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Depletion of yeast RNase III blocks correct U2 3′ end formation and results in polyadenylated but functional U2 snRNA

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Yeast U2 snRNA is transcribed by RNA polymerase II to generate a single non-polyadenylated transcript. A temperature-sensitive yeast strain carrying a disruption in *RNT1*, the gene encoding a homolog of RNase III, produces 3′-extended U2 that is polyadenylated. The U2 3′-flanking region contains a putative stem–loop that is recognized and cleaved at two sites by recombinant GST–Rnt1 protein *in vitro*. Removal of sequences comprising the stem–loop structure blocks cleavage *in vitro* and mimics the effects of Rnt1 depletion *in vivo*. Strains carrying a U2 gene lacking the Rnt1 cleavage site produce only polyadenylated U2 snRNA, and yet are not impaired in growth or splicing. The results suggest that eukaryotic RNase III may be a general factor in snRNA processing, and demonstrate that polyadenylation is not incompatible with snRNA function in yeast.

Keywords: dsRNA/poly(A)/RNA processing/ *Saccharomyces cerevisiae*/snRNA

Introduction

Five small nuclear RNAs (U1, U2, U4, U5 and U6 snRNA) are essential components of the mRNA splicing apparatus (Madhani and Guthrie, 1994; Ares and Weiser, 1995; Staley and Guthrie, 1998). All except U6 are transcribed by RNA polymerase II to produce primary transcripts with 7-methylguanosine (^{7}mG) caps at their 5′ ends, similarly to mRNA transcripts. Unlike mRNA, the 3′ ends of snRNA are not polyadenylated (for review, see Guthrie and Patterson, 1988). In metazoans, snRNA 3′ end formation is influenced by promoter elements and requires specific template sequences downstream of the mature 3′ end (Hernandez, 1985; Yuo *et al*., 1985; Hernandez and Weiner, 1986; Neuman de Vegvar *et al*., 1986; Neuman de Vegvar and Dahlberg, 1990). Once transcribed, the snRNAs are exported to the cytoplasm where they associate with the Sm proteins through a short U-rich sequence called the Sm site, to form small nuclear ribonucleoprotein particles (snRNPs; for review, see Guthrie and Patterson, 1988; Mattaj, 1988; Zieve and Sauterer, 1990). While in the cytoplasm, the snRNA is subject to additional methylation, producing a 2,2,7-trimethylguanosine (TMG) cap, and is imported back to the nucleus where it may associate with additional snRNP-specific proteins to form functional snRNP complexes (Ségault *et al*., 1995).

In yeast, the general features of snRNP biogenesis appear to be conserved. Yeast U1, U2, U4 and U5 snRNAs have TMG caps and are not polyadenylated at their 3′ ends (Wise *et al*., 1983; Ares, 1986; Guthrie and Patterson, 1988). Yeast snRNAs have Sm sequences that are critical for their biogenesis and function (McPheeters *et al*., 1989; Jones and Guthrie, 1990). Homologs of vertebrate Sm proteins have been identified (Rymond *et al*., 1993; Hermann *et al*., 1995; Seraphin, 1995), and shown to support assembly of functional yeast snRNPs *in vivo* (Rymond *et al*., 1993; Roy *et al*., 1995). Unlike the situation in metazoans, mRNA promoter substitutions show that yeast snRNA 3′ end formation does not require special snRNA promoter elements (Patterson and Guthrie, 1987; Miraglia *et al*., 1991; Seraphin *et al*., 1991). Internal snRNA sequences near the 3′ end influence the position and efficiency of 3′ end formation of U2 (Igel and Ares, 1988; Shuster and Guthrie, 1988), but no studies have systematically addressed the role of the 3′-flanking sequences in yeast snRNA 3′ end formation.

Recently, the analysis of mutations that perturb snRNA 3′ end formation in yeast have implicated eukaryotic homologs of bacterial RNase III as *trans*-acting factors in 3′ end formation. In *Schizosaccharomyces pombe* (fission yeast), a mutation (*snm*) that affects the biogenesis and 3′ end formation of several snRNA is suppressed by *pac1*$^+$, a member of the RNase III gene family (Potashkin and Frendewey, 1990; Rotondo *et al*., 1995). In *Saccharomyces cerevisiae*, the disruption of *RNT1* (the budding yeast homolog of the RNase III gene; Abou Elela *et al*., 1996) results in reduced levels of the large form of U5 (U5L) and U2 snRNA in the cell (Chanfreau *et al*., 1997). Further analysis of the processing pathway *in vivo* and *in vitro* revealed that U5L 3′ end formation depends on Rnt1 cleavage in the 3′-flanking sequence (Chanfreau *et al*., 1997). An alternative pathway results in production of the shorter U5 form (U5S) when Rnt1 is depleted (Chanfreau *et al*., 1997). Here we analyze the role of Rnt1 in the processing pathway of U2 snRNA. We show that Rnt1 cleaves a stem–loop structure near the 3′ end of U2, leading to the formation of the correct U2 3′ end. Disruption of *RNT1* leads to the accumulation of extended, polyadenylated U2 snRNA transcripts *in vivo*. Yeast strains carrying a U2 gene from which the Rnt1 processing site has been deleted produce only polyadenylated U2 at normal levels, and show no splicing or growth defects *in vivo*.

Results

Disruption of RNT1 is not lethal, but affects U2 3′ end formation in vivo

Our previous analysis of the *RNT1* gene indicated that the gene is essential, based on tetrad dissections and incubation

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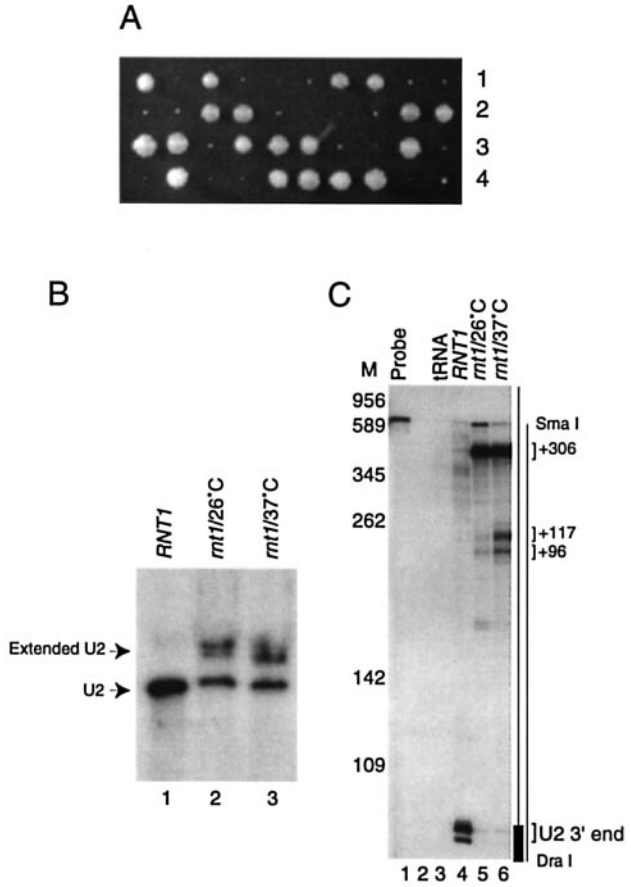

Fig. 1. *In vivo* analysis of U2 snRNA processing in cells carrying a disrupted *RNT1* gene. (**A**) Tetrad dissection of cells carrying the *rnt1* deletion allele lacking the conserved nuclease and double-stranded RNA-binding domains. The cells were dissected on rich media and incubated at 26°C for 7 days. (**B**) Northern blot analysis of U2 snRNA transcripts. The RNA was extracted from wild-type cells (*RNT1*), cells carrying an insertional disruption of the *RNT1* gene at permissive temperature (*rnt1*/26°C), or cells carrying an insertional disruption of the *RNT1* gene at restrictive temperature (*rnt1*/37°C). The RNA was separated on a 4% polyacrylamide gel, transferred to a nylon membrane and hybridized to a radiolabeled oligonucleotide complementary to the U2 5′ end. The position of normal and extended forms of U2 snRNA is indicated on the left. (**C**) Mapping the 3′ end of U2 snRNA using an RNase protection assay. The RNA was extracted from *RNT1*, *rnt1*/26°C or *rnt1*/37°C, hybridized to a probe complementary to the 3′ end of U2 snRNA and 3′-flanking sequences, and digested with RNase T1. The probe was also hybridized to *E.coli* tRNA (tRNA) as a control. Protected fragments were fractionated on a polyacrylamide gel and autoradiographed. The positions of the probe, mature U2 3′ end and extended U2 snRNA are indicated on the right.

at 30°C (Abou Elela *et al*. 1996). Subsequently we found that strains carrying the original *rnt1* disruption, which retains the potential to encode a partially functional protein, could grow at 26°C (Chanfreau *et al*. 1997). To eliminate the possibility that the viability of the *rnt1* insertional disruption is due to residual activity, we created a *rnt1* deletion allele lacking the conserved nuclease and double-stranded RNA-binding domains characteristic of RNase III homologs. We introduced this deletion disruption into a diploid strain, and allowed dissected tetrads to grow on rich medium at 26°C for 7 days. The appearance and co-segregation of a tiny colony phenotype with the His$^+$ marker (Figure 1A) demonstrate that *rnt1* deletion is not lethal, but severely compromises the growth of yeast. These results indicate that, although there are no strictly essential Rnt1 functions, presumably due to alternative but less efficient processing pathways, wild-type growth rates require Rnt1 activity.

In *S.cerevisiae*, the synthesis of U5L and U2 snRNAs is reduced in cells depleted of Rnt1 (Chanfreau *et al*., 1997, see Materials and methods). Extended forms of U2 RNA can be seen in RNA extracted from *rnt1* disruption strains, suggesting that Rnt1 functions in the processing of U2 snRNA (Figure 1B; Chanfreau *et al*., 1997). To determine how U2 processing is affected by the absence of Rnt1, we analyzed the 5′ and 3′ ends of U2 extracted from the *rnt1* disruption strain and compared them with U2 RNA from wild-type cells. Annealing and extension of a primer complementary to U2 showed that RNA from the disruption strain has the correct U2 5′ end (Ares, 1986; data not shown), suggesting that the longer forms of U2 observed in the absence of Rnt1 are the result of 3′ end extension.

To analyze the 3′ end of U2 in the *rnt1* disruption, we performed an RNase protection assay using a probe that spans the 3′ end of U2 and includes sequences downstream (Ares, 1986; Figure 1C). RNA from wild-type cells protects the probe at positions corresponding to the mature 3′ end of U2 snRNA. On the other hand, RNA extracted from *rnt1* disruption cells at both restrictive and permissive temperatures showed three major bands corresponding to U2 RNA extended past the mature 3′ end approximately to positions +96, +117 and +306, as well as two faint bands corresponding to the mature 3′ end. We conclude that efficient U2 3′ end formation at the normal site requires Rnt1. In the absence of Rnt1 activity, 3′ ends are formed efficiently further downstream.

The 3′-extended U2 snRNA formed in the absence of Rnt1 is polyadenylated

The observed polyadenylation of the snRNA-like telomerase RNA of yeast (Chapon *et al*., 1997) and analyses of the 3′-flanking sequences of the U2 gene suggested that the extended U2 might be polyadenylated. To test this, we performed oligo(dT)–cellulose chromatography to separate poly(A)$^+$ and poly(A)$^-$ RNA, and looked at the distribution of correct and extended U2 3′ ends in the fractions using the RNase protection assay (Figure 2A). RNA from wild-type cells contains U2 with the mature 3′ end in the poly(A)$^-$ fraction, and no signal was seen in the poly(A)$^+$ fraction (Figure 2A). RNA from *rnt1* disruption cells has a small amount of correctly matured U2 in the poly(A)$^-$ fraction, whereas the three extended forms of U2 snRNA appear in the poly(A)$^+$ fraction (Figure 2A). Since there are no contiguous runs of A residues encoded in the U2 flanking sequence that could explain the association of extended but non-polyadenylated U2 with oligo(dT)–cellulose, the extended forms of U2 observed in the *rnt1* disruption cells must be polyadenylated.

Recombinant GST–Rnt1 cleaves a stem–loop structure at the 3′ end of U2

A previously identified dyad symmetry in the 3′-flanking sequences of the U2 gene (Ares, 1986) could allow 3′-extended U2 precursors to fold into a stem–loop structure that might be recognized and cleaved by Rnt1. To test this, we incubated RNA transcripts containing the U2

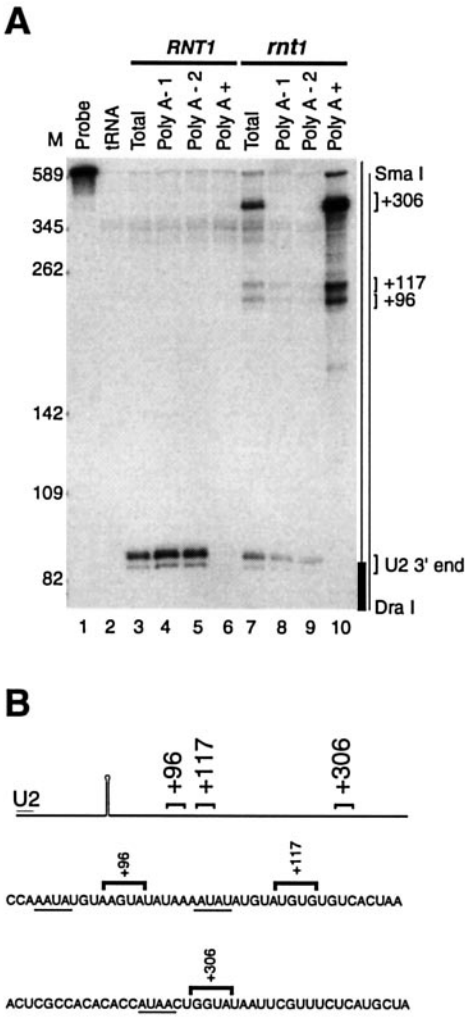

Fig. 2. Disruption of *RNT1* leads to polyadenylation of U2 snRNA. (**A**) Mapping the 3′ end of U2 snRNA in the poly(A)$^-$ and poly(A)$^+$ RNA fractions. Total RNA was extracted from wild-type (lanes 3–6) or *rnt1* disruption cells (lanes 7–10) at permissive temperature, fractionated on an oligo(dT) column, and subjected to the RNase protection assay described in Figure 1. Lane 1 contains undigested probe, lane 2 shows the probe protected after hybridization to tRNA, lanes 3 and 7 show the probe protected by total (unfractionated) RNA, lanes 4 and 8 show the probe protected by the flow-through RNA (poly A$^-$ 1), lanes 5 and 9 show the probe protected by the first wash of the column (poly A$^-$ 2), and lanes 6 and 10 show the probe protected by the eluted fraction (poly A$^+$). The probe, positions of the mature U2 3′ end and the approximate sites of 3′ end formation in the extended forms of U2 snRNA are presented on the right. (**B**) Sequence of the 3′-flanking sequences of the U2 gene. Underlined sequences represent putative poly(A) signals. The brackets indicate the approximate sites of 3′ end formation as determined by the RNA protection assay.

flanking sequences with purified GST–Rnt1 protein (Abou Elela *et al*., 1996) *in vitro*. A synthetic radiolabeled pre-U2 snRNA extending from within the U2 coding region (93 nucleotides from the mature 3′ end) to a position 485 residues downstream was transcribed *in vitro*, gel purified, incubated with increasing amounts of GST–Rnt1 protein and fractionated on a denaturing polyacrylamide gel (Figure 3A). This transcript was cleaved efficiently, generating three products (P1, P2 and P3). The largest fragment (P1) is derived from the 3′ end of the substrate. The second product (P2) represents the 5′ end fragment containing U2 coding sequences plus a small segment of 3′-flanking sequence. The third product (P3) is formed by two cuts on either side of the stem–loop structure (see below).

To map the cleavage sites, we prepared unlabeled pre-U2 substrate and incubated it with GST–Rnt1. The cleavage products were subjected to primer extension using a primer complementary to U2 3′-flanking sequences from position +81 to +97 (relative to the mature end). The primer extension products were compared with a DNA sequence ladder of the U2 3′-flanking sequences generated with the same primer (Figure 3B). Two pairs of closely spaced cleavage sites are observed after incubation with GST–Rnt1. The 5′-most cleavages occur at positions +26 and +27 based on comparison of the reverse transcription products with the sequence. The downstream cleavages occur at positions +61 and +62. Our ability to detect the +26/+27 site using a primer downstream of position +62 indicates that in a fraction of the molecules, the +26/+27 site is cut before the site at +61/+62, although we do not know if this represents an obligatory order of cleavage. These data suggest that Rnt1 cleaves in the stem–loop structure near the U2 3′ end during formation of the natural 3′ end. Since Rnt1 does not produce mature 3′ end *in vitro*, it is likely that other factors (e.g. an exonuclease) are required to generate the mature end.

To determine substrate requirements for Rnt1 cleavage of pre-U2 RNA *in vitro*, we tested a series of transcripts ending beyond or to within sequences required to form the stem–loop structure. Transcripts ending at positions +194, +124, +54 and +42 relative to the mature 3′ end were incubated with GST–Rnt1 protein and fractionated on a denaturing polyacrylamide gel (Figure 4A). As predicted, the RNAs that include the intact stem–loop structure (Figure 4B; +194 and +124 transcripts) are cut into three fragments consistent with cleavage at positions +26/+27 and +61/+62. Surprisingly, the +54 transcript lacking the lower 3′ end half of the stem but retaining the loop and upper stem sequence is cleaved detectably near position +26/+27 (Figure 4B). A shorter substrate lacking the 14 bases forming the top of the structure is not cleaved. Rnt1 is similar to bacterial RNase III in that it requires a double-stranded RNA region to recognize the substrate but can cut at unpaired sites (Chelladurai *et al*., 1991; Abou Elela *et al*., 1996; Chanfreau *et al*., 1997). The cleavage we observe is unusual because a duplex region is thought to be required on both sides of the cleavage site(s). Unless another part of the substrate is folded in to replace the deleted sequence, this cut suggests that key duplex-binding contacts are present 3′ to the cut site. In any case, a minimum of 54 bases beyond the mature U2 3′ end are required for cleavage of pre-U2 by Rnt1 *in vitro*. Efficient cleavage may require the complete stem–loop structure.

Deletion of the stem–loop structure prevents correct 3′ end formation and causes polyadenylation of U2

To examine the requirement for the stem–loop structure at the 3′ end of U2 for accurate processing *in vivo*, we constructed a U2 gene lacking the entire stem. Sequences containing the stem–loop structure from position +15 to position +68 were replaced by 6 bp, introducing a unique

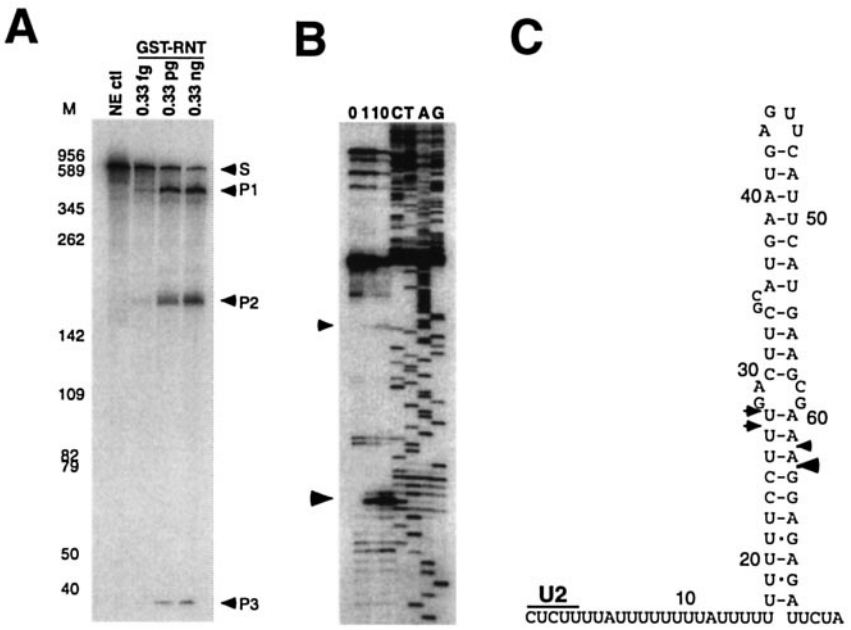

Fig. 3. Rnt1 cleaves a model pre-U2 substrate *in vitro*. (**A**) *In vitro* cleavage of pre-U2 snRNA by purified GST–Rnt1 protein. RNA substrates spanning position –96 to +479 (relative to the mature U2 3′ end) were produced using T3 RNA polymerase and incubated in the absence of the enzyme (NE ctl) or with increasing concentrations of GST–Rnt1 protein. The initial substrate (S) and products (P1, P2 and P3) are indicated on the right. (**B**) Mapping the site of cleavage using primer extension. Primer complementary to the 3′-flanking sequence of U2 snRNA was extended on pre-U2 substrate RNA after incubation with GST–Rnt1 protein for 0, 1 or 10 min. The reference DNA sequence (produced using the same primer) is shown on the left. The product corresponding to the cleaved RNA is indicated by the large arrowhead on the left. Note that bands corresponding to Rnt1 cleavage increase with incubation time while reverse transcription stop sites do not. (**C**) Model of the RNA structure downstream from the mature U2 3′ end. Rnt1 cleavage sites are indicated by arrows.

restriction site. The plasmid carrying the entire U2 gene lacking the stem (pRS315-U2Δstem) was transformed into a plasmid shuffling strain carrying wild-type U2 on a URA3 plasmid. After plasmid shuffling, cells containing only the U2Δstem gene grew normally, indicating that the stem–loop deletion has little effect on U2 function (see below). The effect of the stem–loop deletion on U2 3′ end formation was analyzed using Northern blots and an RNase protection assay. On Northern blots (Figure 5A), RNA from wild-type cells (lane 1) or cells carrying the pRS315-U2 wild-type gene (lane 2) show a single band corresponding to mature U2 snRNA. RNA from the *rnt1* disruption cells (lane 4) contains reduced amounts of mature U2 and abundant extended U2 (see also Figure 1B). Surprisingly, RNA from cells carrying the U2Δstem mutant gene (lane 3) express primarily extended forms of U2, and little detectable correctly matured U2. This result shows that sequences containing the stem–loop structure in the 3′-flanking sequences of the U2 gene are required for correct U2 3′ end formation *in vivo*.

To map the 3′ end formed in the absence of the stem–loop structure, RNA was extracted from cells carrying pRS315-U2 or pRS315-U2Δstem constructs and subjected to an RNase protection assay using a probe derived from the U2Δstem construct (Figure 5B). RNA from cells carrying the wild-type U2 construct produces two bands; the major band corresponds to the correct U2 3′ end. The minor band appears at the position where the U2Δstem probe diverges from the sequence of wild-type RNA and represents the sum total of wild-type U2 transcripts that extend beyond the stem–loop deletion site in the probe. RNA from cells carrying the U2Δstem mutant gene produces four bands corresponding to 3′-extended forms of U2. After considering the size difference due to the stem–loop deletion, we estimate that three of these bands correspond to the sites of polyadenylation mapped in RNA extracted from *rnt1* disruption cells (Figure 1C). The new site of 3′ end formation near +83 may have been activated by sequence context effects due to the proximity of the deletion end point at position 68. The residual amount of mature U2 snRNA in *rnt1* insertional disruption cells (Figure 1B) and its greatly reduced level in cells carrying the U2Δstem mutant suggest either that the *rnt1* insertional disruption produces residual Rnt1 activity, or that a second mechanism of U2 3′ end formation exists that utilizes the sequences within the stem–loop. The very small amount of correctly formed U2 in U2Δstem mutant (seen in Figure 6, lane 6) indicates that a very inefficient mechanism for forming the correct 3′ end exists. We conclude that sequences containing the putative stem–loop in the U2 3′-flanking region are essential for efficient formation of correct U2 3′ ends *in vivo*.

To determine whether the extended U2 transcripts derived from the U2Δstem gene were polyadenylated, we separated total RNA into poly(A)$^-$ and poly(A)$^+$ fractions and subjected them to the RNase protection assay (Figure 6). RNA from cells expressing the U2Δstem mutant gene produces an extremely small amount of correctly terminated poly(A)$^-$ U2. All four of the abundantly expressed extended forms, including the newly activated site around +83, are polyadenylated. These results show that blocking Rnt1 cleavage of pre-U2 snRNA by deletion of the stem–loop cleaved by Rnt1 leads to polyadenylation of extended U2 snRNA.

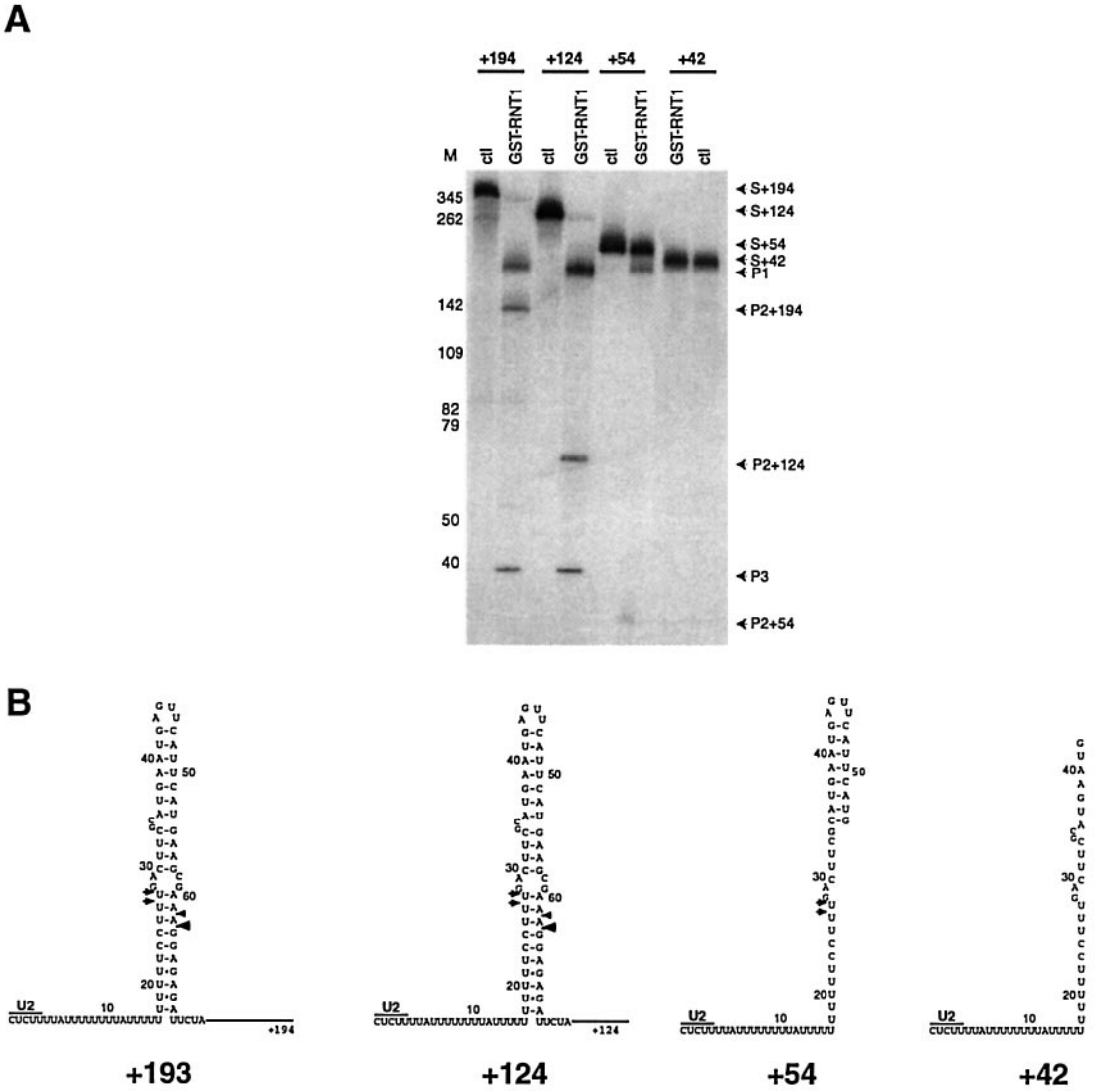

Fig. 4. Contribution of pre-U2 3′-flanking sequences to cleavage by GST–Rnt1. (**A**) *In vitro* cleavage of model pre-U2 substrates containing progressive 3′ deletions. Radiolabeled RNA substrates were produced by T7 RNA polymerase transcription using DNA templates ending at positions +193, +124, +54 and +42. The different substrates were incubated in the absence (ctl) or presence (GST–Rnt1) of GST–Rnt1 protein for 10 min, phenol extracted and loaded on a polyacrylamide gel. Initial substrate (S) and the different products (P1, P2 and P3) are indicated on the right. (**B**) Model for the RNA structure formed by the different pre-U2 substrates. Pairing of G+54 could occur with either C+35 (as shown in the +54 model) or C+33 (as shown in the +193 and +124 models). Rnt1 cleavage sites are indicated by arrows.

Polyadenylation of U2 does not affect splicing or cell growth

To examine the functional effect of U2 3′ end polyadenylation, we looked for splicing or growth defects in cells expressing the U2Δstem gene, in which virtually all of the U2 RNA is polyadenylated (see Figure 6). We grew cells carrying pRS315-U2 or pRS315-U2 Δstem constructs at 18, 26 or 37°C. Surprisingly, no growth defects were detected in the cells producing polyadenylated U2 snRNA (Figure 7A). We have also compared the splicing efficiency of U3 snoRNA in cells carrying the wild-type U2 plasmid with those carrying the U2Δstem mutant and observe no difference (Figure 7B). Based on several observations (Seraphin and Rosbash, 1989; Wells and Ares, 1994), growth of yeast is reduced when wild-type U2 RNA levels are reduced <10 fold, arguing that the small residual amount of correctly terminated U2 (Figure 6) is unlikely to be supporting growth of the U2Δstem mutant. These data indicate that there is little if any functional consequence of the polyadenylation of U2 snRNA in yeast.

Discussion

Yeast U2 snRNA is transcribed normally as a single non-polyadenylated RNA (Ares, 1986). We have found that U2 3′ end formation requires the eukaryotic RNase III-like enzyme Rnt1. Depletion of yeast Rnt1 *in vivo* results in the accumulation of extended and polyadenylated U2 snRNA (Figures 1 and 2). Recombinant yeast Rnt1 cleaves a stem–loop structure downstream of the mature U2 3′ end *in vitro* (Figures 3 and 4). *In vivo*, expression of a U2 gene lacking the stem–loop produces polyadenylated U2 snRNA (Figure 6). Despite the nearly complete polyadenylation of the U2 population in the cell, no defects in mRNA splicing or cell growth could be detected (data not shown). The involvement of yeast Rnt1 in the formation

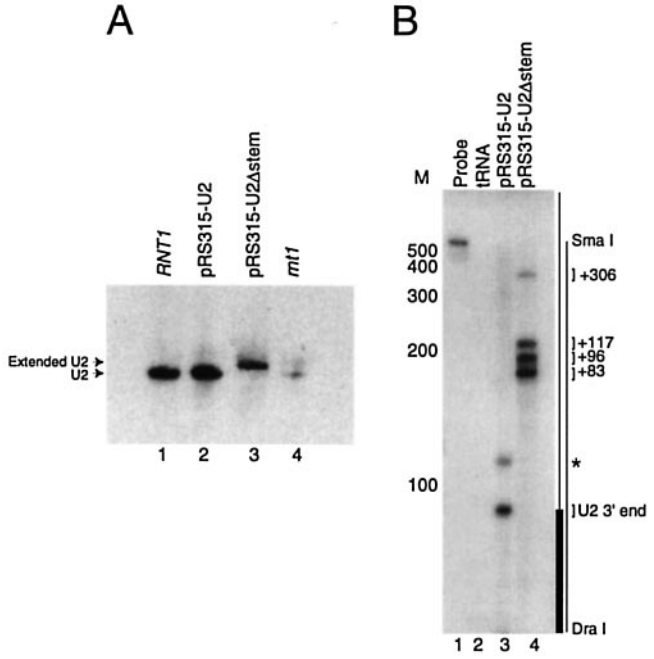

Fig. 5. Sequences including the 3′-flanking stem–loop are required for correct 3′ end formation *in vivo*. (**A**) Northern blot of U2 snRNA transcripts. RNA was extracted from wild-type cells (lane 1, *RNT1*), cells dependent on U2 snRNA expressed from plasmid pRS315 carrying a wild-type U2 gene (lane 2, pRS315-U2), cells dependent on U2 snRNA expressed from a plasmid carrying a U2 gene lacking the stem–loop sequences in the 3′ flank (lane 3, pRS315-U2Δstem) and cells carrying a disrupted *RNT1* gene (lane 4, *rnt1*), run on a 4% acrylamide gel, blotted and hybridized to radiolabeled oligonucleotide complementary to the 5′ end of U2 snRNA. Normal and extended forms of U2 snRNA are indicated on the left by arrows. (**B**) Mapping 3′ ends of U2 snRNA produced from the U2Δstem mutant gene. RNA from pRS315-U2 (lane 3) or pRS315-U2Δstem cells (lane 4) was extracted, hybridized to an RNA probe complementary to the 3′ end and flanking sequences of the Δstem mutant (indicated at right), and digested with RNase T1. The mature U2 3′ end and the ends of the extended forms are indicated with brackets on the right. Numbering of positions of the extended forms is indicated using the wild-type U2 sequence. The asterisk indicates cleavage due to sequence divergence between the U2Δstem probe and transcripts from the wild-type U2 gene. Undigested probe (lane 1) and probe digested after hybridization to tRNA (lane 2) are included as controls.

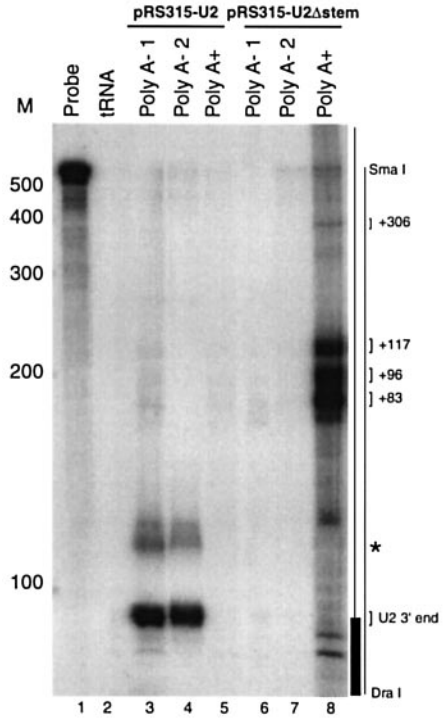

Fig. 6. Deletion of the Rnt1 cleavage sites leads to polyadenylation of U2 snRNA. Total RNA extracted from pRS315-U2 (lanes 3–5) or pRS315-U2Δstem cells (lanes 6–8) was fractionated on oligo(dT)–cellulose, and both poly(A)$^-$ ('flow-through', poly A$^-$ 1, lanes 3 and 6; 'first wash', lanes 4 and 7) and poly(A)$^+$ RNA fractions (lanes 4 and 8) were subjected to the RNase protection assay as in Figure 5. The mature U2 3′ end and the ends of the extended forms are indicated by brackets on the right. The numbering of positions of the extended forms is indicated using the wild-type U2 sequence. The asterisk indicates cleavage due to sequence divergence between the U2Δstem probe and transcripts from the wild-type U2 gene. Undigested probe (lane 1) and probe digested after hybridization to tRNA (lane 2) are included as controls.

of U2 snRNA 3′ ends identifies this enzyme as a *trans*-acting factor in U2 biogenesis. These results suggest a general role for RNase III-like enzymes in snRNA biogenesis.

3′ End formation of both U2 and U5L spliceosomal snRNA is mediated by Rnt1

Rnt1 recently was shown to cleave a stem–loop structure within pre-U5 transcripts, leading to the formation of U5L snRNA (Chanfreau *et al*., 1997). Here we add pre-U2 snRNA to the list of eukaryotic RNase III substrates and suggest snRNA processing as a general role for this enzyme in addition to its role in pre-rRNA processing (Abou Elela *et al*., 1996). In each of these cases, the role of Rnt1 seems limited to the production of site-specific cleavages to generate processing intermediates, which are trimmed later to the mature ends, possibly by exonucleases. The cleavage of Rnt1 in both pre-U5 and pre-U2 snRNA occurs within sequences able to form a stem–loop structure downstream of the mature 3′ end. These sequences are

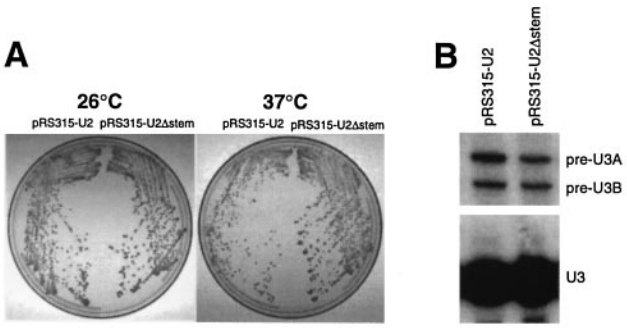

Fig. 7. Polyadenylation of U2 snRNA does not affect cell growth or splicing of pre-U3 RNA. (**A**) Deletion of Rnt1 cleavage sites in pre-U2 RNA does not cause an obvious growth defect. Cells dependent on a wild-type U2 gene (pRS315-U2, left half of each plate) or the mutant U2 gene lacking the Rnt1 cleavage sites (pRS315-U2Δstem, right half of each plate) were grown on rich medium (YEPD) at 26°C (left plate) and 37°C (right plate). (**B**) Deletion of Rnt1 cleavage sites in pre-U2 RNA does not cause an obvious splicing defect. RNA was extracted from cells carrying pRS315-U2 (left lanes, top and bottom panels) or pRS315-U2Δstem cells (right lanes, top and bottom panels) annealed to a primer complementary to the second exon of U3A and U3B snoRNA and extended with reverse transcriptase. Bands corresponding to unspliced pre-U3A and pre-U3B (top panel) and spliced U3 (U3A+U3B, bottom panel) are indicated on the right. The two panels represent two different exposure times of the same gel (the top panel was exposed for 4 days, the bottom panel overnight).

necessary and sufficient for efficient Rnt1 cleavage of pre-U2 (Figures 3 and 5) and pre-U5 (Chanfreau *et al*., 1997).

Rnt1 cleavage of a stem–loop structure downstream of U2 snRNA and U5 snRNA mature 3′ ends leads to two different end products. In the case of U5 snRNA, the choice of cleavage site by Rnt1 on either side of the stem–loop structure leads to the production of the U5S or U5L snRNA (Chanfreau *et al*., 1997). In contrast, cleavage of the U2 stem–loop leads to a single U2 3′ end. This difference may be due the nature of the structure of these two substrates: removal of the bottom 3′ half of the U5 stem–loop appears to inactivate further cleavage of the substrate by Rnt1 (Chanfreau *et al*., 1997). On the other hand, a series of deletions within the U2 stem indicates that only 8 bp from the top of the stem are required for cleavage at the +25/+26 site in the pre-U2 substrate (Figure 4). This observation, plus the efficient generation of doubly cleaved pre-U2 substrate (Figures 3 and 4), suggests that, unlike U5, U2 precursor cleaved at either the distal or proximal site eventually will produce the same mature 3′ end, after the action of a still undefined exonuclease.

Evolutionary variation in snRNA 3′ end formation mechanisms

Our results extend and reinforce the conclusion that 3′ end formation of yeast snRNA is generally an RNA processing event, rather than a transcription termination event, as appears to be the case in metazoans. In vertebrates, 3′ end formation is linked to transcription initiation and depends on the promoter sequence (Hernandez and Weiner, 1986; Neuman de Vegvar *et al*., 1986). The differences in the mechanism of snRNA 3′ end formation between yeast and vertebrates may be a reflection of different demands for mature snRNA. The splicing machinery in vertebrates is required to process much higher numbers of intron-containing mRNAs than in yeast (Wise *et al*., 1983; Dahlberg and Lund, 1987; Guthrie and Patterson, 1988). Alternatively, yeast may require more stringent co-regulation of splicing and ribosome production than do vertebrate cells. The ribosomal protein genes, translation factors and U3 snoRNA constitute a major class of intron-containing genes in yeast and there may be regulatory reasons for using common factors such as Rnt1 for the biogenesis of both ribosome and spliceosome components.

The *S.pombe* RNase III homolog *pac1*$^+$ is involved in the 3′ end formation of U2, U5 and U4 snRNAs based on the ability of extra copies of the *pac1*$^+$ gene to rescue a temperature-sensitive mutation called *snm1* in which U2 and U4 transcripts are 3′ extended, and a long version of U5 snRNA is absent (Potashkin and Frendewey, 1990; Rotondo *et al*., 1995), similarly to the situation in budding yeast (Chanfreau *et al*., 1997; this work). These results, plus the potential of RNA derived from the 3′-flanking sequences of *S.pombe* U2 and U4 to form extended stem–loops similar to other RNase III substrates (M.Ares, unpublished), indicate that the direct involvement of RNase III in snRNA metabolism may be widespread. Although no effects on the accumulation of U4 snRNA can be seen upon depletion of Rnt1 *in vivo* (Chanfreau *et al*., 1997), it remains possible that Rnt1 may be involved in its processing. The differing results in the two yeasts may be explained by differences in the jobs performed by *S.pombe* and *S.cerevisiae* RNase III, or by the other particular activities available to process the 3′ ends in the absence of RNase III. We prefer the latter explanation since there is evidence for different alternative processing pathways in the absence of Rnt1: in the case of U5, U5L snRNA is not formed while the level of the fully functional U5S snRNA is unaffected (Chanfreau *et al*., 1997); in the case of U2, functional U2 is 3′ extended and polyadenylated (Figure 6). Although the two yeasts diverged from each other and from metazoans in the long distant past, the multiple mechanisms of snRNA 3′ end formation may overlay one another in different ways in different organisms, so that the consequences of disrupting an efficient pathway are buffered.

Polyadenylation of U2 snRNA

Preventing normal processing of U2 snRNA by Rnt1 results in the accumulation of extended and polyadenylated U2 RNA (Figures 2 and 6). Analysis of sequences downstream of the Rnt1 cleavage site reveals three potential polyadenylation signals (Figure 2B). When Rnt1 is depleted, these polyadenylation signals are activated, leading to cleavage and polyadenylation of pre-U2 snRNA (Figure 2). Rnt1 may prevent polyadenylation of snRNA either by processing the polyadenylated 3′ end or by removing the polyadenylation signals before they are recognized by the polyadenylation machinery. Analysis of the 3′ end of yeast telomerase RNA reveals a population of polyadenylated ends and shows that a mixed population of poly(A)$^-$ and poly(A)$^+$ RNA is produced (Chapon *et al*., 1997). The adenylation of telomerase RNA is dependent on the normal mRNA adenylation machinery and *cis*-acting polyadenylation elements (Chapon *et al*., 1997). Evidence indicates that polyadenylated telomerase RNA transcripts are processed into stable non-polyadenylated RNA (Chapon *et al*., 1997). The polyadenylated population of U2 RNA we observe in the U2Δstem mutant appears to be stable, and is not used efficiently as a precursor for the formation of U2 RNA with the normal wild-type 3′ end.

Because there are no instances of polyadenylated snRNAs in any system, we expected a defect in mRNA splicing and cellular growth. However, when we examined the growth and splicing of cells dependent on the U2Δstem mutant, we did not find any major defects. Northern blot analysis and the RNA protection assay indicate that virtually all the U2 snRNA in these cells are polyadenylated (see Figures 5 and 6), yet no growth defects or U3 snoRNA splicing defect are detected (Figure 7). We conclude that extension and polyadenylation of the 3′ end of U2 does not interfere with the splicing machinery. Thus, despite their apparent absence in nature, polyadenylation of functional snRNAs is not forbidden.

Materials and methods

Plasmids and strains

Yeast was grown and manipulated according to standard procedures (Rose *et al*., 1990; Guthrie and Fink, 1991). Wild-type cells used in the experiment are strain HI227 (*MATa, leu2, trp1, ura3-52, lys 2*Δ*, his 3-d200, prb1-1122, pep4-3, prc1-407*). The *rnt1* cells are HI227 carrying chromosomal disruption of *RNT1* made by insertion of a *HIS3* fragment (Abou Elela *et al*., 1996). Cells carrying an insertional disruption in the

RNT1 gene can grow slowly at 25°C (Chanfreau *et al*. 1997). To exclude the possibility that this phenotype is due to partial activity of the disrupted gene, we deleted sequences encoding the nuclease and double-stranded RNA-binding domains of Rnt1. Briefly, a 618 bp *Avr*II fragment of the *RNT1* gene was removed and replaced with the 1.8 kb *HIS3 Bam*HI fragment by Klenow fill in and blunt end ligation to create *rnt1Δ::HIS3*. A DNA fragment containing *rnt1Δ::HIS3* was introduced into yeast strain SS330/SS328 (*MATa/Mat α ade2-101/ade2-101, his3-d200/his3-d200, ura3-52/ura3-52*). Southern blots of the His^+ transformants identified those heterozygous for the disruption. Tetrad dissection was performed on rich media and the plates were incubated at 26°C for 7 days.

Plasmids carrying wild-type U2 snRNA were constructed by cloning a *Pvu*II–*Sma*I fragment spanning the entire U2 gene in the *Sma*I site of pRS315. The U2Δstem mutant lacking the putative stem–loop in the U2 3′-flanking sequences was made by site-directed mutagenesis (Kunkel *et al*., 1989) using the oligonucleotide 5′-GGTTGCGTGGTATATAGA-AGCTAGCAAAAATAAAAAAATAAAAGAGCG-3′. This mutation replaces 53 bp (+15 to +68) with 6 bp containing an *Nhe*I restriction site. Yeast strain d2 (Yan and Ares, 1996) carries a deletion in the chromosomal U2 gene, and wild-type U2 on a *URA3* plasmid was used as a recipient for introduction of either the pRS315-U2 or pRS315-U2Δstem plasmid (carrying the *LEU2* marker). After selection for loss of the wild-type U2 gene on 5-fluoro-orotic acid, isogenic strains differing only by the deletion in the U2 flanking sequence were obtained.

RNase protection assay

A probe complementary to the 3′ end of wild-type U2 snRNA was derived from T7 transcription of a plasmid carrying the *Sma*I–*Hin*dIII fragment of the U2 gene (Ares, 1986). A probe complementary to U2Δstem RNA was produced by T7 transcription of the pRS315-U2Δstem plasmid described above. In both cases, the templates were cleaved at the *Dra*I site in the U2 coding region near the 3′ end, and are complementary to the region from within the U2 coding region 97 bases upstream of the mature 3′ end, to 480 downstream of the mature 3′ end. Total yeast RNA (5 μg) was incubated at 42°C for 12 h with ~10^5 c.p.m. of probe in 80% formamide hybridization buffer (Melton *et al*., 1984). The hybridization mix was digested with 100 U/ml RNase T1 for 1 h at 30°C, and the protected fragments were separated on denaturing acrylamide gels.

Primer extension

Primer extension reactions to map the pre-U2 cleavage sites of Rnt1 *in vitro* were performed using 1 μg of *in vitro* transcribed RNA and 0.2 ng of ^{32}P end-labeled oligonucleotide as described earlier (Abou Elela *et al*., 1996). The oligonucleotide used is complementary to the U2 3′-flanking sequences, 'U2/3′ end': 5′-TTACATATTGGTTGC-3′. Primer extension to evaluate U3 splicing was performed as described previously (Ares and Igel, 1990).

Northern blot analysis

Northern blots were done essentially as described before (Chanfreau *et al*., 1997). Briefly, 4–5 μg of total RNA were loaded on 4% polyacrylamide denaturing gels and electro-transferred to a nylon membrane (Hybond-N, Amersham). The membrane was hybridized to the radiolabeled oligonucleotide probe complementary to the branchpoint interaction region of U2 RNA 'L15′: 5′-CAGATACTACACTTG-3′, in 6× SSPE, 0.2% SDS and 2× Denhardt's solution at 42°C. Filters were washed in a solution containing 2× SSPE and 0.1% SDS at the same temperature and exposed to autoradiography.

Isolation of RNA and poly(A)$^+$ RNA selection

Total RNA was prepared as described earlier (Rose *et al*., 1990). Briefly, cells were grown on YEPD rich medium, pelleted at 4000 r.p.m. and disrupted using glass beads. The RNA was extracted with phenol/chloroform and ethanol precipitated. The oligo(dT) selection was carried out as described by Perbal (1988). The RNA was dissolved in H_2O, heated to 65°C for 3 min and fractionated on a Pharmacia oligo(dT) type 7 column (Pharmacia Biotech Inc., Québec, Canada).

In vitro cleavage assay

The radiolabeled RNA used as a substrate for Rnt1 was generated by either T7 or T3 RNA polymerase in the presence of [α-^{32}P]UTP. The RNA substrate used for the *in vitro* cleavage assay described in Figure 3 was generated from a T3 promoter of the plasmid pRS426-U2 3′ end. This plasmid was constructed by inserting a *Dra*I–*Sma*I fragment of U2 snRNA (Ares, 1986) in the *Sma*I site of pRS426 (Christianson *et al*., 1992). All other substrates were produced from a T7 promoter of the plasmid pRS316-U2 3′ end. To make this plasmid, the U2 *Dra*I–*Sma*I fragment was released from the plasmid pRS426-U2 3′ end using the linker restriction sites *Hin*dIII and *Bam*HI and inserted in the plasmid pRS316 using the same sites. For the *in vitro* cleavage assay, 5000 c.p.m. of radiolabeled RNA were incubated at 30°C with GST–Rnt1 protein, prepared as described earlier (Abou Elela *et al*., 1996), in 30 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 5 mM spermidine, 30 mM KCl, *Escherichia coli* tRNA (0.1 mg/ml) in a total volume of 10 μl. The reaction was stopped by the addition of 100 μl of TE buffer pH 8 and the RNA was extracted using phenol/chloroform, ethanol precipitated, and loaded on a denaturing 8% acrylamide gel.

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