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Splicing of the U6 RNA precursor is impaired in fission yeast pre-mRNA splicing mutants

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ABSTRACT

U6 RNA is a member of a class of small abundant stable nuclear RNAs that are essential for splicing. In all species examined so far, the U6 RNA is a RNA polymerase III transcript. The U6 gene of the fission yeast *Schizosaccharomyces pombe* is unusual in that it is interrupted by an intron whose structure is similar to those found in pre-mRNAs. As part of our previous analysis of three *S. pombe* temperature sensitive pre-mRNA splicing mutants we examined their spliceosomal snRNA content. In contrast to the other snRNAs, the amount of U6 RNA is reduced at the restrictive temperature in all three of the mutants compared to the wild type. To investigate the cause of this reduction we have analyzed the efficiency of splicing of the U6 RNA precursor (U6 pre-RNA) in the pre-mRNA splicing mutants. At the restrictive temperature the ratio of unspliced U6 precursor to mature RNA is elevated in the mutants compared to the wild type grown under identical conditions, indicating a defect in U6 pre-RNA splicing. In this regard, the U6 RNA precursor behaves similarly to pre-mRNAs. Unspliced U6 pre-RNA was also detected in wild type cells under certain growth conditions.

INTRODUCTION

U6 RNA is a member of a class of small nuclear RNAs (snRNAs) found in all eukaryotic cells. The snRNAs associate with proteins in small nuclear ribonucleoprotein particles (snRNPs), usually with one snRNA per snRNP. The exception is the U4/U6 snRNP, which contains both U4 and U6 RNAs (1,2) held together by base pairing (3,4). The U6 RNA, along with the U1, U2, U4 and U5 RNAs, is required for the splicing of mRNA precursors (pre-mRNAs) and is a constituent of the spliceosome (reviewed in refs. 5–8). Studies of splicing complex assembly *in vitro* suggest that the U4 and U6 RNAs dissociate at the same time as or just prior to the cleavage/ligation reaction at the 5′ splice site (9–12). This results in the release of the U4 RNA, probably as a ribonucleoprotein (RNP), while the U6 RNA remains in the spliceosome through the cleavage/ligation at the 3′ splice site and is eventually found in a RNP complex with U2 and U5 RNAs on the excised intron.

Several properties of the U6 RNA set it apart from the other snRNAs. In vertebrates the U6 RNA has been shown to have a 5′ 'cap' (13) that is different from the characteristic 2,2,7-trimethylguanosine (m_3G) cap found on most other snRNAs (14). Also, despite similarities in promoter structures (15–17), vertebrate U6 RNAs are transcribed by RNA polymerase (Pol) III; whereas, the other snRNAs are transcribed by Pol II (18) . The sequence of the U6 RNA of the budding yeast *Saccharomyces cerevisiae* is very similar to the vertebrate homologs (4) and suggests that it is transcribed by Pol III. In the fission yeast *S. pombe* the U6 RNA is also likely to be a Pol III transcript because its gene has a T/A rich sequence in the 5′ flanking region and its 3′ end maps to a stretch of T residues (19). Surprisingly, however, the *S. pombe* U6 gene is interrupted by an intron whose

structure is typical of introns found in fission yeast pre-mRNAs (19). No other snRNA gene from any source has been shown to possess an intron, including the U2, U3, and U4 RNAs of *S. pombe* (20,21,22).

We recently isolated three *S. pombe* temperature sensitive (*ts*$^-$) pre-mRNA splicing mutants (*prp1*, *prp2* and *prp3*) (23). An analysis of the spliceosomal snRNA content in the *prp*$^-$ mutants indicated normal amounts of U1, U2, U4 and U5 RNAs. However, the steady state level of U6 RNA was slightly reduced in *prp1*, *prp2* and *prp3*, compared to the wild type, after a short period at the nonpermissive temperature (23) . The fact that the U6 intron is structurally identical to those of pre-mRNAs (19) suggests that the U6 RNA precursor might be spliced by the same mechanism as pre-mRNAs. It would then follow that the reduction in U6 RNA that we observe in the *prp*$^-$ mutants is the result of a defect in the splicing of the U6 pre-RNA. To test these hypotheses we have investigated the splicing of the U6 pre-RNA in *prp1*, *prp2* and *prp3*. Our results show a temperature sensitive accumulation of unspliced U6 pre-RNA and an elevated ratio of precursor/mature U6 RNA in the *prp*$^-$ mutants compared to the wild type. Thus, splicing of the U6 pre-RNA is impaired in the pre-mRNA splicing mutants.

MATERIALS AND METHODS

Yeast strains and growth conditions

All *S. pombe* strains used in this study were derived from strains 972 (h^-) and 975 (h^+) introduced by U. Leupold. The temperature sensitive pre-mRNA splicing mutants have been described (23). Yeast were grown in YE medium (0.5% yeast extract, 3% glucose) at 23°C for the permissive temperature or 37°C for the nonpermissive temperature.

Oligodeoxonucleotides

U6-SC, 5′-TC(A/T)TCTCTGTATTG-3′, is derived from the sequence of the *S. cerevisiae* U6 gene (4) and is complementary to nts 31−44 of *S. pombe* U6 RNA with one mismatch at position 32. The other oligonucleotides are derived from the sequence of the *S. pombe* U6 gene and are listed with their sequences and the nucleotides to which they are complementary within the U6 pre-RNA (19): U6I-1, 5′-GACTCGAACCTTGGTAA-3′, 61−77; U6I-2, 5′-GGTAAATATTGTTACTTAC-3′, 47−65; U6E2-1, 5′-GTCATCCTTGTGCAGGG-3′, 105−121; and U6E2-2, 5′-TTTCTCTCAA-TGTCGCAGTG-3′, 121−140. The oligonucleotides were synthesized and 5′ end-labeled with [γ-^{32}P]ATP as described (23).

Antisense RNA probes

The U6 antisense RNA probe was prepared by transcription of the human U6 gene (clone kindly provided by G. Kunkel) with T7 RNA polymerase (New England BioLabs) and [α-^{32}P]UTP (New England Nuclear) under the conditions recommended by the manufacturer.

Northern analysis

RNA was extracted from the cells by disruption with glass beads followed by phenol/chloroform/isoamylalcohol extractions (24) and ethanol precipitation. Total RNA was fractionated on 10% polyacrylamide (19:1, acrylamide:bisacrylamide)/8 M urea gels. RNA was then electroblotted (Hoeffer Scientific Industries) to Gene Screen Plus (New England Nuclear) as described by the manufacturer. RNA blots were hybridized with either RNA or oligonucleotide probes and washed as described (23).

Primer extension assay

Total RNA (20 μg) and the appropriate oligonucleotide (1×10^6 dpm, approximately 2

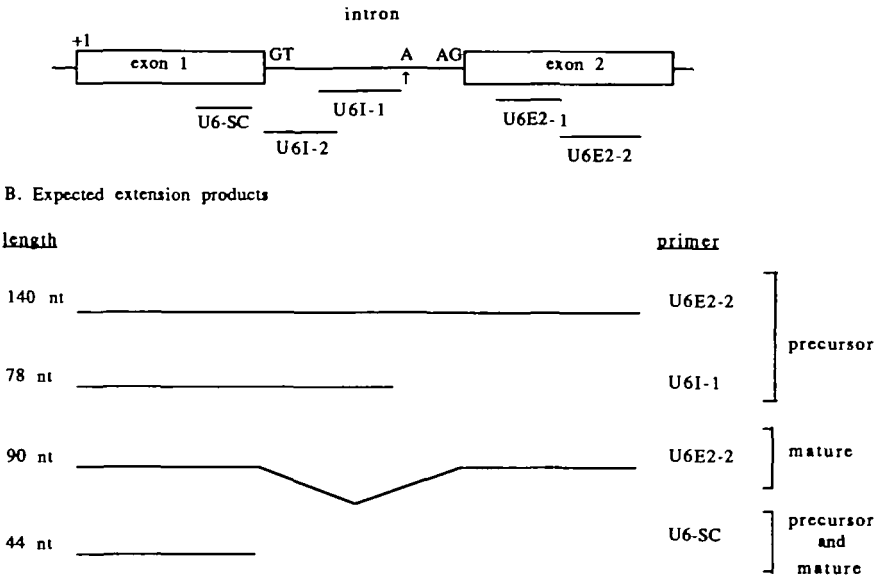


Figure 1. Probes used in northern blot analysis and primer extensions. A. Oligonucleotides used for probes. U6-SC is derived from the sequence of the *S. cerevisiae* U6 gene (4) and recognizes 14 nts within exon 1 of the *S. pombe* transcript. U6I-1 and U6I-2 are intron-specific probes that recognize 17 and 19 nts, respectively, of the intron of the *S. pombe* U6 pre-RNA. U6E2-1 and U6E2-2 are exon specific probes that recognize 17 and 20 nts, respectively, of exon 2 of *S. pombe* U6 RNA. The arrow marks the expected branch point. The U6I-1 probe should hybridize just upstream of this site. B. Diagrammatic representation of the extension products expected from mature or precursor RNA when U6I-1, U6E2-2 or U6-SC are used as primers.

pmol) were heated in 10 μl of 100 mM Tris (pH 7.5), 150 mM KCl and 6 mM $MgCl_2$ at 85°C for 10 min, followed by a gradual lowering of the temperature to 37°C over a period of 1 hr and then incubation at this temperature for 30 min. An equal volume of reverse transcription mix (1 mM dATP, 1 mM dTTP, 1 mM dGTP, 1 mM dCTP, 0.2 mg/ml BSA (nuclease free, BRL), 10 mM dithiothreitol and 157 units of Moloney Murine Leukemia Virus reverse transcriptase (BRL)) was added and the reaction was incubated at 37°C for 30 min. The reactions were treated with 10 μg of heat treated RNase A (Sigma) for 15 min at 37°C, phenol extracted, chloroform extracted, ethanol precipitated and run on either an 8% or 10% polyacrylamide/urea sequencing gel (25). Controls employing several different dilutions of the total RNA were included in each experiment to be certain that the oligonucleotides were present in excess.

RESULTS

Decreased U6 RNA content in the pre-mRNA splicing mutants

We have previously shown by northern blot analysis that the amounts of the spliceosomal snRNAs U1, U2, U4 and U5 are the same in the *prp*$^-$ mutants as in the wild type (23). However, the concentration of U6 RNA in the mutants is reduced relative to the wild type after a 2 hour (hr) shift to the elevated temperature (23). As an initial investigation of U6 RNA processing in the *prp*$^-$ mutants we decided to confirm our previous

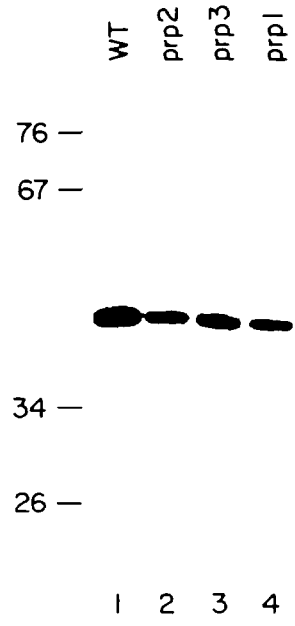


Figure 2. Primer extension analysis of U6 RNA in the pre-mRNA splicing mutants. Total RNA was prepared from the wild type (WT) or mutants (*prp1*, *2*, and *3*) grown to mid-log at 23°C and then shifted to 37°C for 2 hr. The RNA (20 μg/reaction) was used as template for primer extension analysis with U6-SC (see Fig. 1) as primer. The sizes and positions of DNA markers are indicated on the left. Exposure to X-ray film was at −70°C with an intensifying screen for 1 hr.

observation by a different assay method. The wild type and the three *ts*$^-$ mutants were grown to mid-log stage at 23° and then shifted to 37°C for 2 hr. Total RNA was prepared and used as template for primer extension reactions with an oligonucleotide primer, U6-SC (Fig. 1A), complementary to the *S. cerevisiae* U6 RNA (4). This primer hybridizes to nucleotides 31−44 of *S. pombe* U6 RNA with one mismatch at position 32 (19) and gives rise to an extension product of 44 nt (Fig. 1B). The primer recognizes both the mature U6 RNA and its precursor(s). As shown in figure 2, the steady state level of U6 RNA in *prp1*, *prp2* and *prp3* is slightly lower than the wild type after 2 hr at the nonpermissive temperature. The steady state amounts of U6 RNA at the permissive temperature are the same in the *prp*$^-$ mutants and the wild type (data not shown). These results support our earlier northern analyses (23). We detect a small but reproducible temperature-dependent reduction in U6 RNA content in the *prp*$^-$ mutants compared to that present in the wild type grown under identical conditions.

U6 pre-RNA splicing defect

If the diminished U6 RNA levels in the *prp*$^-$ mutants result from a block in the splicing of the precursor, then *prp1*, *prp2* and *prp3* should exhibit temperature sensitive defects in U6 pre-RNA splicing. The best evidence for a processing defect in an analysis of steady state RNA pools is an increase in the precursor/mature ratio (26−28). Since Tani and Ohshima did not observe U6 pre-RNA in the wild type (19) under normal growth conditions, we initially wanted to see if any U6 precursor could be detected by northern blotting in

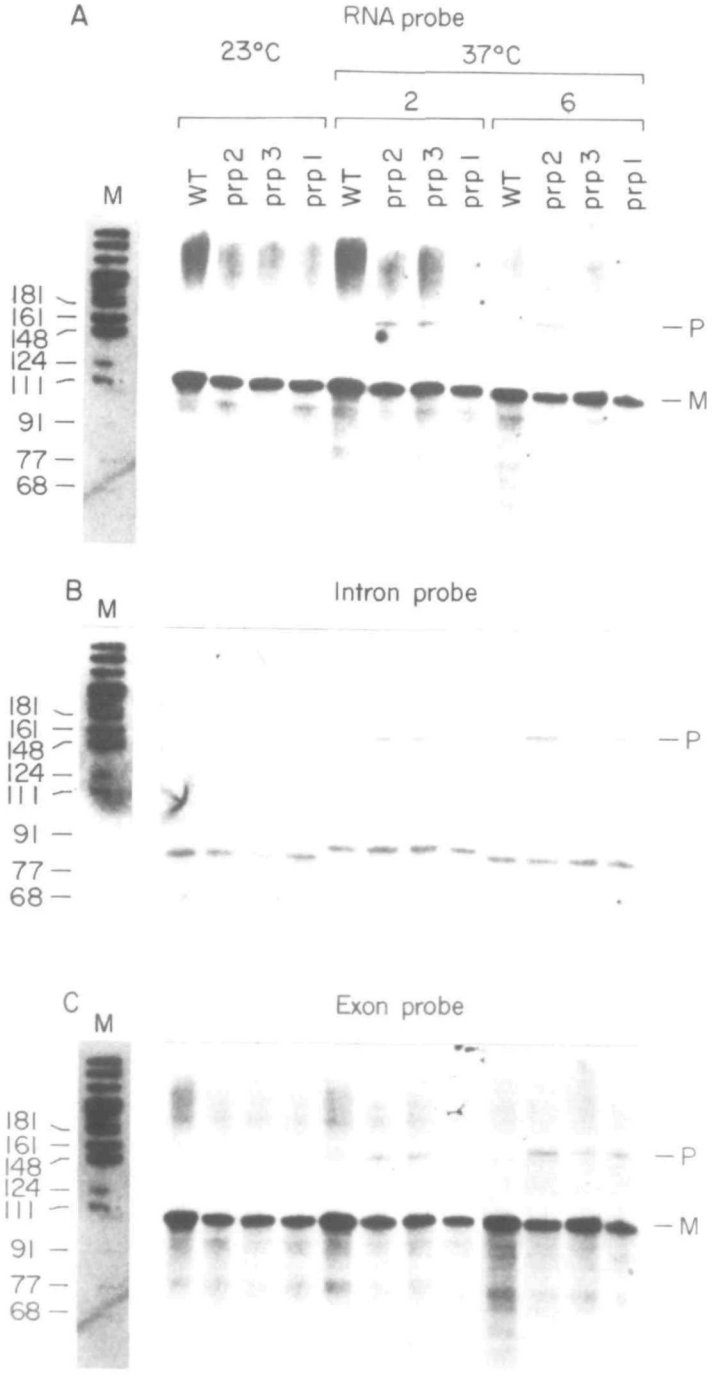
the *prp*$^-$ mutants at the nonpermissive temperature. The probe for this experiment was a human U6 antisense RNA, which recognizes an RNA of the expected size in *S. pombe* (23). At 23°C only a band of the size expected for mature U6 RNA was seen (Fig. 3A). However, after a 2 hr shift to 37°C a longer transcript (about 150 nucleotides, nt) appears in both the wild type and mutants. The larger RNA is the size expected for the unspliced precursor. After 6 hr at 37°C, the accumulation of the presumptive precursor persists in the mutants, but it is no longer observed in the wild type.

To determine whether the larger U6 RNA was an unspliced precursor, two oligonucleotides (U6I-1 and U6I-2), which specifically recognize the *S. pombe* U6 intron (Fig. 1A), were used as probes on the same blot shown in figure 3A. No intron-containing species were detected in the wild type or the mutants with the U6I-1 probe at 23°C (Fig 3B). Several bands smaller than that expected for the U6 pre-RNA were seen in all the lanes, but these were not observed when the blot was reprobed with U6I-2 (data not shown). We therefore do not believe that these bands represent smaller intron-containing RNAs such as the intermediate lariat or final intron product. The intensities of these nonspecific signals indicate that equal amounts of RNA were loaded in each lane. After the cells were shifted to 37°C for 2 hr, an intron-containing RNA of 150 nt appeared in both the wild type and mutants. This RNA was seen exclusively in the *prp*$^-$ mutants after 6 hr at 37°C. When the autoradiograms from the U6I-1 (Fig. 3B) and anti-U6 RNA probings (Fig. 3A) of the same blot are aligned by superimposing the molecular weight markers, the longer transcript detected with the anti-U6 RNA probe corresponds exactly with that of the intron containing precursor. Identical results were obtained with the U6I-2 probe (data not shown).

As further support for these results, the intron-specific oligonucleotide was stripped from the membrane and the blot was reprobed with the exon-specific probe U6E2-1, which is perfectly complementary to the fission yeast U6 RNA (Fig. 1). The same pattern of precursor and mature transcripts was obtained with U6E2-1 as with the anti-U6 RNA probe (compare Fig. 3A and Fig. 3C).

The northern analysis demonstrates an impairment in U6 pre-RNA splicing in *prp1*, *prp2* and *prp3*. The appearance of unspliced precursor alone is insufficient evidence to make this conclusion. However, after the shift to 37°C the levels of mature U6 RNA in the mutants remained approximately equal or decreased slightly compared to 23°C, while the amounts of U6 pre-RNA increased from undetectable levels at 23°C to a moderate accumulation at 37°C. Therefore, the precursor/mature ratio increased, indicating a temperature sensitive defect in U6 pre-RNA splicing in the *prp*$^-$ mutants. By the same criterion the shift from 23°C to 37°C is also slightly deleterious for U6 pre-RNA splicing in the wild type. After 2 hr at 37°C the precursor content increased in the wild type over 23°C levels; whereas, the amount of mature U6 RNA appeared to remain constant. However, in contrast to the *prp*$^-$ mutants, this effect is transient in the wild type. By 6 hr at 37°C, precursor was no longer detected in the wild type and there was no significant change in the quantity of mature RNA. Thus, after 6 hr at the nonpermissive temperature a defect in U6 pre-RNA splicing is clearly discernible in *prp1*, *prp2* and *prp3* relative to the wild type. Even after 2 hr at 37°C a visual comparison of the precursor/mature ratios indicates that the impairment in splicing is more severe in the mutants than the wild type. The amount of mature U6 RNA in the wild type after 2 hr at 37°C was slightly greater than in the mutants, while the wild type precursor level was the same or slightly less than in *prp1*, *prp2* and *prp3* (Fig. 3 A and C).

To confirm the conclusions of the northern analyses, we performed a series of primer



extension experiments. The wild type and mutants were grown as described above and total RNA prepared as template for the reverse transcription reactions. Data for 23°C and after a 6 hr incubation at 37°C are shown (fig. 4). The U6I-1 oligonucleotide recognizes only intron-containing species. An extension product of 78 nt is expected from this primer if unspliced precursor is present (Fig. 1B). As with the northern experiments, after 6 hr at 37°C, no precursor was detected in the wild type, even by the more sensitive primer extension assay (Fig. 4A). A band indicative of the unspliced U6 pre-RNA was seen in each of the *prp*$^-$ mutants. The U6I-1 primer extension, also detected a small amount of unspliced U6 pre-RNA at 23°C. The amount of precursor at the permissive temperature was the same in the wild type and the mutants. Longer exposures of the northern blots (Fig. 3) confirm this result (data not shown).

When the exon-specific oligonucleotide U6E2-2 is used as a primer, an extension product of 140 nt is expected from unspliced precursor and a shorter cDNA of 90 nt is produced from mature U6 RNA (Fig. 1B). Again, U6 pre-RNA was detected in the mutants but not the wild type after a 6 hr shift to 37°C (Fig. 4B). The primer extension experiments reproducibly indicated a greater accumulation of precursor in *prp1* and *prp2* than in *prp3* after growth for 6 hr at the nonpermissive temperature.

DISCUSSION

Our previous northern blot analysis of snRNA in three temperature sensitive pre-mRNA splicing mutants (*prp1*, *prp2* and *prp3*) showed normal amounts of U1, U2, U4 and U5 RNAs but slight reductions in the steady state levels of U6 RNA in the mutants relative to the wild type after a short period at 37°C (23). We confirmed this observation here by primer extension assay (Fig. 2). In addition, we have demonstrated that U6 pre-RNA splicing is impaired in the mutants at the nonpermissive temperature. The ratio of precursor/product in steady state RNA has been used to compare the splicing efficiencies of *cis*-acting mutations (26–28). We have used the same approach to investigate the affect of *trans*-acting mutations on U6 pre-RNA splicing. Temperature sensitive U6 splicing defects were indicated in *prp1*, *prp2* and *prp3* by an elevated U6 precursor/mature RNA ratio at 37°C compared to the wild type. The correlation between the reduction in U6 RNA and the U6 pre-RNA splicing defect suggests that the lower U6 RNA content in the *prp*$^-$ mutants is caused by a partial or complete block in the splicing of the U6 precursor. However, a steady state analysis can not unequivocally establish a cause and effect relationship between inefficient splicing and reduced U6 RNA content. For example, the splicing impairment alone might be insufficient to alter the steady state level of U6 RNA. The mature U6 RNA could be unstable in the mutants, perhaps as a consequence of the pre-mRNA splicing defect. In this case the effect would have to be specific for

Figure 3. Northern blot analysis of U6 RNA in the pre-mRNA splicing mutants. The wild type (WT) and mutants (*prp1*, *2*, and *3*) were grown to mid-log at 23°C and then shifted to 37°C. Total RNA prepared from the 23°C cultures or after a shift to 37°C for 2 or 6 hr was fractionated (20 mg/lane) on a polyacrylamide/urea gel and electroblotted onto a nylon membrane. The northern blot was probed with (A) an antisense RNA derived from the human U6 gene, (B) U6I-1 (see Fig. 1) and (C) U6E2-1 (see Fig. 1). P and M on the right of each panel mark the positions of precursor and mature RNA, respectively. The lanes shown separately on the left (M) are electroblotted ^{32}P-DNA size markers (*Msp* I digested pBR322). The sizes in nucleotides of selected fragments are given on the far left. Exposure to X-ray film was at −70°C with an intensifying screen for 1 week (panel A) and 24 hr (panels B and C).

U6 RNA, since the other snRNAs, including U6's snRNP partner U4 RNA, are normal in *prp1*, *prp2* and *prp3* (23).

Tani and Ohshima (19) in their analysis of U6 RNA in *S. pombe* were unable to detect the unspliced U6 pre-RNA in total RNA from wild type cells grown under normal *S. pombe* conditions. In this investigation we have taken advantage of the splicing defects in the *prp*$^-$ mutants to demonstrate that an intron-containing U6 pre-RNA is made in *S. pombe*. We have also shown that unspliced U6 pre-RNA can be detected in the wild type under certain growth conditions. At 23°C a small amount of precursor was seen in primer extension experiments with an intron-specific primer (Fig. 4A). The inability of Tani and Ohshima to detect U6 pre-RNA in cells grown at 30°C might have been the result of splicing efficiencies in rapidly growing cells being too high to permit appreciable accumulation of unspliced precursor. At 23°C the growth rate of *S. pombe* is approximately one half that at 30°C. Our data imply a reduced U6 splicing efficiency at 23°C that suggests that the rate of U6 pre-RNA splicing might be sensitive to growth rate. We also observed an increase in unspliced U6 pre-RNA relative to the mature RNA signal shortly after shifting cells from growth at 23°C to 37°C (Fig. 3). This precursor accumulation is transient in the wild type; by 6 hr after the temperature shift, no U6 pre-RNA is detectable. These observations could reflect a mild heat shock associated with shifting the cells to an elevated temperature, consistent with Tani and Ohshima's claim that detectable amounts of U6 precursor are produced after a brief shift to 43°C (19). Other studies have shown an inhibition of pre-mRNA splicing as a consequence of heat shock (29–32). Unlike U6 splicing, we did not observe a transient decrease in the efficiency of pre-mRNA splicing in the wild type upon shifting from 23°C to 37°C (23). Because of the differences in abundance between pre-mRNA and the U6 precursor, it is difficult to make conclusions from these data. A 3–6 times longer exposure of the northern blot is required to visualize a pre-mRNA signal in the mutants compared to the U6 pre-RNA. We have never seen pre-mRNA in total RNA from the wild type under any growth conditions. Our previous assays (23) might not have been sufficiently sensitive to detect a transient accumulation of pre-mRNA in response to temperature shift.

The U6 intron has a structure very typical of *S. pombe* pre-mRNA introns (19). Its unusual feature is that it is located within an snRNA. One of our questions in this study was whether the behavior of the U6 intron was similar to pre-mRNA introns. If the U6 intron were spliced identically to pre-mRNA introns, then it should be removed by a two step, lariat intermediate mechanism in the same type of spliceosome and requiring the same components as pre-mRNAs. The fact that U6 pre-RNA splicing is impaired in mutants that are partially (*prp3*) or completely (*prp1* and *prp2*) blocked in pre-mRNA splicing is consistent with the hypothesis that U6 splicing shares with pre-mRNA splicing a requirement for the *prp1*$^+$, *prp2*$^+$ and *prp3*$^+$ gene products. In addition, the primer extension analyses (Fig. 4) indicate that the severity of the U6 splicing defect is greater in *prp1* and *prp2* than in *prp3*, which is the same pattern observed for the α- and β-tubulin pre-mRNAs (23). However, we need more information on the requirements and pathways of both pre-mRNA and U6 pre-RNA splicing in *S. pombe* before we can say with confidence that they use the same mechanism and require the same factors. An alternative explanation is that the U6 splicing defect in the *prp*$^-$ mutants is a secondary effect. For example, a U6-specific splicing factor might be encoded by an intron-containing pre-mRNA. Or, the U6 pre-RNA could be spliced by a unique process that is sensitive to the level of pre-mRNA splicing in the cell.

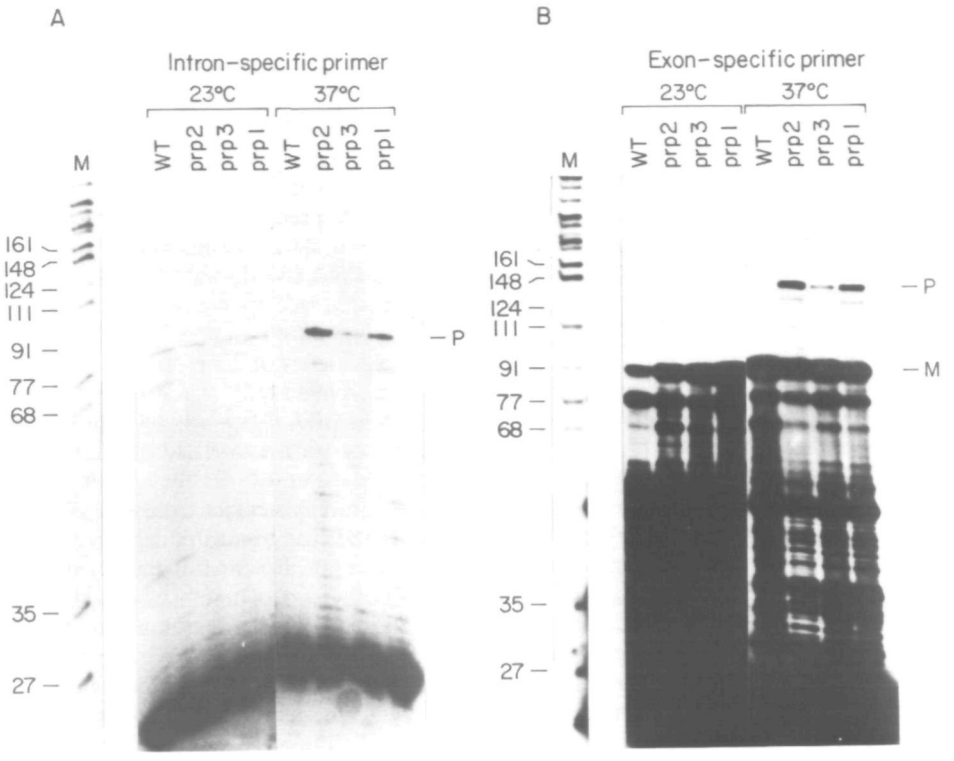


Figure 4. Primer extension analysis of U6 precursor and mature RNAs in the pre-mRNA splicing mutants. The wild type (WT) and mutants (*prp1*, *2*, and *3*) were grown as described in the legend to Fig. 3. Total RNA was prepared from the 23°C cultures and after a 6 hr shift to 37°C. The RNA (20 mg/reaction) was used as template for primer extension analysis. Primers were (A) U6I-1 and (B) U6E2-2 (see Fig. 1). M and P mark the positions of the extension products produced from the mature and precursor RNAs, respectively. DNA markers (*Msp* I digested pBR322) are displayed in separate lanes (M) to the left of each panel with the sizes in nucleotides of selected fragments indicated. Exposure to X-ray film was at −70°C with an intensifying screen for 24 hr.

One difference between the accumulation of pre-mRNAs and that of the U6 precursor in the *prp*$^-$ mutants is that near normal amounts of mature U6 RNA are present even after six hours at the nonpermissive temperature. By comparison, we detect no mature β-tubulin mRNA in *prp1* and *prp2* after only one hour at 37°C (23). A likely explanation for this observation is that the snRNAs are more stable than mRNAs. For example, the U5 homolog of *S. cerevisiae*, snR7, has been shown to be extremely stable (33). Five generations are required to deplete the snR7 pool sufficiently to see an effect on pre-mRNA splicing. The snRNAs in mammalian cells have also been shown to be metabolically stable (34). Therefore, it is likely that most of the mature U6 transcript observed at 37°C was synthesized prior to the temperature shift. Because of the differences in stabilities of mRNA and U6 RNA and their precursors, it is difficult to compare the efficiency of splicing for these two types of RNA in the *prp*$^-$ mutants. It could be that the U6 pre-RNA is spliced by the same mechanism as pre-mRNAs, but it is less sensitive to the *prp1*, *prp2* and *prp3* mutations than the pre-mRNAs that we have tested (23).

The amount of unspliced precursor accumulated in the *prp*$^-$ mutants at 37°C appears to be low, considering the fact that U6 RNA is a fairly abundant RNA in *S. pombe* and is probably transcribed at a high rate. Instability of the unspliced precursor may be the explanation. The stability of snRNAs is probably largely due to their packaging into snRNPs (35,36). Since the U6 intron is located within a region predicted to base pair with U4 RNA (4,37), it is unlikely that the unspliced U6 precursor would be assembled into a U4/U6 snRNP and it may therefore be degraded.

The U6 RNA genes of vertebrates (15−17) and probably of *S. cerevisiae* (4) are transcribed by Pol III. If the *S. pombe* U6 RNA is also a Pol III transcript, then its precursor represents a unique example of the splicing of a pre-mRNA type intron that is embedded in a Pol III transcript. Most of what is known about pre-mRNA splicing suggests that the splicing machinery primarily recognizes structures within the intron (8,38,39); although context effects involving exon sequences have been reported (40). Therefore, it might be expected that a pre-mRNA type intron could be spliced from a Pol III transcript. However, Sisodia et al. (41) have shown that an intron-containing pre-mRNA whose transcription was directed by a 5S RNA promoter (a Pol III promoter) was not spliced in mammalian cells. Several explanations could reconcile these results with those for the splicing of the *S. pombe* U6 RNA precursor. U6 promoters are structurally distinct from the 5S RNA promoter (15−17) , and different factors are required for the transcription of these two Pol III RNAs (14) . The U6 promoter also shares several *cis*-acting elements with the other snRNAs, which like pre-mRNAs, are Pol II transcripts. Therefore, it is possible that the U6 primary transcript may be exposed to the same nuclear environment as pre-mRNAs. Alternatively, despite its structure, the *S. pombe* U6 gene may be transcribed by RNA polymerase II. These questions must be addressed by further experiments in both the yeast and vertebrate systems. A search for mutants that specifically affect the splicing of U6 RNA may reveal a unique regulatory role for the unusual placement of a pre-mRNA type intron in a snRNA.

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