleotides were synthesized on the Applied Biosystems 380-A DNA synthesizer, then gel purified and passed through a Sep-Pak (Lo *et al.*, 1984). To prepare the oligonucleotides for use as probes for the Northern blots, they were 5′ end-labeled using [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase (Maniatis *et al.*, 1982). Labeled DNA was separated from unincorporated [γ-^{32}P]ATP by centrifugation through a Sephadex G-50 column (Maniatis *et al.*, 1982). The following oligonucleotides were used in this study: β-tubulin I4 (31mer), 5′-GTTT-TAAGCAACCGAACACTTGTAAGAAAGC-3′; β-tubulin E4/5 (30mer), 5′-CTTTCCACCAGCGGCCTCGTTAAAATAAAC-3′; U1 (14 mer), 5′-TGCCAGGTAAGTAT-3′, complementary to nucleotides (nts) 1−14 of human U1 RNA (Krainer and Maniatis, 1985); U2 (15mer), 5′-CAGATACTACACTTG-3′, complementary to nts 28−42 of human U2 RNA (L15 of Black *et al.*, 1985; gift of N.Hernandez, Cold Spring Harbor); U4 (17mer), 5′-AGTTTTCAACTAGCAAT-3′, complementary to nts 56−72 of *S.pombe* U4 RNA (D.Tollervey, personal communication); U5 (15mer), 5′-CTGGTAAAAGGCAAG-3′, complementary to nts 90−104 of *S.cerevisiae* snR7 RNA (Patterson and Guthrie, 1987); *sup9*$^+$ intron (26mer), 5′-TAGATGACTAGAATACAGGATTCAAG-3′ (gift of I.Willis and D.Söll, Yale). The U6 probe was a T7 antisense RNA produced from a clone of the human U6 RNA gene (gift of N.Hernandez, Cold Spring Harbor).

Bacterial plasmid construction

The 1.8 kb *Hin*dIII fragment of pDB(NDA2)1 (a gift of M.Yanagida) carrying the α1-tubulin gene (*nda2*, Toda *et al.*, 1984) was inserted into the *Hin*dIII site of the plasmid Bluescribe M13 (Stratagene) to produce pBSα1. pBSα1 was cut with *Kpn*I and then religated to remove a 723 bp fragment at the 3′-end of the α1-tubulin insert. The result was pBSα1.1, which contains all of exon 1, the entire intron and part of exon 2 (see Figure 5A).

Synthesis and preparation of complementary-strand probes

pBSα1.1 was linearized with *Acc*I or *Fnu*DII, phenol extracted and ethanol precipitated prior to transcription. T7 RNA polymerase (New England BioLabs) and [α-^{32}P]UTP (New England Nuclear) was used to prepare ^{32}P-labeled antisense RNA under the conditions recommended by the manufacturer. One μg of plasmid DNA was used per reaction. The transcription mix was treated with 20 μg/ml of RNase-free DNase (Promega Biotec) for 15 min at 37°C. Antisense RNA was then phenol extracted, chloroform extracted and ethanol precipitated. To gel purify the probe, the RNA was run on a 4% polyacrylamide/urea sequencing gel (Maxam and Gilbert, 1980). The gel was exposed to Kodak X-ray film for 1 min and the band was cut out of the gel and eluted in 2 M ammonium acetate, 0.1% SDS for 3−4 h at 37°C. The antisense RNA was then ethanol precipitated and used immediately in ribonuclease protection assays.

Ribonuclease protection assays

RNase protection assays were done with ^{32}P-labeled T7 RNA polymerase transcripts under conditions previously described (Zinn *et al.*, 1983). The RNA samples were fractionated on 8% polyacrylamide/urea sequencing gels (Maxam and Gilbert, 1980). Gels were exposed to Kodak X-ray film for 1 and 3 days at −70°C with an intensifying screen.

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Note added in proof

While this paper was in proof, we became aware of the discovery by Tani and Ohshima [Tani,T. and Ohshima,Y. (1989) *Nature*, **337**, 87–90] of a pre-mRNA-like intron in the gene for the *S.pombe* U6 RNA. A block in the splicing of the U6 preRNA would explain the reduction in U6 RNA that we observed in the three splicing mutants (Figure 5B). We have recently obtained evidence that confirms this explanation. (J.Potashkin and D.Frendewey, manuscript in preparation).