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Pac1p, an RNase III homolog, is required for formation of the 3′ end of U2 snRNA in *Schizosaccharomyces pombe*

DEWANG ZHOU,[1] DAVID FRENDEWEY,[2] and SUSAN M. LOBO RUPPERT[1]

[1]Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA
[2]Regeneron Pharmaceuticals, Inc., Tarrytown, New York 10591-6707, USA

ABSTRACT

Like its homologs in higher eukaryotes, the U2 snRNA in *Schizosaccharomyces pombe* is transcribed by RNA polymerase II and is not polyadenylated. Instead, an RNA stem-loop structure located downstream of the U2 snRNA coding sequence and transcribed as part of a 3′ extended precursor serves as a signal for 3′-end formation. We have identified three mutants that have temperature-sensitive defects in U2 snRNA 3′-end formation. In these mutants, the synthesis of the major snRNAs is also affected and unprocessed rRNA precursors accumulate at the restrictive temperature. Two of these mutants contain the same G-to-A transition within the *pac1* gene, whereas the third contains a lesion outside the *pac1* locus, indicating that at least two genes are involved. The *pac1*$^+$ gene is codominant with the mutant allele and can rescue the temperature-sensitive phenotype and the defects in snRNA and rRNA synthesis, if overexpressed. In vitro, Pac1p, an RNase III homolog, can cleave a synthetic U2 precursor within the signal for 3′-end formation, generating a product that is a few nucleotides longer than mature U2 snRNA. In addition, U2 precursors are cleaved and trimmed to the mature size in extracts made from wild-type *S. pombe* cells. However, extracts made from *pac1* mutant cells are unable to do so unless they are supplemented with purified recombinant Pac1p. Thus, the 3′ end of *S. pombe* U2 snRNA is generated by a processing reaction that requires Pac1p and an additional component, and can be dissociated from transcription in vitro.

Keywords: Pac1p; RNA processing; RNase III

INTRODUCTION

The spliceosomal small nuclear RNAs (snRNAs) in vertebrates, U1–U5, are members of a special class of RNA polymerase II transcription units with distinct promoter structures and 3′ end-forming signals (reviewed by Dahlberg & Lund, 1988; Lobo & Hernandez, 1994). Unlike the majority of RNA polymerase II transcripts, namely messenger RNAs (mRNAs), the snRNAs are not translated and have unusual 2, 2, 7-trimethyl guanosine cap structures. The snRNA promoters characteristically contain a proximal sequence element (PSE) that binds the TATA-box binding protein (TBP)-containing complex SNAPc/PTF (Sadowski et al., 1993; Yoon et al., 1995). In addition, the 3′ ends of the RNA polymerase II transcribed snRNAs are formed by a mechanism different from that which generates the 3′ ends of mRNAs (Hernandez, 1985; Yuo et al., 1985; Ciliberto et al., 1986; Neuman de Vegvar et al., 1986).

The 3′ ends of snRNAs are generated by a two-step mechanism: precursors with a few extra nucleotides at their 3′ ends are synthesized and transported to the cytoplasm where they are exonucleolytically shortened to the mature size (Chandrasekharappa et al., 1983; Madore et al., 1984a, 1984b). In vertebrates, 3′-end formation of snRNA precursors requires a short conserved sequence, the 3′ box, located 9–19 nt downstream of the RNA coding region (Hernandez, 1985; Yuo et al., 1985; Ciliberto et al., 1986; Hernandez & Weiner, 1986; Neuman de Vegvar et al., 1986). This signal is rather tolerant to mutation, and deletion of part of the 5′ or 3′ end of the sequence, while markedly decreasing 3′-end formation, does not entirely eliminate it (Hernandez, 1985; Yuo et al., 1985; Neuman de Vegvar et al., 1986; Ach & Weiner, 1987). Several lines of evidence suggest that in vertebrates the 3′ ends of

Reprint requests to: Susan M. Lobo Ruppert, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, 844 BBRB, 845 19th Street South, Birmingham, Alabama 35294, USA; e-mail: sruppert@bmg.bhs.uab.edu.

snRNA precursors are formed either by termination of transcription or by a processing event intimately coupled to transcription. First, 3′-end formation requires a compatible snRNA promoter (Hernandez and Weiner, 1986; Neuman de Vegvar et al., 1986). If the snRNA promoter is replaced by a mRNA promoter, the snRNA 3′ end is not formed. Instead transcription continues past the normal 3′ end and the transcripts are polyadenylated using cryptic or natural polyadenylation sites. Second, long read-through transcripts formed in vitro in isolated mouse nuclei are not precursors of mature U1 and U2 RNA molecules (Lobo & Marzluff, 1987). Third, in vitro synthesized snRNAs with long 3′ extensions are not processed to the mature length when they are injected into *Xenopus* oocytes (Murphy et al., 1982; Westin et al., 1984). The study of vertebrate snRNA 3′-end formation has been hampered by the fact that these genes are inefficiently transcribed in vitro in extracts otherwise competent for mRNA gene transcription. None of the factors involved in 3′-end formation has been identified, and the mechanism remains unknown.

In contrast to the vertebrate snRNAs, the 3′ ends of two snRNAs, U2 and U5L, in the budding yeast *Saccharomyces cerevisiae* are formed by a processing event unlinked to transcription (Chanfreau et al., 1997; Abou Elela & Ares, 1998). U5L is one of two forms of U5 RNA in *S. cerevisiae*, the other being U5S; the two forms differ in length at their 3′ ends. U2 and U5L snRNA accumulation is impaired in cells that have a temperature-sensitive mutation in the *RNT1* gene. Purified, recombinant Rnt1p, a homolog of bacterial RNase III, can cleave putative RNA stem-loop structures formed by U2 and U5 3′-flanking regions in vitro. Whole-cell extracts made from a wild-type yeast strain endonucleolytically cleave long synthetic precursors to generate correct U5L snRNA 3′ ends, whereas extracts derived from *rnt1* mutant cells are unable to do so. When such an extract is supplemented with purified Rnt1p, the processing of U5 precursors is restored, strongly implicating Rnt1p in the formation of the mature U5L 3′ end (Chanfreau et al., 1997). Depletion of Rnt1p in vivo also blocks correct U2 3′-end formation and results in the accumulation of polyadenylated U2 snRNAs (Abou Elela & Ares, 1998). In contrast to U2 and U5L, in the *rnt1* mutant cells the steady-state levels of U1, U4, and U5S are increased at the restrictive temperature (Chanfreau et al., 1997), suggesting that their 3′ ends may be generated by a different mechanism. This is supported by the observation that a shortened version of *S. cerevisiae* U1 snRNA, expressed from an autonomously replicating plasmid, is processed by Rnt1p-dependent and Rnt1p-independent pathways (Seipelt et al., 1999).

The snRNAs in the fission yeast, *Schizosaccharomyces pombe* (Tollervey, 1987; Tollervey & Mattaj, 1987; Brennwald et al., 1988; Porter et al., 1988, 1990; Dandekar & Tollervey, 1989; Small et al., 1989; Tani & Ohshima, 1989; Selinger et al., 1992), resemble their mammalian counterparts more closely in size and sequence than those in *S. cerevisiae* (reviewed by Guthrie & Patterson, 1988). In addition, a low proportion of genes in *S. cerevisiae* contains introns, and snRNAs in *S. cerevisiae* are less abundant than in *S. pombe* and vertebrates (Tollervey, 1987; Tollervey & Mattaj, 1987). Compared to their vertebrate homologs, relatively little is known about snRNA synthesis in *S. pombe*. A temperature-sensitive mutant, *snm1*, has been identified that forms extended snRNA transcripts at the restrictive temperature (Potashkin & Frendewey, 1990). Although the *snm1*$^+$ gene has not been identified, the mutant phenotype can be completely rescued by overexpression of Pac1p, a *S. pombe* protein with 25% amino acid identity to bacterial RNase III (Rotondo et al., 1995). Like bacterial RNase III, Pac1p cleaves RNA hairpin structures in vitro (Rotondo et al., 1997). However, the possibility that Pac1p may directly process *S. pombe* snRNA precursors has not been examined.

In this article, we identify the sequence element required for 3′-end formation of *S. pombe* U2 snRNA. This sequence element lies immediately downstream of the U2 coding region and, when transcribed as part of a longer precursor RNA, it is predicted to fold into a stable stem-loop structure. Mutations that disrupt the putative stem-loop structure abrogate 3′-end formation in vivo. Compensatory mutations that restore base pairing also restore 3′-end formation. We have isolated three mutants that are temperature sensitive (ts) for snRNA 3′-end formation. These three ts mutants are also defective in the processing of ribosomal RNA (rRNA) precursors at the restrictive temperature. Two of our mutants and *snm1* contain the same lesion within their *pac1* genes. Our third mutant contains a wild-type *pac1*$^+$ gene, indicating that an additional component may be required for snRNA 3′-end formation in *S. pombe.* Furthermore, we show that purified, recombinant *S. pombe* Pac1p is capable of cleaving the RNA stem-loop structure formed by the U2 3′-flanking region in vitro. Lastly, we demonstrate that a whole-cell extract made from a wild-type strain can process a synthetic U2 precursor to the mature size, whereas a similar extract made from a *pac1* mutant strain cannot do so unless it is supplemented with recombinant Pac1p.

RESULTS

Identification of the sequence element required for 3′-end formation of *S. pombe* U2 snRNA

It had previously been observed that 64 bp of 3′-flanking sequence was sufficient to generate mature U2 3′ ends in vivo (Dandekar & Tollervey, 1991). This sequence along with the last 43 nt of the U2 snRNA coding region was duplicated downstream of a U2 gene

marked by insertion of a 6-bp *Sac*I site as shown in Figure 1A. A series of seven clustered point mutations were introduced into the first copy of the 64-bp 3′-flanking sequence as described in Materials and Methods. The rationale for this approach is that a mutation in the first copy of the signal for 3′-end formation should result in the synthesis of a longer transcript that forms a stable 3′ end at the second copy of the signal. We transformed a *leu*$^-$ *S. pombe* strain (SP130) with the control and mutant U2 constructs. Total RNAs were isolated from *leu*$^+$ transformants and analyzed by RNase T1 protection of antisense RNA probes. The antisense probes used in this experiment are complementary to the marked U2 gene or to the marked mutant genes in pSP-MU2D as shown in Figure 1A, and extend from upstream of the start site of transcription to downstream of the duplication. This allows the simultaneous mapping of the 5′ and 3′ ends of the same RNA molecule. RNAs made from either single colonies or pools of ten colonies were assayed in parallel (data not shown) and gave the same results, indicating that the copy number of the pSP1 vector is essentially constant.

Figure 1B shows the RNase T1 protection analysis of steady-state RNAs derived from the different U2 constructs in vivo. Band e-U2 results from protection of 147 nt of the probe by endogenous U2 RNA and is seen in all lanes, including lane 1, in which RNA was isolated from cells transformed with the pSP1 vector alone and assayed with a probe complementary to the marked U2 gene with wild-type 3′-flanking sequence. Figure 1B, lane 2, shows the size of the protected fragments derived from the marked U2 transcripts (m-U2) and endogenous U2 RNA (e-U2), as this sample was obtained from colonies that contained the marked U2 gene, but without the downstream duplication. When the last 43 nt of the RNA coding sequence and the 64 nt of 3′-flanking region are duplicated, only the correct U2 3′ ends are seen (Fig. 1B, lane 3), indicating that the second, more downstream copy of the putative signal is not used. The mutations M1–M3 (Fig. 1B, lanes 4, 5, and 6) abrogate formation of the correct 3′ end, and a longer transcript, corresponding to 3′-end formation at the second copy of the signal, is seen (ext.-U2). The mutants M4–M6 (Fig. 1B, lanes 7, 8, and 9) show a reduced proportion of correct 3′ ends and some extended transcripts. All the RNA from mutant M7 forms the correct 3′ end; no extended transcript is detected (Fig. 1B, lane 10).

Primer extension analysis was carried out with an oligonucleotide complementary at its 3′ end to the *Sac*I insertion in the marked U2 gene. This oligonucleotide does not serve as a primer for reverse transcription of the endogenous U2 snRNA (our unpubl. data). All the transcripts from the transformed genes are correctly initiated (Fig. 1C), indicating that the longer RNAs derived from mutants M1–M6 are extended at their 3′ ends. In each case where an extended transcript is observed, the level of the mature RNA is diminished. However, for some mutants, such as M1 and M3, there does not appear to be a direct correlation between the levels of the mature and extended transcripts. Because the extended transcripts derived from different mutants vary in sequence at the sites of the substitutions, they may not be equally stable in vivo. The results of the linker-scanning mutation analysis indicate that a relatively large signal for 3′-end formation, spanning approximately 45 nt, lies within the U2 3′-flanking sequence.

The signal for U2 snRNA 3′-end formation is an RNA stem-loop structure

The mfold program (Zuker, 1994) was used to create the minimal energy RNA secondary structure model of the 64 nt of U2 3′-flanking sequence shown in Figure 2A. In this structure, 45 nt form a hairpin with a 21-bp stem and a 3-nt apical loop. The stem contains seven non-Watson–Crick base pairs: 4 G:U, a C:U, an A:A and a U:U. The latter three pairs are shown as a symmetrical bulge, which may not distort the helix. If the hairpin were important for 3′-end formation, mutations that disrupt base pairing should debilitate the process of 3′-end formation. Indeed, mutants M1–M6 that disrupt the hairpin structure gave rise to 3′ extended transcripts as shown above (Fig. 1B, lanes 4–9). Furthermore, the region mutated in M7 lies outside the hairpin structure and RNAs transcribed from this mutant form correct 3′ ends. Thus, our data are consistent with a model in which the sequence immediately downstream of the U2 snRNA coding sequence is transcribed and folded into a hairpin structure that is required for 3′-end formation. As a further test of this model we constructed mutants M2C and M3C in which the putative hairpin structure that was disrupted in M2 and M3, respectively, is restored by compensatory base substitutions (Fig. 2A). Thus, mutant M2C contains the substitutions shown in blue type in Figure 2A, which include the original substitutions present in M2 as well as additional base changes complementary to them. Similarly, M3C contains the base changes shown in red type (Fig. 2A) and includes the original substitutions present in M3. These constructs were introduced into *S. pombe* cells as before; RNA was isolated and analyzed by RNase T1 protection using homologous antisense RNA probes. The results are shown in Figure 2B. The production of extended transcripts seen with mutants M2 and M3 (Fig. 2B, lanes 2 and 4) is abrogated by the compensatory base changes in mutants M2C and M3C (Fig. 2B, lanes 3 and 5). Instead all the transcripts derived from M2C and M3C form the correct U2 3′ ends. These data confirm that the signal for 3′-end formation of U2 snRNA is an RNA stem-loop structure.

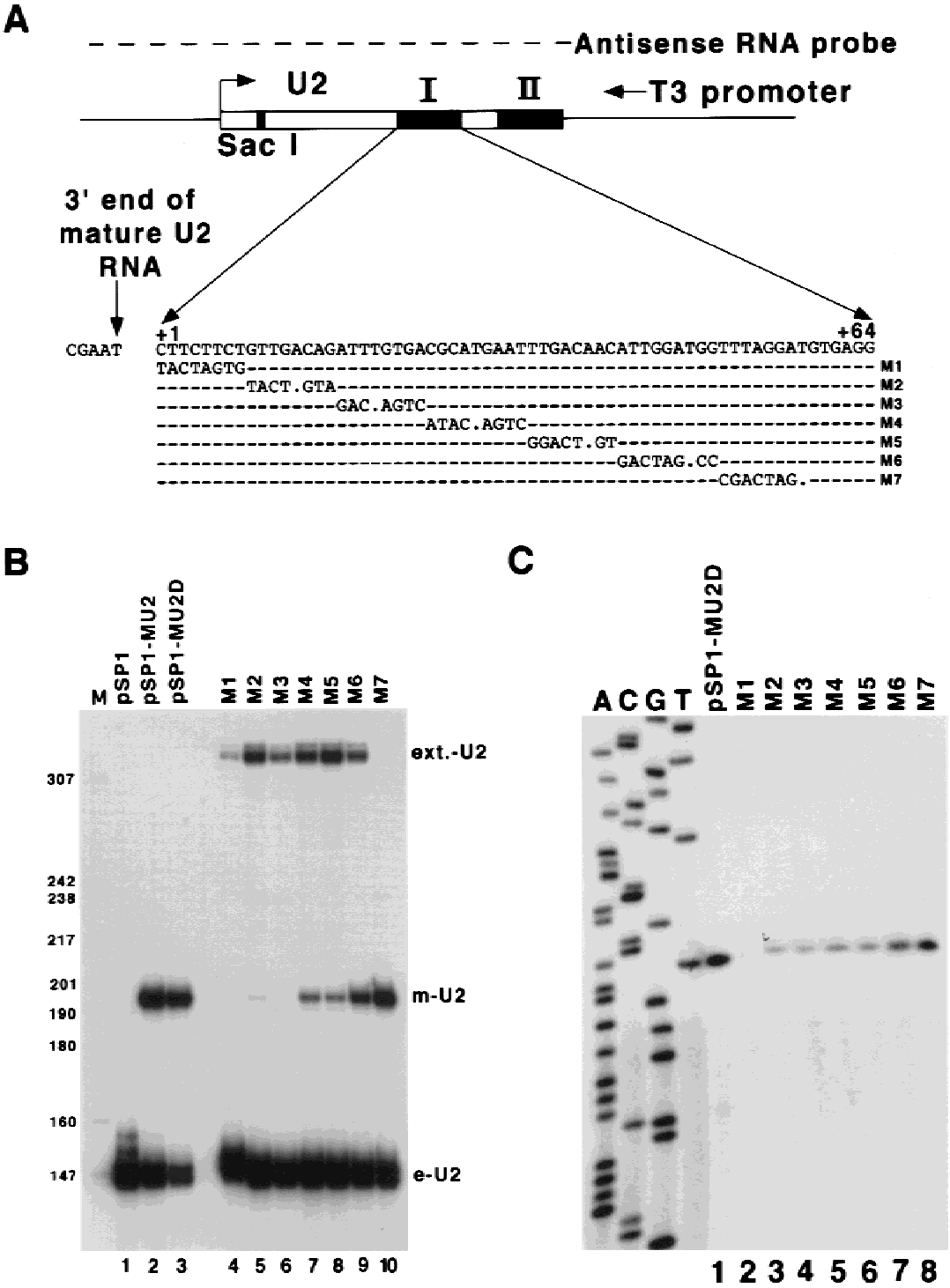

FIGURE 1. Identification of the sequence element required for U2 3′-end formation. **A**: Diagram of the DNA constructs. The parent construct, pSP-MU2D, is shown. Plasmid pSP-MU2 is identical to pSP-MU2D except that it lacks the duplication. The sequences mutated in the first copy of the U2 3′-flanking region are also shown. The DNA constructs were transformed into the *leu*$^{-}$ *S. pombe* strain SP130. Total RNAs were purified from *leu*$^{+}$ transformants. For each mutant, a homologous antisense RNA probe was synthesized with T3 RNA polymerase using the corresponding DNA construct, linearized upstream of the transcription start site, as template. **B**: RNase T1 protection assay of U2 snRNAs. Equal amounts (0.6 μg) of total RNA were used. RNAs in lanes 1, 2, and 3 were from cells transformed with pSP1 vector, pSP1-MU2, and pSP1-MU2D, respectively. RNAs in lanes 4–10 were from cells transformed with DNA constructs containing mutated, marked U2 genes M1–M7. M: molecular weight marker. **C**: Primer extension analysis of the marked U2 transcripts. A 5′-labeled primer extending from nt 60 to the *Sac*I site in the marked U2 gene was hybridized to equal amounts (15 μg) of total RNA extracted from cells transformed with plasmid pSP1-MU2D (lane 1) or constructs containing mutated, marked U2 genes M1–M7 (lanes 2–8). The primer was extended with AMV reverse transcriptase, and the cDNA products were fractioned on a 6% acrylamide/8 M urea sequencing gel. The reference DNA sequence shown on the left, lanes A, C, G, and T, was generated using the same oligonucleotide.

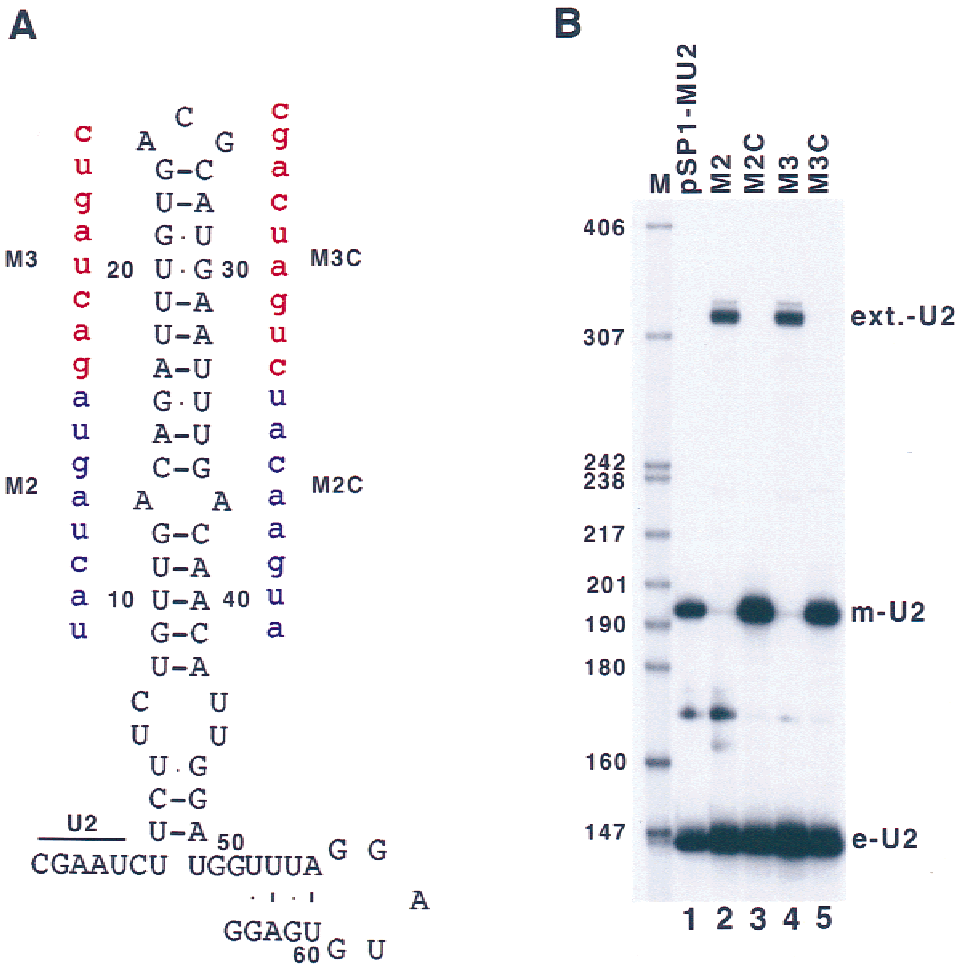

FIGURE 2. The signal for U2 3′-end formation is an RNA stem-loop structure. **A**: RNA secondary structure. The mfold program (Zuker, 1994) was used to fold the RNA sequence from the 64 nt of the U2 3′-flanking region. The nucleotides mutated in M2 and M3 are shown in blue and red, respectively, on the left side of the stem-loop structure. The sequences of the compensatory mutants M2C and M3C that restore base pairing to mutants M2 and M3 include the blue and red sequences on both sides of the stem-loop structure, respectively. **B**: RNase T1 protection assay. The antisense RNA probes were prepared as in Figure 1. Equal amounts (0.6 μg) of total RNA were used. The RNAs were from cells transformed with plasmid pSP1-MU2 (lane 1), constructs containing M2 and M3 (lanes 2 and 4), or constructs containing mutants M2C and M3C (lanes 3 and 5). M: molecular weight marker.

Isolation of mutants with temperature-sensitive defects in U2 snRNA 3′-end formation

Having defined the *cis*-acting elements necessary for U2 snRNA 3′-end formation, we wanted to identify the *trans*-acting factors involved by isolating strains deficient in the process. Four hundred ts mutants, obtained as described in Materials and Methods, were screened for defects in U2 3′-end formation at the restrictive temperature. Each of the ts mutants was grown to mid-log phase at room temperature (25 °C) and then shifted to the restrictive temperature (36 °C) for 5 h. Total RNA was isolated and the 3′ ends of the endogenous U2 snRNAs were analyzed by RNase T1 protection of an antisense RNA probe that is complementary only to the U2 3′-flanking region, not to the coding sequence. This probe can detect only transcripts that extend beyond the correct U2 3′ end. RNA from *snm1* serves as a positive control and RNAs from the wild-type strain, SP972, and ts180 (a mutant chosen at random from our collection) served as negative controls in this assay. RNAs from three mutants, ts35, ts138, and ts170, protected an antisense RNA probe fragment of 230 nt (Fig. 3A, lanes 5, 7, and 9) as did RNA from *snm1*. Of these mutants, ts138 and ts170 were isolated from two independent banks that were generated by EMS mutagenesis and ts35 from a bank that was produced by MNNG mutagenesis.

Ts35, ts138, and ts170 were back-crossed to wild-type strains thrice and tetrad analysis showed that in all cases the ts growth defect (*ts*$^-$) segregated 2:2, indicating that each strain contains a single mutation. In addition, RNA from the progeny of the four spores of several tetrads were analyzed using RNase T1 protec-

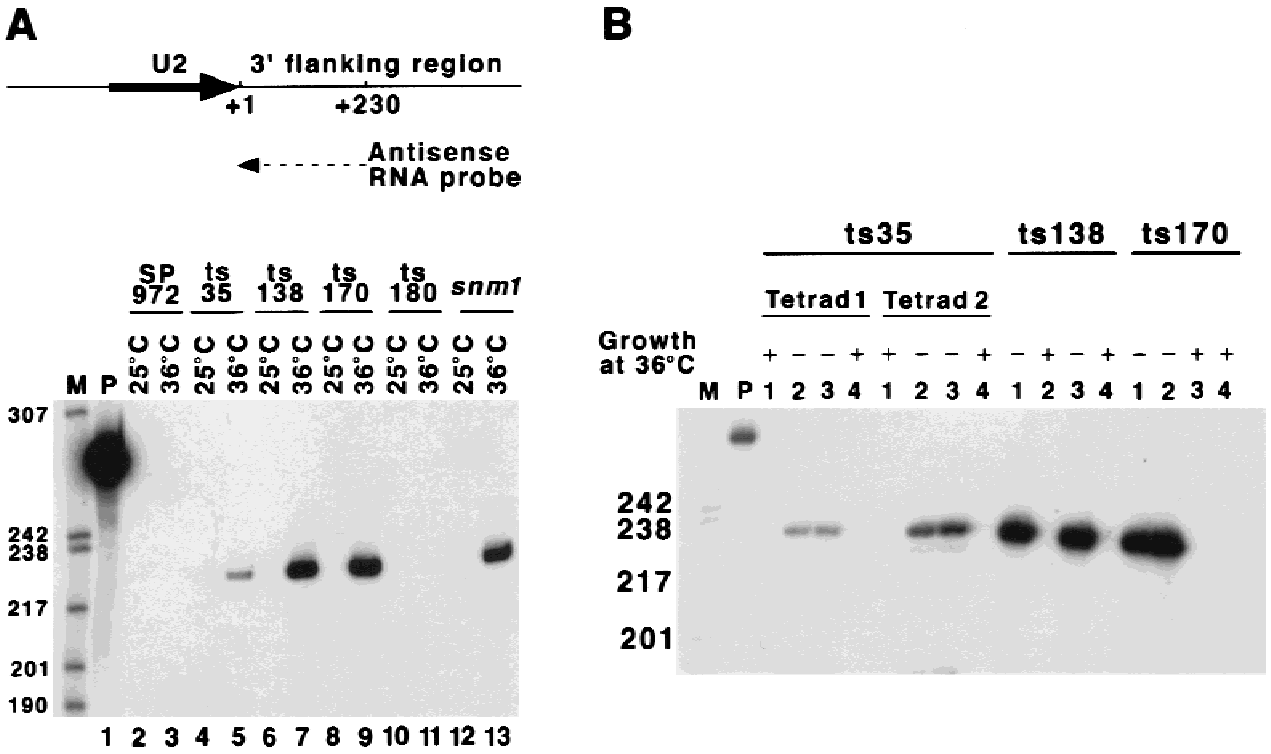

FIGURE 3. Isolation of ts mutants defective in U2 snRNA 3′-end formation. **A**: Screen for ts mutants defective in U2 snRNA 3′-end formation. A total of 400 ts mutants were screened. Each ts mutant was grown to mid-log phase at room temperature, and half of the culture was then shifted to 36 °C for 5 h. Cellular RNAs were purified and analyzed by the RNase T1 protection assay using an RNA probe complementary only to the U2 3′-flanking region. Equal amounts (0.6 μg) of total RNA from each mutant were used. M: molecular weight marker. P: antisense RNA probe. **B**: The *ts*$^-$ phenotype cosegregates with the defect in U2 snRNA 3′-end formation. ts35, ts138, and ts170 were mated to wild-type strains and allowed to undergo meiosis. Tetrads were dissected and total RNA was prepared from the progeny of each of the four spores after a shift to 36 °C for 5 h. RNase T1 protection assays were done as in Figure 3A. M: molecular weight marker. P: antisense RNA probe.

tion of the antisense U2 3′-flanking probe. In each case, only RNA derived from the two *ts*$^-$ spores protected a 230-nt antisense RNA probe fragment (Fig. 3B). Complementation analysis showed that ts138 and ts170 belong to the same complementation group whereas ts35 belongs to a different group.

The synthesis of all the major snRNAs is affected in ts35, ts138, and ts170

To determine whether or not the defects in ts35, ts138, and ts170 were specific for U2 snRNA, expression of the major snRNAs in these mutants was analyzed by Northern hybridization using oligonucleotide probes complementary to the mature snRNA sequences. RNA from the *snm1* strain (Potashkin & Frendewey, 1990) was used as a positive control, and RNAs from wild-type strain SP972 and two ts mutants from our collection, ts171 and ts180, served as negative controls. The levels of mature U1, U2, and U4 snRNAs in ts138, ts170, and *snm1* at the restrictive temperature (Fig. 4A, lanes 4, 6, and 14) were reduced relative to those of the controls (Fig. 4A, lanes 8, 10, and 12). In contrast, ts35 expressed elevated levels of these snRNAs at 36 °C (Fig. 4A, lane 2). The levels of mature U3 snRNA in the mutants were similar to those of the controls at both temperatures. A longer exposure showed that all three ts mutants and *snm1* expressed extended U1-, U2-, U3-, U4-, and U5-specific transcripts at 36 °C. In *S. pombe* there are normally two U5 transcripts, a major species, U5S, of 114 nt and a minor species, U5L, of 140 nt. In ts138, ts170, and *snm1* both U5 species were reduced in amount at 36 °C in comparison to the controls (Fig. 4A, compare lanes 4, 6, and 14 with lanes 8, 10, and 12), whereas in ts35 the levels of both U5 species increased at 36 °C (Fig. 4A, lane 2). A longer version of the RNA polymerase III-transcribed U6 snRNA (Fig. 4A, lanes 2, 4, 6, and 14) is present in all three ts mutants and *snm1* at 36 °C. The U6 gene in *S. pombe* contains an intron (Tani & Ohshima, 1989), removal of which requires components of the pre-mRNA splicing machinery (Potashkin & Frendewey, 1989; Urushiyama et al., 1996). The longer version of U6 snRNA seen in Figure 4A corresponds to unspliced U6 precursor as it hybridizes to an intron-specific probe (data not shown). As snRNAs are involved in pre-mRNA splicing, the accumulation of the unspliced U6 precursor may be a secondary consequence of the

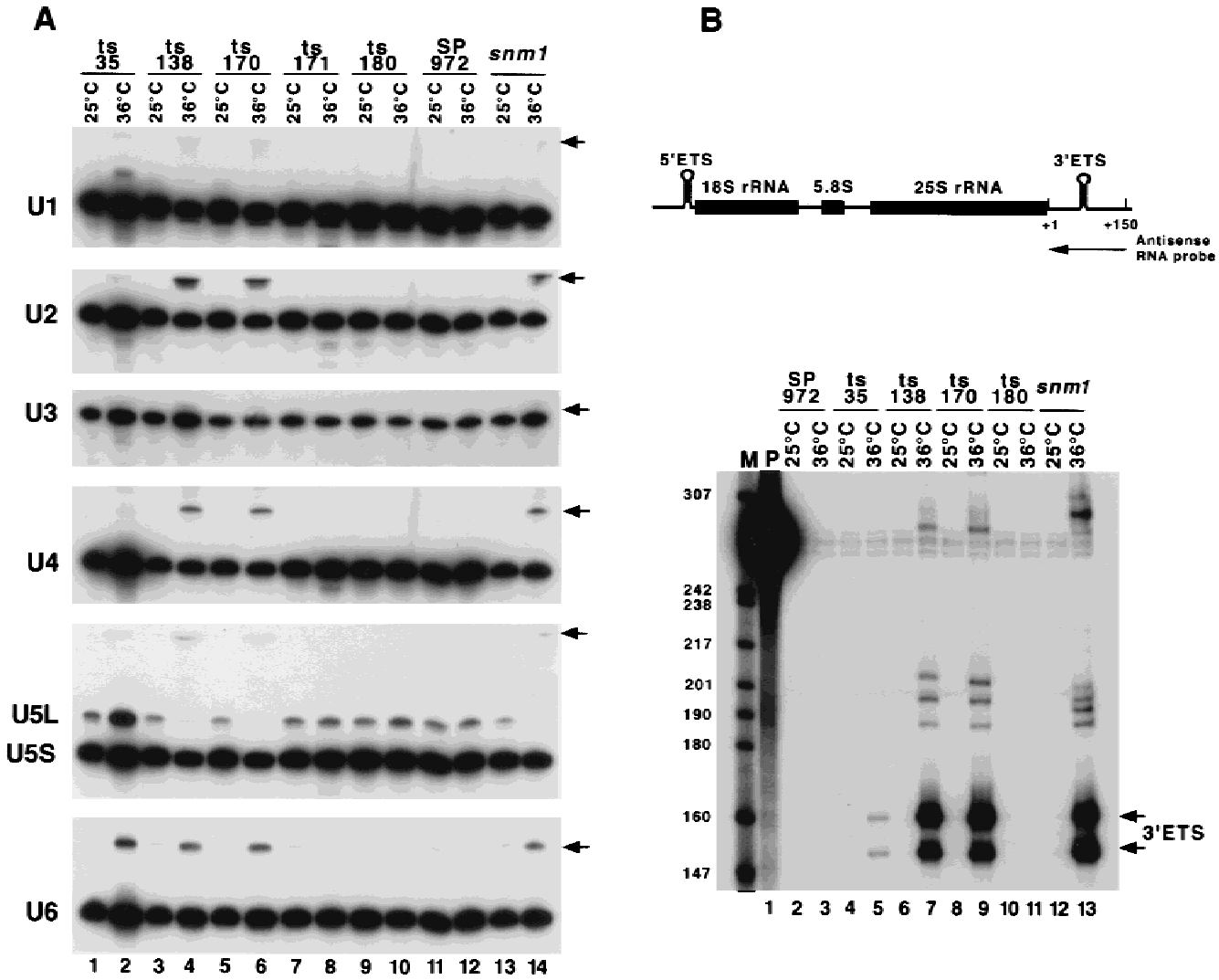

FIGURE 4. Characterization of the ts mutants. **A**: Northern hybridization. ts35, ts138, and ts170 were grown to mid-log phase at room temperature. Half of each culture was then shifted to 36 °C for 5 h. Cellular RNAs were purified and run on a denaturing 10% acrylamide gel. RNAs from ts171, ts180, and the wild-type strain SP972 served as negative controls, and RNAs from *snm1* served as a positive control. Equal amounts (15 μg) of total RNA from each strain were used. Probes used for Northern blot were γ^{32}P-ATP-labeled oligonucleotides specific for U1, U2, U3, U4, U5, and U6 snRNAs. The hybridization and washing of the Northern blots was performed as described (Potashkin & Frendewey, 1990). The same filter was sequentially reprobed with the different oligonucleotides. **B**: RNase T1 protection assay of rRNA precursors. pSP-ETS was used to make a RNA probe complementary only to the 3′ ETS of rRNA. RNAs from ts180 and wild-type strain SP972 served as negative controls. Equal amounts (0.6 μg) of total RNA from each strain were used. M: molecular weight marker. P: antisense RNA probe.

defect in snRNA synthesis. Thus, while the synthesis of all the major U snRNAs is simultaneously affected in ts35, ts138, ts170, and *snm1,* ts35 has distinctly different patterns of expression of the snRNAs than the other mutants.

Processing of rRNA precursors is impaired in mutants deficient in snRNA synthesis

In *S. cerevisiae*, the processing of snRNA and rRNA precursors is deficient in the *rnt1* mutant cells (Abou Elela et al., 1996; Chanfreau et al., 1997; Abou Elela & Ares, 1998). We therefore examined the possibility that the processing of rRNA precursors might be impaired in our ts mutants. An antisense RNA probe complementary only to the 3′ ETS (external transcribed spacer) was used in an RNase T1 protection assay to monitor the processing of rRNA precursors at the normal and restrictive temperatures in each mutant. As shown in Figure 4B, at 36 °C pre-rRNA precursors accumulated dramatically in ts138, ts170, and *snm1* (lanes 7, 9 and 13) in comparison to the controls (lanes 3 and 11). Again, ts35 behaved differently from the other mutants as only a small amount of precursor accumulated in

this mutant at the restrictive temperature (Fig. 4B, lane 5).

Ts138 and ts170 have the same point mutation within the *pac1* coding sequence

The *pac1*$^+$ gene, one of two genes in *S. pombe* that encode proteins with similarities to bacterial RNase III, was isolated as a suppressor of the *snm1* mutation (Rotondo et al., 1995). Pac1p was shown to cleave double-stranded RNA structures and to cut a pre-rRNA 3′ ETS substrate at known in vivo processing sites (Rotondo et al., 1997). The identification of an RNA hairpin structure as the signal for 3′-end formation of U2 snRNA and the isolation of mutants simultaneously deficient in the synthesis of snRNAs and rRNAs suggested that the *pac1* locus could be mutated in ts35, ts138, or ts170. To examine this possibility, genomic DNA was isolated from these ts strains and a wild-type strain (SP972). The *pac1* genes were amplified by PCR with Pfu polymerase and sequenced on both strands using the oligonucleotides described in Materials and Methods. Surprisingly, ts138 and ts170 contain the same point mutation within the Pac1p coding sequence, a transition from G to A at nt 1024, whereas the *pac1* genes in ts35 and strain SP972 are wild type (Xu et al., 1990; Iino et al., 1991). We subsequently sequenced the *pac1* locus from the *snm1* strain and found the same G-to-A transition as in ts138 and ts170. The mutant *pac1* allele in ts138, ts170, and *snm1* encodes a protein with an alanine-to-threonine substitution at position 342 and is henceforth referred to as *pac1-A342T*. As shown in Figure 5A, the alanine residue at position 342 is one of the most highly conserved residues in double-stranded RNA binding domains (dsRBDs) (Bycroft et al., 1995; Kharrat et al., 1995). Thus, in *S. pombe*, mutations in at least two genes affect U2 snRNA 3′-end formation: one is the *pac1*$^+$ gene, and the other is the gene mutated in ts35.

If the point mutation in *pac1* is the cause of the ts phenotype in ts138 the defect should be rescued by expression of the wild-type gene. We therefore transformed an autonomously replicating plasmid containing the wild-type *pac1*$^+$ gene into strain ts138. We also integrated a single copy of the wild-type *pac1*$^+$ gene into the genome of ts138 and confirmed that integration had occurred homologously by Southern analysis (data not shown). As shown in Figure 5B, the *pac1*$^+$-containing plasmid, which is present in 3 to 10 copies per cell, completely rescues the ts growth phenotype. However, a single integrated wild-type copy of the *pac1*$^+$ gene only partially alleviates the temperature sensitivity of ts138. The ts138 strain containing a wild-type *pac1*$^+$ gene integrated adjacent to the mutant *pac1* gene (ts138 + *pac1*$^+$-INT) has a mean doubling time at 36 °C of 2 h 50 min. This is intermediate between the 5 h 50 min mean doubling time of ts138 transformed with vector alone (ts138 + pDblet) and the 2 h 20 min mean doubling time for ts138 transformed with a multicopy *pac1*$^+$ containing plasmid (ts138 + pD*pac1*$^+$, Fig. 5B). ts138 + pD*pac1*$^+$ has the same mean doubling time at 36 °C as the wild-type strain (SP972). RNase T1 protection assays confirmed that the defects in U2 3′-end formation and in rRNA processing were completely corrected by transformation of ts138 with the *pac1*$^+$-containing plasmid, but only partially corrected by integration of a single wild-type *pac1*$^+$ gene (Figs. 5C and 5D, compare lanes 5 with lanes 3 and 4). Thus, the mutant *pac1* gene is codominant with the wild-type *pac1*$^+$ gene and overexpression of the wild-type gene is required to completely rescue the ts phenotype.

Purified Pac1p cleaves the signal for U2 snRNA 3′-end formation in vitro

To determine if the Pac1p enzyme can cleave the stem-loop structure formed by RNA from the U2 3′-flanking region, we incubated internally labeled pre-U2 RNA transcripts with purified HIS-Pac1p (Fig. 6). In this experiment, an excess of U2 precursor was used and the optimal concentration of Pac1p (0.045 μg/mL) was determined by titration (data not shown). As shown in

FIGURE 5. The *pac1*$^+$ gene is mutated in ts138 and ts170. **A**: Alignment of double stranded RNA binding domain (dsRBDs) sequences. The highly conserved alanine residue is in bold type, and is the amino acid mutated to T in ts138, ts170 and *snm1*. Sequences are identified with their accession numbers as follows: Cb rnc, L21436; Ec rnc, X02946; Sc rnt: *S. cerevisiae* Rnt1p, U21016; Sp pac; Pac1p, X54998; Sp orf, orf SPAC8A4.08c on *S. pombe* chromosome I, Z66569; Ce orf, orfCELK12H4.1, L14331; Ce orf2, orfCELF26E4.13, 387642; Dr orf, AC005448; Xlrbpa-2, Q91836. **B**: Rescue of the ts phenotype by the *pac1*$^+$ gene. ts138 was transformed with vector pDblet alone (ts138 + pDblet) or plasmid pD*pac1*$^+$, which contains the wild-type *pac1*$^+$ gene (ts138 + pD*pac1*$^+$), or with a wild-type *pac1*$^+$ gene integrated homologously in its genome (ts138 + *pac1*$^+$-INT). The cells were streaked on MM + ade plates and grown either at room temperature or at 36 °C for 2 days. **C**: RNase T1 protection assay of U2 snRNA. ts138 strains transformed with vector pDblet alone, or plasmid pD*pac1*$^+$, or integrated with a wild-type *pac1*$^+$ gene were grown in MM + ade liquid medium to mid-log phase. After a shift to 36 °C for 5 h, the total RNAs were purified and analyzed by the RNase T1 protection assay as in Figure 3A. RNA from a wild-type strain SP972 (also purified after a shift to 36 °C for 5 h) served as a negative control. Equal amounts of total RNA (0.6 μg) from each strain were used. M: molecular weight marker. P: antisense RNA probe. **D**: RNase T1 protection assay of pre-rRNA. The same RNA samples as in **C** were analyzed by an RNase T1 protection assay using an RNA probe complementary to the 3′ ETS of pre-rRNA as in Figure 4B. M: molecular weight marker. P: antisense RNA probe.

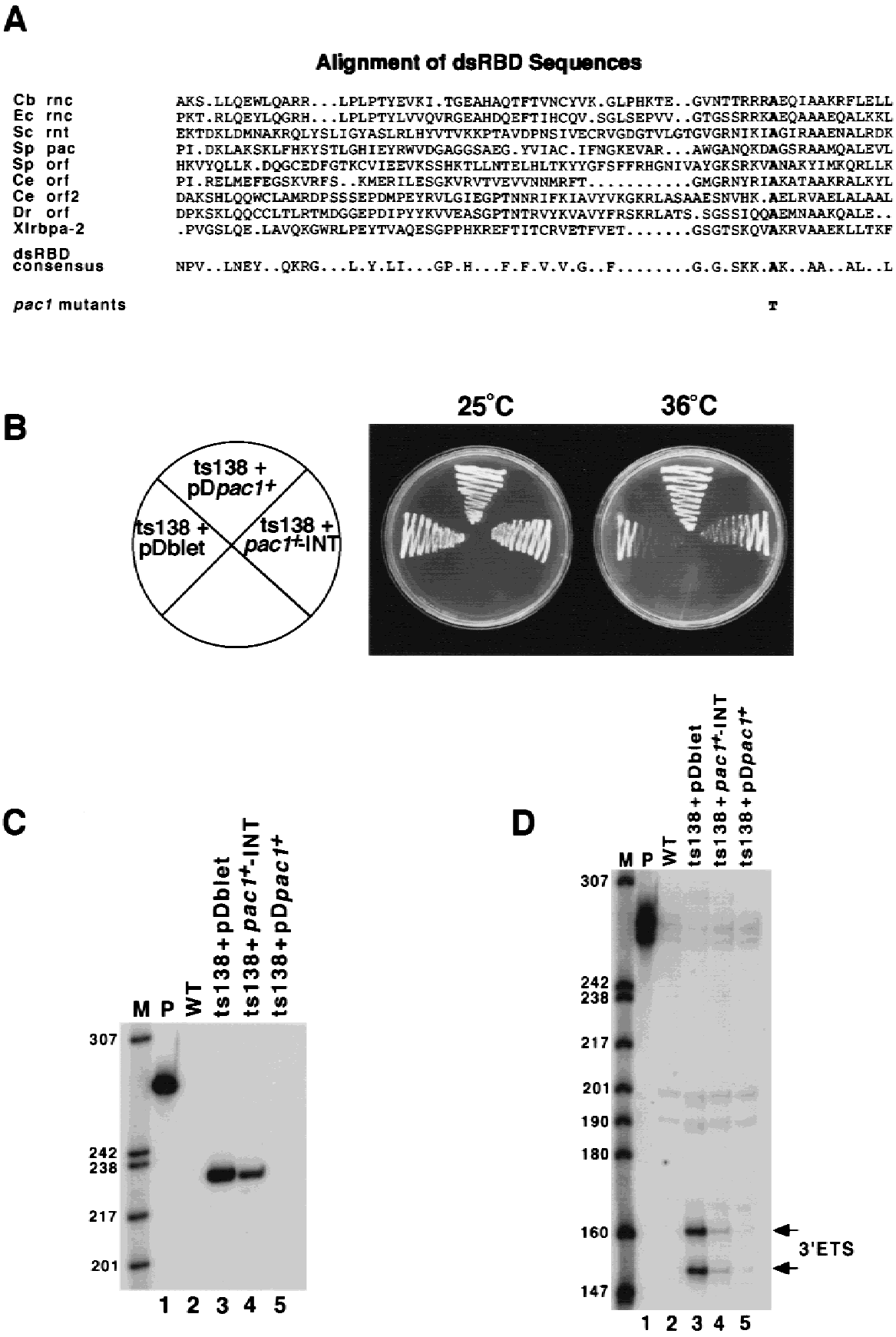

FIGURE 5. (*Legend on facing page.*)

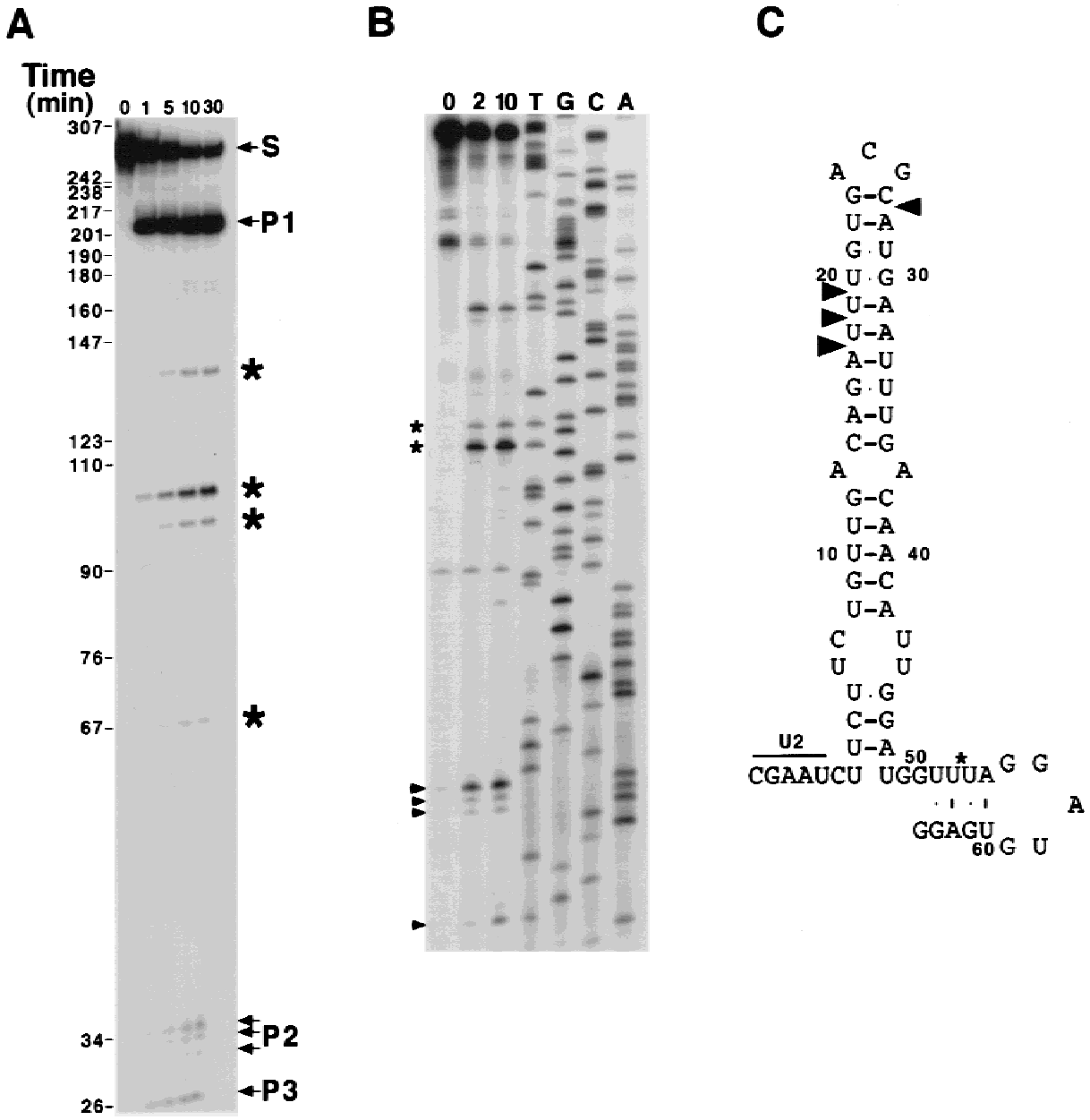

FIGURE 6. Pac1p cleaves a pre-U2 snRNA precursor in vitro. **A**: In vitro cleavage of pre-U2 snRNA precursor by purified HIS-Pac1p. Internally labeled pre-U2 transcripts were incubated with purified HIS-Pac1p for the times (min) indicated. The initial substrate (S) and products (P1, P2, and P3) are indicated on the right. Products marked with * correspond in size to HIS-Pac1p cleavages within the U2 coding region. **B**: Mapping the site of cleavage using primer extension. A primer complementary to the 3′-flanking sequence of U2 snRNA was extended on pre-U2 substrate RNA after incubation with HIS-Pac1p for 0, 2 or 10 min. The reference DNA sequence (produced using the same primer) is shown on the right. Arrowheads on the left indicate the products corresponding to Pac1p cleavages in the 3′-flanking stem-loop structure. Pac1p cleavage sites within the U2 coding region are indicated by *. Note that bands corresponding to Pac1p cleavage increase with incubation time whereas reverse transcription stops do not. **C**: Cleavage sites within the RNA secondary structure in the U2 3′-flanking region. Arrows indicate Pac1p cleavage sites. In addition, a possible Pac1p cleavage site in the small stem-loop is marked by *.

Figure 6A the major product P1 (214 nt) has the size expected for Pac1p cleavage within the stem-loop structure. At the same time four minor products (marked with *), corresponding in size to Pac1p cleavage within the mature U2 snRNA sequences, are also observed. The Pac1p cleavage sites were mapped by primer extension using an oligonucleotide complementary to the 3′-flanking sequence of U2 snRNA. The cleavage sites in the stem-loop structure are indicated with arrowheads in Figures 6B and 6C. Based on the sizes of products P2 and P3 (approximately 23–34 nt long) in Figure 6A, there appears to be another Pac1p cleav-

age site (marked with * in Fig. 6C) within the minor stem-loop in the U2 3′-flanking region. In Figure 6B, we obtained two primer extension products (indicated by *) that correspond to Pac1p cleavage within the last stem-loop of the mature U2 snRNA. In mature U2 snRNA there are three stem-loops, approximately 12 bp in length, that are potential substrates for Pac1p, although under our assay conditions, they are less efficiently cleaved than the stem-loop structure within the U2 3′-flanking sequence.

Extracts from *pac1* mutant cells fail to generate intermediate or the mature-size U2 products in vitro

To determine whether the deficiency in U2 3′-end formation in ts138 was due to reduced activity of the mutant *pac1* enzyme, a synthetic radiolabeled U2 precursor was incubated in extracts prepared from wild-type and ts138 cells grown at the permissive temperature. As shown in Figure 7, in the wild-type extract an intermediate (I) appears first and is converted into mature-size U2 snRNA with increasing incubation time. In extracts made from ts138, however, neither the intermediate or mature-size U2 snRNA was produced. This lack of processing is attributable to reduced Pac1p activity because the production of the intermediate and mature-size U2 snRNA was restored when the same extract was supplemented with purified HIS-Pac1p (Fig. 7). RNA fragments, indicated by * in Figure 7, that are seen in extracts from wild-type and mutant cells even in the absence of added Pac1p, are probably due to contaminating nuclease activities. Taken together, our in vitro data support a direct role for Pac1p in U2 3′-end formation in vivo.

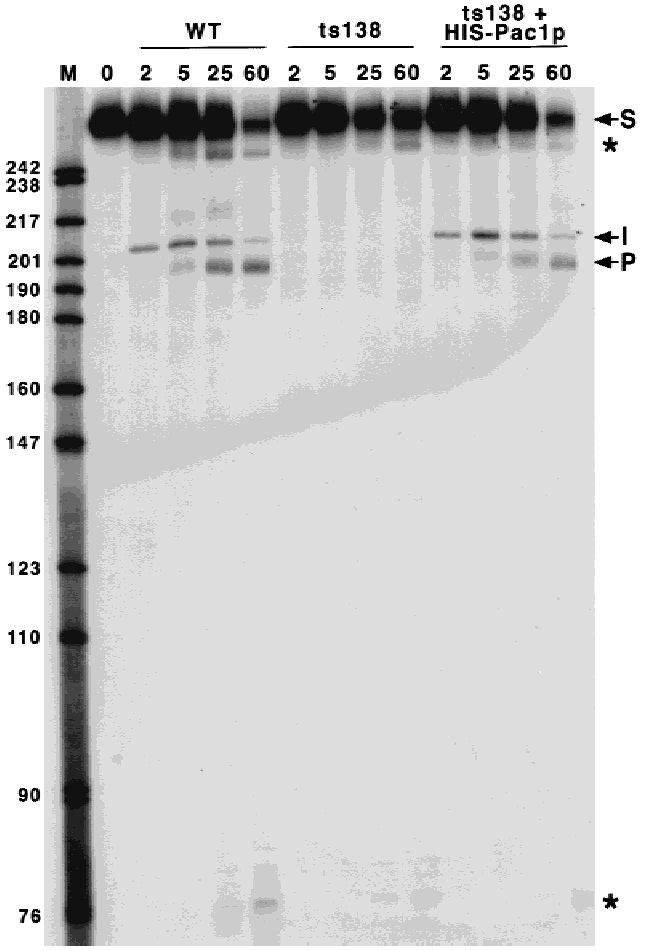

FIGURE 7. Pac1p is required for correct U2 3′-end processing in vitro. Internally-labeled pre-U2 transcripts with 3′ extensions were incubated in whole-cell extracts made from wild-type (WT) or mutant ts138 strains for the time (min) indicated. The products were loaded on a denaturing 6% acrylamide gel. The initial substrate (S), intermediate (I), and the product (P) are indicated on the right. M: molecular weight marker.

DISCUSSION

We have identified an RNA stem-loop structure that is encoded within the U2 snRNA 3′-flanking sequence as a signal for 3′-end formation. This signal is recognized and cleaved by Pac1p, one of two RNase III orthologs (Rotondo & Frendewey, 1996) in *S. pombe*.

The *pac1*$^+$ gene was previously shown to suppress the ts phenotype of the *snm1* mutant when overexpressed from a multicopy vector (Rotondo et al., 1995). However, based on the observation that integration of a single wild-type copy of the *pac1*$^+$ gene into the genome of the *snm1* strain did not rescue the ts phenotype, it was concluded that *pac1*$^+$ was not the *snm1*$^+$ gene. We have identified three mutants, ts35, ts138, and ts170, that have ts defects in snRNA 3′-end formation. Sequencing of the *pac1* genes from these strains and from *snm1* showed that ts138, ts170, and *snm1* harbored the same G-to-A transition within the Pac1p coding region. As was observed for *snm1*, the growth phenotype of ts138 is rescued by overexpression of *pac1*$^+$ from a multicopy vector. However, we observed that a single copy of this gene integrated homologously into the genome of the mutant partially corrects the growth defect of ts138 (Fig. 5). We also found that the defect in snRNA 3′-end formation (and rRNA processing) was partially alleviated by a single copy of *pac1*$^+$ and completely overcome by expression of multiple copies of this gene. Thus, the mutant allele, *pac1-A342T,* is codominant with the wild-type allele.

Although there are several possible mechanisms for codominance, we favor the idea that Pac1p may function as a dimer, for the following reasons. First, *Escherichia coli* RNase III, which is related to Pac1p in terms of its structure, cleavage specificity, and function in rRNA synthesis, has been shown to work as a dimer (Court, 1993). Secondly, the crystal structure of the second dsRBD of the *Xenopus laevis* RNA binding protein A, namely domain Xlrbpa-2, complexed to double-stranded

RNA was solved recently (Ryter & Schultz, 1998). It showed that two molecules of this protein interact with each other and with double-stranded RNA simultaneously. The Xlrbpa-2 domain contains the same highly conserved residues found in Pac1p and in *E. coli* RNase III, suggesting that all these proteins may interact with dsRNA in a similar manner. Thus, the codominance of the mutant and wild-type *pac1* alleles in our experiments may be explained by the formation of inactive dimers that contain one molecule each of the wild-type and mutant Pac1 proteins. Overexpression of the wild-type protein would be required to titrate out the mutant protein and form sufficient numbers of wild-type dimers to rescue the phenotype of the mutant.

Xlrbpa-2 spans 16 bp of dsRNA and interacts with two successive minor grooves and across the intervening major groove on one face of a primarily A-form dsRNA (Ryter & Schultz, 1998). Xlrbpa-2 has a α–β–β–β–α fold that was also observed in the NMR structures of RNase III and the *Drosophila* protein, *staufen* (Bycroft et al., 1995; Kharrat et al., 1995). A short peptide segment just preceding and including the N-terminal portion of the C-terminal α-helix of Xlrbpa-2 provides all of the major groove interactions. Within this short peptide, residues K163, K164, and K167 contact dsRNA directly whereas residue A166 does not. Interestingly, this A residue is one of the most highly conserved residues in dsRBDs (Fig. 5A). In ts138, ts170, and *snm1,* the substitution of a T at the equivalent A residue in Pac1p (amino acid residue 342) causes defects in snRNA 3′-end formation and in rRNA synthesis. Perhaps the conservation of this A residue may reflect a role in positioning the conserved K residues in a conformation favoring interaction with RNA. Substitution of a T residue at this position may sterically hinder this conformation or may affect the interaction of the protein with RNA because of the polar nature of the side chain. In contrast, a hydrophobic side chain may be tolerated at this position as the Rev3 mutation at the homologous position in *E. coli* RNase III, an A-to-V change, suppresses a second site mutation in the ribosomal protein S12 (Nashimoto & Uchida, 1985; Nashimoto et al., 1985).

We noticed that Pac1p cleaves aberrantly within the mature U2 snRNA sequences (Fig. 6). At the concentration used in our experiments (0.045 μg/mL) less than 1% of an *S. pombe* 3′ ETS RNA template was cleaved (our unpubl. results). At a fourfold higher concentration, which is required for efficient cleavage of the 3′ ETS in vitro, a large proportion of U2 precursor RNA is cleaved within the U2 snRNA coding sequences (our unpubl. data). Thus, in vitro, the stem-loop structure in the U2 3′-flanking sequence is a better substrate for Pac1p than is the U2 coding sequence or the 3′ ETS. Many proteins associate with nascent pre-rRNA, and ribonucleoprotein complexes, rather than naked pre-rRNAs, may be the actual cellular substrates for rRNA processing (Pinol-Roma, 1999). In support of this view is the demonstration that the S12 ribosomal protein in *E. coli* interacts genetically with RNase III (Nashimoto & Uchida, 1985; Nashimoto et al., 1985). Possibly, proteins associated with snRNA precursors modulate the specificity of Pac1p and prevent it from cleaving within the mature sequences in vivo.

We have shown that Pac1p is required for the processing of rRNA precursors in *S. pombe* cells and Rotondo et al. (1997) have demonstrated that the 3′ ETS is cleaved by this enzyme in vitro at known in vivo processing sites (Melekhovets et al., 1994). As was shown for the extended U2 transcripts in the *S. cerevisiae rnt*1 mutant (Abou Elela & Ares, 1998), the long U2 transcripts seen in ts138 at the restrictive temperature are polyadenylated (data not shown). Although an RNase III-like protein, Rnt1p, is also required for formation of snRNA 3′ ends and cleavage of the 3′ ETS of rRNA precursors in *S. cerevisiae*, there are some differences in snRNA and rRNA synthesis between the two yeasts. In the *S. cerevisiae rnt1* mutant, the accumulation of U2 and U5L RNAs is reduced at the normal and restrictive temperatures, whereas the processing of rRNA precursors is deficient only at the restrictive temperature (Abou Elela et al., 1996; Chanfreau et al., 1997). In contrast, in the *pac1-A342T* mutants described here, the defects in snRNA and rRNA synthesis are manifest only at the restrictive temperature. Thus the relative affinities of snRNA and rRNA precursors for each RNase III homolog may be different, or the differences between the yeasts may reflect the nature of the mutations within their respective RNase III homologs. The sequence of the *rnt1* mutation has not been published. In *rnt1* cells the steady-state levels of U1, U4, and U5S snRNAs is increased at the restrictive temperature (Chanfreau et al., 1997), whereas in the *pac1-A342T* mutants, the synthesis of these snRNAs is decreased (Fig. 4). Thus, in *S. cerevisiae* there appears to be an alternative pathway for formation of the 3′ ends of RNA polymerase II-transcribed snRNAs. Such a pathway either does not exist or is very minor in *S. pombe,* and 3′-end formation of the major snRNAs in this organism appears to require Pac1p. In addition, at least one other component is involved in snRNA synthesis in *S. pombe*, namely the factor mutated in ts35. As purified Pac1p, alone, can cleave U2 precursors in vitro, the role of the factor mutated in ts35 is not clear and awaits the cloning of the corresponding gene.

Our results show that in *S. pombe*, as in *S. cerevisiae* (Chanfreau et al., 1997; Abou Elela & Ares, 1998), the formation of the 3′ ends of snRNAs can be dissociated from transcription. Studies in plants (Connelly & Filipowicz, 1993) and sea urchins (Wendelburg & Marzluff, 1992) indicate that the two processes may also be separable in these organisms. In contrast, in vertebrates 3′-end formation is intimately coupled to tran-

scription from snRNA promoters (Hernandez & Weiner, 1986; Neuman de Vegvar et al., 1986). However, there are some similarities between the yeast and vertebrate systems. In *S. cerevisiae* (Noble & Guthrie, 1996) and in extracts from *S. pombe* cells (Fig. 7), cleavage of snRNA precursors by an RNase III homolog gives rise to intermediates with short extensions that are subsequently trimmed away. Such intermediates in snRNA metabolism were first observed in mammalian cells (Chandrasekharappa et al., 1983; Madore et al., 1984a, 1984b). Is an RNase III-like enzyme also involved in vertebrate snRNA synthesis? If so, how is the activity of such an enzyme linked to transcription from snRNA promoters? These are among several intriguing questions about snRNA synthesis that have yet to be answered.

MATERIALS AND METHODS

Yeast strains

The *S. pombe* strains used in this work are as follows: FYC1, wild-type strain (972 h^-); SP130 (*h^- ade6-M210 leu1–32*); Strain 445 (*h^+ ade6-M210 leu1–32 ura4-D18*); Strain 446 (*h^- ade6-M210 leu1–32 ura4-D18*); Strain 447 (*h^+ ade6-M216 leu1–32 ura4-D18*); Strain 448 (*h^- ade6-M216 leu1–32 ura4-D18*); Q1309 (*h^- ade6-M616*) and Q1310 (*h^+ ade6-M616*); ts138 (*h^- ade6-M616 ura4-D18 pac1-A342T*); and the *snm1* mutant, JP44 (*h^- leu1–32 snm1-1^{ts}*).

The standard protocols for *S. pombe* were used (Gutz et al., 1984; Moreno et al., 1991; Alfa et al., 1993)

Plasmids

The *S. pombe* U2 snRNA gene, containing 150 nt of 5′ sequence and 64 nt of 3′ sequence, was amplified from genomic DNA by PCR, cloned into the *Bam*HI site in pUC119, and verified by sequencing. The two primers used for PCR were U25′ (5′-ACTGGATCCTTTTTGTTAATTTCATGGCCC-3′), and U23′ (5′-ACGGGATCCCCTCACATCCCAAACCATCCA-3′). The *Bam*HI sites are underlined. The resulting plasmid, pUC-U2, was used to mark the U2 gene by site-directed mutagenesis (Kunkel, 1985) by introduction of a *Sac*I site between nt 40 and 41 in the coding region as described below. The resulting construct, pUC-MU2, contains the marked U2 gene. The insert of pUC-MU2 was then excised and ligated into the *Bam*HI site of the vector pSP1 (Cottarel et al., 1993) to generate the construct pSP-MU2. pSP-U2D was constructed by cloning a *Pst*I-*Xho*I PCR fragment, containing the last 43 nt of the U2 gene and 64 nt of the 3′-flanking sequence, into pSP1. The PCR primers used were 3F5′ (5′-ATGCTGCAGACTGGTTTCTTGCTATT-3′) and 3F3′ (5′-CCACTCGAGACTCACATCCTAAACCATCC-3′), with the *Pst*I and *Xho*I sites underlined. Insertion of the marked U2 gene into the *Bam*HI site of pSP-U2D generated pSP-MU2D.

The construct pSP-U2 3′FL contains a 230-bp U2 3′-flanking sequence PCR fragment cloned between the *Bam*HI and *Sal*I sites of pSP1. The two primers used were U2 3′FLF (5′-ACTGGATCCCTTCTTCTGTTGACAGATTTGTGA-3′), and U2 3′FLR (5′-CACGTCGACCCTCACATCCTAAACCA TCCA-3′) with the *Bam*HI and *Sal*I sites underlined. A DNA fragment containing the 3′ external transcribed spacer (3′ETS) of the *S. pombe* ribosomal RNA gene was also obtained by PCR and cloned into the *Bam*HI site of pSP1. The resulting plasmid was named pSP-ETS. The two PCR primers were ETS5′ (5′-ACTGGATCCTCCATTAAGGAACCATCATCT-3′), and ETS3′ (5′-ACTGGATCCTTCCCCCTTCCTCCTCTCC TGCTT-3′); the *Bam*HI sites are underlined.

The *pac1* genes from the wild-type strain SP972, ts35, ts138, ts170, and *snm1* strains were amplified by PCR and sequenced on both strands using the oligonucleotides PACF1 (5′-TACAGCATTCTTGGATACTT-3′), PACF2 (5′-CCGCCA TTACCACCTTTAC-3′), PACR1 (5′-GACCTTTTAACGATCG AAACC-3′) and PACR2 (5′-GGCAGAATAACTGAGGACAA-3′). The *pac1*$^+$ gene was excised from plasmid pIRT31-*pac1*$^+$ (Rotondo et al., 1995) by digestion with *Sac*I and *Hin*dIII, and subcloned into vector pDblet (Brun et al., 1995). The resulting plasmid was called pD*pac1*$^+$. To integrate the wild-copy *pac1*$^+$ gene into the genome of ts138, the *ars* fragment in the plasmid pD*pac1*$^+$ was removed by *Bam*HI digestion and the remaining portion of the plasmid was religated. This plasmid, named *pac1*$^+$-INT, was then digested with *Xba*I and used to transform ts138 cells.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed by the method of Kunkel (1985). The plasmids pUC-U2 and pUC-MU2 were used to make uracil-substituted single-stranded DNA templates. The oligonucleotide used to mark the U2 gene was 5′-CAAGTGTAGTATGAGCTCTGTTCTTTTCAG TT-3′; the *Sac*I site is underlined. The oligonucleotides used to introduce clustered point mutations into the U2 3′-flanking region contained a *Spe*I site (underlined) to facilitate identification of the mutants. Their sequences were as follows:

M1: 5′-GACGCCGAATTACTAGTGGTTGACAGA-3′;
M2: 5′-CCGAATCTTCTTCTTACTAGTAATTTGT G-3′;
M3: 5′-CTTCTGTTGACAGGACTAGTCCGCATGAA-3′;
M4: 5′-GACAGATTTGTGAATACTAGT CTTGACAAC-3′;
M5: 5′-GTGACGCATGAATGGACTAGTATTGGATGG-3′;
M6: 5′-GAATTTGACAAC GACTAGTCCTTTAGGATG-3′;
M7: 5′-GACAACATTGGATGGCGACTAGTGTGAGG-3′.

Oligonucleotides used to make mutations compensatory to those in M2 and M3 were M2C: 5′-GTGACGCATGAATT ACAAGTAATTGGATGG-3′ and M3C: 5′-ACTAGTACGATA GTCTTGACAACATTGGATGG-3′. All the mutations were confirmed by DNA sequencing. The linker-scanning and compensatory mutant U2 genes were then excised and subcloned into plasmid pSP-U2D.

Isolation of ts mutants

A bank of 200 ts mutants was made by mutagenizing strain SP130 with *N*-methyl-*N*″-nitro-*N*-nitrosoguanidine (MNNG). Mutagenizing the same strain with ethylmethane sulfonate (EMS) made another bank of 50 mutants. In each case the fraction of viable cells after exposure to the mutagen was 20%. A bank of 150 ts mutants, also generated by EMS mu-

tagenesis, was kindly provided by Dr. Dhar (Brown et al., 1995). These banks of ts mutants were screened for defects in 3′-end formation at the restrictive temperature as described below. Genetic analysis was performed using standard techniques (Gutz et al., 1984; Moreno et al., 1991; Alfa et al., 1993).

Yeast transformation and RNA isolation

To identify the sequence element for U2 3′-end formation, DNA constructs were transformed into *S. pombe* cells by the alkali cation method (Okazaki et al., 1990). *S. pombe* cells were grown in selective minimal medium to mid-log phase and total RNA was purified using the hot acidic phenol method (Ausubel et al., 1993).

To screen the banks of ts mutants, each strain was inoculated into 10 mL of YEA medium and grown to mid-log phase at 25 °C. Half of the culture was then shifted to 36 °C for 5 h, and the remaining half was grown at 25 °C for 5 h. Subsequently the cells were pelleted by centrifugation, frozen, and total cellular RNA was prepared as described above.

Analysis of RNA

The probes described in the figure legends were used to analyze the 3′ ends of U2 transcripts by RNase T1 protection assays according to the protocol of Lobo and Hernandez (1989). The 5′ ends of the U2 transcripts were mapped by primer extension using AMV reverse transcriptase (Lobo & Hernandez, 1989). The oligonucleotide used for primer extension, 5′-AACTGAAAAGAACAGAG-3′, was complementary to bases 44–60 of the marked U2 gene. The RNase T1 protection assay was also used to screen the banks of ts mutants to identify mutants that are defective for U2 snRNA synthesis at the restrictive temperature, and to analyze the effect of temperature shift on the processing of *S. pombe* ribosomal RNA precursors.

snRNAs from ts mutants were also analyzed by Northern hybridization. Fifteen micrograms of total RNA from each mutant grown at either 25 °C or shifted to 36 °C for 5 h was fractionated on a 10% polyacrylamide-8 M urea gel. The RNAs were then transferred to Zeta-bind GT (Bio-Rad) membranes by semi-dry electroblotting. The hybridization and washing of the blots was performed as described (Potashkin & Frendewey, 1990).

In vitro 3′-end processing

T3 RNA polymerase was used to synthesize U2 precursor RNA with a 3′ extension, using a PCR product as template. The U2 PCR product extended from 4 nt upstream of the U2 coding sequence to nt +64 in the 3′-flanking region, with an additional 9 nt of non-U2 gene sequence derived from the vector. The two PCR primers were PRL5′ (5′-ATTAACCCTCACTAAAGGGAATTCTCTCTTTGCCTTTTGGCT-3′) and U2 3′FR (5′-CACGTCGACCCTCACATCCTAAACCATCCA-3′). The RNA was labeled by incorporation of α-^{32}P CTP (3,000 Ci/mmol) in the transcription reaction. *S. pombe* whole-cell extracts were prepared as described (Woontner & Jaehning, 1990; Woontner et al., 1991). The purification of HIS-Pac1p was described previously (Rotondo et al., 1997). In vitro processing was done at 30 °C in a total volume of 20 μL containing 3 μL *S. pombe* whole-cell extract (150 μg protein), purified Pac1p (0.36 μg/mL), 30 mM Tris-HCl at pH 7.4, 2.5 mM dithiothreitol (DTT), 2.0 mM ATP, and 5 mM $MgCl_2$. At different time points 5-μL aliquots were withdrawn and added to 200 μL of Stop Buffer [200 mM NaCl, 25 mM ethylene diamine tetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS)]. RNAs were then extracted with phenol-chloroform, precipitated with ethanol and analyzed on 6% acrylamide-8 M urea gel.

In vitro cleavage by Pac1p

Internally labeled U2 precursor (30,000 cpm), synthesized by T3 RNA polymerase as described above, was incubated at 30 °C with purified Pac1p (0.045 μg/mL) in a 20-μL reaction that contained 30 mM Tris-HCl (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 1 μM poly(C). At different time points 5-μL aliquots were added to 5 μL of gel-loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 12 mM EDTA). The RNAs were denatured by boiling for 1 min and loaded on a 6% acrylamide-8 M urea gel. To map the Pac1p cleavage sites in the U2 3′-flanking region, unlabeled U2 precursor was synthesized and incubated with purified Pac1p. The products were then extracted with phenol-chloroform, precipitated with ethanol, and used for primer extension by AMV reverse transcriptase using the oligonucleotide U23′FR (described above). A reference DNA sequence was generated with the same oligonucleotide.

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