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Nonpolyadenylated RNA Polymerase II Termination Is Induced by Transcript Cleavage*

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Although the termination of transcription and 3′ RNA processing of the eukaryotic mRNA has been linked to a polyadenylation signal and a transcript cleavage process, much less is known about the termination or processing of nonpolyadenylated RNA polymerase II transcripts. An efficiently expressed plasmid-based expression system was used to study the termination and processing of *Schizosaccharomyces pombe* U3 small nucleolar RNA (snoRNA) transcripts *in vivo*. The termination assay was linked to cell transformation, and restriction fragment length polymorphism was used to determine levels of plasmid-derived U3 snoRNA. Mutation analyses *in vivo* indicate that the maturation of the 3′ end is not directly dependent on an external *cis*-acting sequence or structure; rather, it is dependent on a transcript cleavage that can occur hundreds or even thousands of nucleotides downstream of the mature U3 snoRNA sequence. Similarly, termination is dependent on the same transcript cleavage that is localized in a hairpin structure that normally follows the 3′ end of the U3 snoRNA but that also can be moved hundreds or thousands of nucleotides downstream. Both processes, however, can be induced simultaneously and equally efficiently with a single unrelated Pac1 endonuclease-labile structure. The results support a "reversed torpedoes" model in which a single cleavage allows exonucleases and/or other protein factors access to the transcript leading to transcription termination in one direction and RNA maturation in the other direction.

In eukaryotic cells, the diversity in their DNA-dependent RNA polymerases appears also to be reflected in the mechanisms of transcription termination that each polymerase utilizes. Although studied most intensely, the mechanism(s) of Pol[2] II transcript termination is probably the least understood. In the case of pre-mRNA transcripts, 3′ end processing and termination are normally coupled; the same RNA signals that direct 3′ end processing also promote termination downstream of the processing site. At least two alternative models, which are not necessarily mutually exclusive, have been proposed (1). The first, often referred to as the "anti-terminator" model, suggests that polyadenylation triggers a change in polymerase-associated factors that leads to a less processive RNA polymerase; an alternate model suggests that the transcribing RNA polymerase is "torpedoed," being attacked by an exonuclease that gains access at its free end when the pre-mRNA is cut at the polyadenylation site.

In addition to mRNA, Pol II is responsible for the synthesis of many noncoding RNAs, including small nuclear and nucleolar RNAs (snRNA and snoRNA). Many of these are encoded by discreet transcriptional units, but the 3′ ends are not polyadenylated. In *Saccharomyces cerevisiae*, the termination of nonpolyadenylated transcripts has been reported to require two RNA-binding proteins, Nrd1 and Nab3, which appear to bind specifically to two common RNA sequence motifs, GUA[AG] and UCUU (2, 3). Both genetic and physical data (4) suggest that a heteromeric complex of these RNA-binding proteins recognizes sequences in the nascent snoRNA transcripts and directs Pol II to terminate, perhaps somewhat reminiscent of Pol I termination (5). In the later case, however, the transcription termination factor (6) actually has been shown to bind to repeated DNA sequence elements and postulated to promote a pause in transcription (7). Numerous other protein factors also have been implicated in nonpolyadenylated Pol II termination including a helicase (Sen1) and subunits of the APT complex (Pti1, Ref2, Ssu72, and Swd2), which associate with mRNA cleavage/polyadenylation factor Pta1 (8–13). Because these factors do not affect all genes in an equal manner, Buratowski and co-workers (14) recently suggested that Pol II may choose between distinct termination mechanisms.

In a further study of U3 snoRNA function in pre-rRNA maturation, we have isolated and examined the expression of the U3 snoRNA genes of *Schizosaccharomyces pombe*. This fission yeast contains two U3 snoRNA encoding genes that are almost equally expressed (15). In the course of our analyses we used targeted mutagenesis to assess both upstream and downstream sequence requirements. Finding them to be shorter than previously reported in *S. cerevisiae*, we undertook a more detailed analysis, including an examination of elements critical to 3′ end processing and the termination of transcription. Surprisingly, as observed with polyadenylated transcripts, in *S. pombe* 3′ end processing and transcript termination are coupled and dependent on transcript cleavage, which simultaneously initiates both processes.

EXPERIMENTAL PROCEDURES

Strains and Vectors—Escherichia coli strain C490 (recA$^-$, rk$^-$, mk$^-$, thr$^-$, leu$^-$, met$^-$) was used as a host for pTZ19R (16) and pFL20 (17) plasmid recombinants. *S. pombe* (h$^-$, leu1-32, ura4-D18) cells were used as a host to express the pFL20 yeast

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[2] The abbreviations used are: Pol, polymerase; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; bp, base pair(s); RT, reverse transcription; contig, group of overlapping clones.

shuttle vector recombinants. Bacterial clones were grown at 37 °C in LB-ampicillin broth or LB-ampicillin agar, and yeast transformants were grown with aeration under selective conditions in minimal medium containing 0.64% yeast nitrogen base, 0.5% dextrose, 80 μg/ml leucine, 0.36 mg/ml potassium acetate, and 0.5 mg/ml potassium dihydrogen phosphate (18, 19). Growth rates were determined using the absorbancy of cultures at 550 nm.

Preparation and Expression of Mutant U3 snoRNA Genes—*S. pombe* genomic and plasmid DNA were extracted from logarithmically growing cells essentially as described by Hoffman and Winston (20). Cultures (10 ml) with absorbancies of 0.4–0.6 at 550 nm were harvested by centrifugation, washed with water, and resuspended in 200 μl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), 200 μl of phenol:chlorophorm:isoamyl alcohol (25:24:1), and 0.3 g of acid-washed glass beads. The cells were disrupted by vortex for 3 min, and after centrifugation and ethanol precipitation, the aqueous phase was treated with RNase A (25 ng/μl) for 30 min. Ammonium acetate (120 mM) was added, and the DNA was again precipitated with ethanol.

The U3B snoRNA gene region was prepared by PCR amplification using primers complementary to spacer sequences preceding and following adjacent open reading frames as described in Fig. 1. The 4.7-kilobase pair amplified fragment was cloned in pTZ19R after digestion with HindIII restriction endonuclease and the recombinant DNA (pTZ19U3B) was used as a DNA template for further manipulations. After double digestion with BstUI and BamHI, the U3B gene-containing fragment was subcloned into a multicopy yeast shuttle vector, pFL20, to form a recombinant (pFL20U3B), which contained the U3B gene coding sequence together with a 1868-base pair (bp) upstream region and a 2133-bp downstream region. Shortened gene segments were prepared using PCR amplification and appropriate complementary primers with BamHI adapter sequences at their ends when required. These included pFL20U3B_3.3, which contained 927 bps upstream and 2133 bps downstream as well as pFL20U3B_0.5, which contained only 69 bps upstream and 198 bp downstream. Plasmid DNA preparations for characterization or yeast transformation were based on the methods of Birnboim and Doly (21).

For more detailed sequence analyses, base substitutions were introduced using a two-step PCR-based strategy as described previously (22). Again, when appropriate, primers with BamHI adapter sequences at their ends were used to prepare the final fragments; after digestion with BamHI endonuclease, the mutant gene sequences were cloned directly into the pFL20 yeast shuttle vector using its BamHI site. In other instances, the vector was digested with PvuII endonuclease or double digested with BamHI and PvuII and fragments with blunt or mixed ends were ligated, as required. All of the mutations were confirmed by automated DNA sequencing (CBS-DNA Facility, University of Guelph), and the pFL20 recombinants were used to transform *S. pombe* cells by the method of Okazaki *et al.* (19). To ensure a reproducible result, at least three transformants were chosen for each mutant sequence.

Preparation and Analysis of Cellular U3 snoRNAs and Precursors—To assay the total cellular U3 snoRNA or precursor RNA, logarithmically growing cells were disrupted by vortex with an equal volume of glass beads (23), and cellular RNA was prepared using the protocol of Steele and coworkers (24). The relative amounts of U3 snoRNA or pre-snoRNA were determined by RT-PCR after treatment with 0.1 unit/μl of RNase-free DNase (25). The RT reactions (20 μl) were performed using 1–2 μg of DNase-treated RNA, 0.2 mM dNTPs, 60 pmol of U3 snoRNA-specific (5′-ACACGTCAGAAAACACC-3′) or precursor-specific (5′-ATGCACATCAAGTTATAAC-3′) primer, and 40 units of Moloney murine leukemia virus reverse transcriptase (Fermentas Inc., Hanover, MD), incubated for 2 h at 37 °C. For PCRs (50 μl) 0.1–10% of the RT reaction product was used with 0.2 mM dNTPs, 30 pmol of each primer, and 1 unit of *Taq* polymerase for 30 cycles.

Individual U3 snoRNA components were assayed using restriction fragment length polymorphism. Whole cell nucleic acid was extracted from logarithmically growing cultures with sodium dodecyl sulfate/phenol as previously described (24), and the U3 snoRNA was purified by fractionation on 8% denaturing polyacrylamide gels and elution by homogenization in SDS buffer after staining with methylene blue (26). U3 cDNA was prepared from the gel-purified RNA by RT-PCR (27) using Moloney murine leukemia virus reverse transcriptase and primers specific for the 5′ and 3′ ends of the *S. pombe* U3 snoRNAs (5′-ATCGACGATACTCCATAG-3′ and 5′-ACACGTCAGAAAACACC-3′), respectively, as described above. [α-^{32}P]dCTP (2–5 μCi) was added to the PCR to label the DNA products. The reaction mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and the labeled copy DNA was precipitated with ethanol and finally digested with MboI endonuclease (Invitrogen); fragments were fractionated on 12% nondenaturing polyacrylamide gels and detected by autoradiography. For quantitative analyses, the images were captured using a Umax Astra 600P scanner (Umax Technologies, CA) and quantified using Molecular Analyst PC software (Bio-Rad).

Ligation-mediated Terminal Analyses—U3 snoRNA 3′ ends were determined by a three step ligation-mediated PCR amplification protocol essentially as described by Hitchcock *et al.* (28). A 5′ end phosphorylated (pCAGGAAACAGCUAUGAC) RNA adapter oligonucleotide (300 pmol) was first ligated to the 3′-hydroxyl groups total cellular RNA (1 μg) using 10 units of T4 RNA ligase (Fermentas, Inc.) in 15 μl of ligation buffer. The ligated RNA was heat-inactivated (75 °C, 10 min) and diluted in reverse transcriptase reaction mix containing 60 pmol of complementary primer. The solution was denatured at 85 °C for 5 min, cooled to 42 °C, and incubated with 40 units of Moloney murine leukemia virus reverse transcriptase (Fermentas Inc.) for 2 h at 37 °C. The resulting cDNA solution was diluted to 20 μl, and a 2-μl aliquot was used for PCR amplification, with 30 pmol of complementary primer, 30 pmol of U3 snoRNA-specific primer (5′-ATCGACGATACTCCATAG-3′), and 2.5 units *Taq* DNA polymerase in 50 μl of buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0 containing 1% Triton X-100).

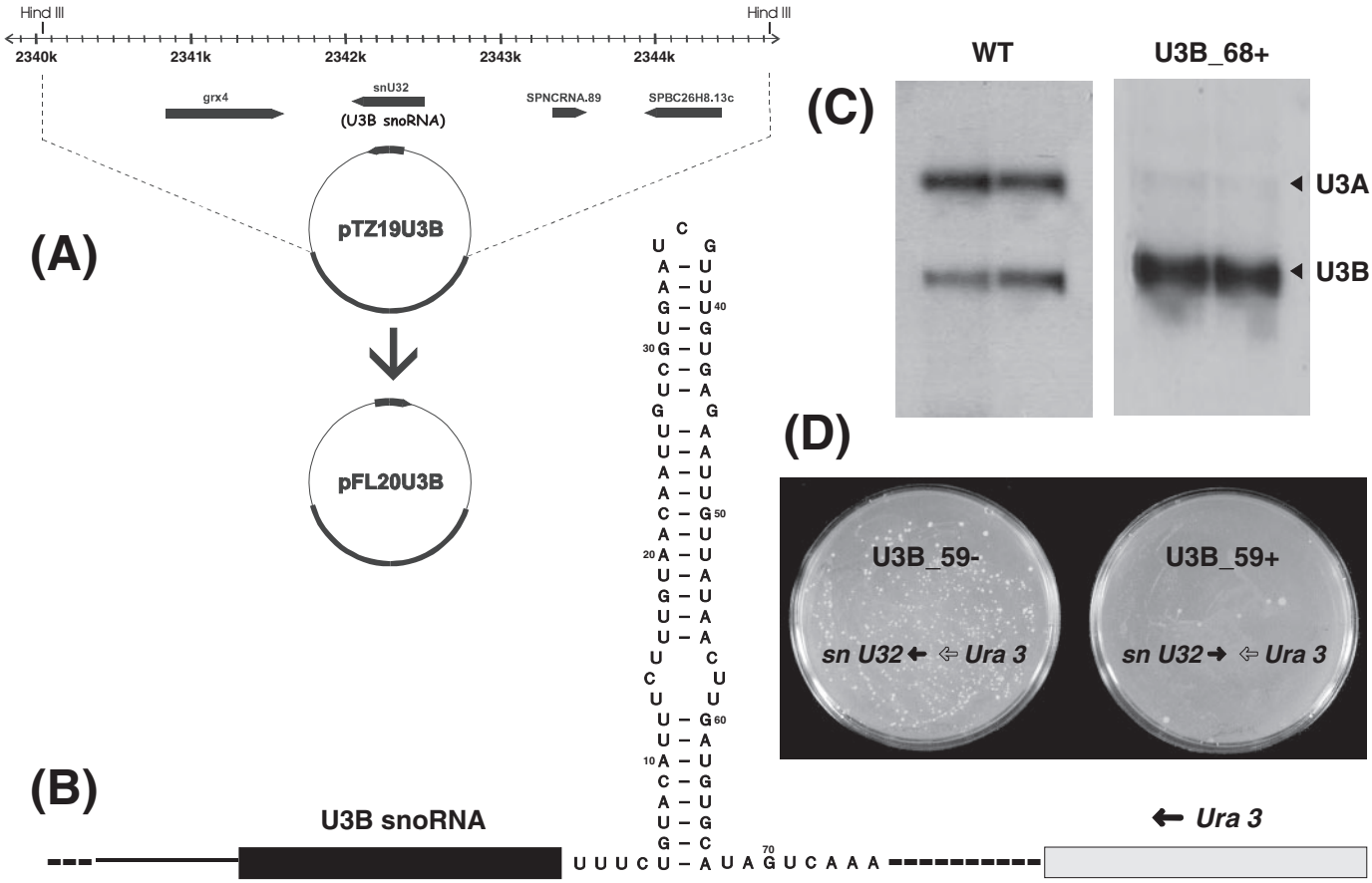

FIGURE 1. **Isolation and manipulation of the U3B snoRNA gene of *S. pombe*.** *A*, primers, upstream and downstream of a HindIII fragment containing snU32, the gene encoding the U3B snoRNA of *S. pombe* cells, were used to amplify this region using genomic DNA prepared from *S. pombe* (h^-, leu1–32, ura4-D18) as a template; the initial sequence was taken from pJ5566 as described in the *S. pombe* gene bank. The amplified DNA was cloned in pTZ19R (pTZ19U3B) and used as a template to prepare alternate constructs. The products were cloned downstream of the selective marker (*Ura* 3) in a high copy yeast shuttle vector (pFL20) for *in vivo* expression analyses. *B*, the downstream region of the U3 snoRNA gene is characterized by an extended hairpin structure. *C*, RT-PCR-based assay for *S. pombe* U3 snoRNAs. U3 snoRNA was prepared from normal or transformed cells by SDS/phenol extraction, and the relative distribution of the constituent RNAs was determined by restriction fragment length polymorphism. The whole cell RNA was fractionated on an 8% denaturing polyacrylamide gel, and labeled cDNA of the purified U3 snoRNA fraction was prepared by RT-PCR. The labeled DNA was digested with MboI endonuclease, and fragments were fractionated on 12% native polyacrylamide gels. The gels represent examples of RNA (*left lane*) genomic DNA (*right lane*) from untransformed cells (*left panel*) or cells transformed with an efficiently expressed normal U3B snoRNA gene (*right panel*). *WT*, wild type. *D*, frequency of transformation can be dramatically affected by the direction of U3 snoRNA gene expression. A U3B snoRNA gene containing an abbreviated 3′ end stem sequence was cloned into both orientations, and the constructs were used to transform *S. pombe* cells.

A 5-μl aliquot of the PCR products was characterized by fractionation on a denaturing 8% polyacrylamide gel. The remainder was phenol:chloroform-purified, ethanol-precipitated, and cloned in the pTZ19R vector for subsequent DNA sequence analyses.

Pac1 Ribonuclease Digestion Analyses—RNA structures for digestion analyses were prepared by *in vitro* transcription using T_7 RNA polymerase (29, 30). Template sequences forming hairpin structures in the downstream regions of the *S. pombe* gene encoding the U3B snoRNA and rDNA were prepared by PCR amplification and cloned in both the normal and inverse orientation using pTZ19R (31). Transcription reactions were performed with 0.4–2 μg of a DNA template in the presence of 2 mM ATP, CTP, GTP, and UTP and 100 units of T_7 RNA polymerase using N4 buffer (20 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.1, 1 mM spermidine, 0.01% Triton X-100) in a final volume of 100 μl. After incubation at 37 °C for 4 h, the RNA was precipitated with 2.5 volumes of cold salted ethanol. The transcripts were then purified on a 6% or 8% denaturing polyacrylamide gel and labeled at the 5′ end using bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP (32), after dephosphorylation with calf intestinal phosphatase (27). The phosphatase was heat-inactivated for 10 min at 75 °C in presence of 5 mM EDTA (pH 8.0) before the RNAs were labeled, and the labeled RNAs were again purified on a 6 or 8% denaturing polyacrylamide gel.

Pac1 RNase was prepared, and cleavage reactions were performed essentially as described by Rotondo and Frendewey (33). The i*n vitro* synthesized and labeled RNAs were digested with appropriate amounts of the Pac1 RNase for 60 min at 30 °C in 30 μl of buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol, and 30 mM Tris-HCl, pH 8.1. The reactions were stopped with the addition of an equal volume of loading buffer (formamide containing 0.05% xylene cyanol and 0.05% bromo-phenol blue), the solution was heated for 2 min at 90 °C (5 min at 65 °C), and 5-μl aliquots were applied directly to a 6 or 8% (29:1; acrylamide:bisacrylamide) polyacrylamide gel containing 8.3 M urea. RNA partially digested with T1 ribonuclease or hydrolyzed

TABLE 1
U3B snoRNA genes with alternative downstream sequences

Plasmid[a]	Downstream sequence	Orientation[a]	Transformation efficiency[b]	Growth rate[c]	U3B snoRNA[d]
U3B_2135+	2135	Opposite	8475	4.9 ± 0.3	83.6 ± 6.1
U3B_604+	604	Opposite	8775	4.2 ± 0.2	79.7 ± 4.2
U3B_393+	393	Opposite	10020	4.5 ± 0.4	88.0 ± 1.7
U3B_198+	198	Opposite	11100	4.7 ± 0.2	93.7 ± 1.2
U3B_87+	87	Opposite	6705	4.7 ± 0.1	91.4 ± 0.8
U3B_68+	68	Opposite	6165	4.4 ± 0.1	87.5 ± 1.2
U3B_59+	59	Opposite	90	NA[e]	NA
U3B_45+	45	Opposite	90	NA	NA
U3B_32+	32	Opposite	75	NA	NA
U3B_18+	18	Opposite	135	NA	NA
U3B_198−	198	Same	11700	4.1 ± 0.1	93.7 ± 4.5
U3B_59−	59	Same	6750	7.4 ± 0.3	40.0 ± 6.3
U3B_45−	45	Same	6900	8.0 ± 0.3	41.7 ± 5.4
U3B_32−	32	Same	7275	7.4 ± 0.3	42 ± 6.4
U3B_18−	18	Same	7050	7.7 ± 0.4	45 ± 7.4

[a] Direction of U3 snoRNA transcription is opposite (+) to or the same (−) as the *Ura3* gene.
[b] Number of transformants per μg of plasmid DNA.
[c] Doubling time in hours.
[d] Amount of U3B snoRNA as a percentage of the total U3 snoRNA.
[e] NA, not applicable.

with base (34) also was applied as length markers; after fractionation, the fragments were detected by autoradiography.

RESULTS

An efficiently expressed plasmid associated gene system was used to study the expression and maturation of U3 snoRNA encoded by the *S. pombe* snU32 locus (chromosome 2, contig pJ5566, *S. pombe* gene data base). As summarized in Fig. 1, the U3B snoRNA encoding region in the *S. pombe* genome was PCR-amplified and cloned in the pTZ19R vector. For *in vivo* analyses the normal or altered gene sequences were subcloned into a multi-copy number yeast shuttle vector (pFL20), which subsequently was used to transform *S. pombe* cultures. The gene was expressed under its own promoter elements, and the increased plasmid-derived U3B snoRNA sequence could be readily detected using RT-PCR amplification. As previously reported (15), in normal cells U3A snoRNA was found to make up ~55–60% of the total U3 snoRNA population; when cells were transformed with the plasmid-associated gene, there was a dramatic change, and the U3B snoRNA then made up more than 85% of the cellular U3 snoRNA population (Fig. 1*C*). This reflected the greatly elevated gene dosage consistent with an efficient expression of the plasmid-associated sequence.

Initial mutational analyses were focused on the maturation of U3 snoRNA *in vivo*, in particular the role of the conserved stem structure downstream of the mature U3 snoRNA sequence (Fig. 1). As also illustrated in Fig. 1*D*, in the course of these analyses, we observed a surprisingly variable transformation frequency with efficient transformation for many constructs but very few with others (Table 1). Furthermore, subsequent analyses of the few transformants in the second group revealed unanticipated recombination with none corresponding to the initial construct (results not shown). With more examples, a strong correlation was recognized between the transformation frequency and the direction of U3B snoRNA gene insertion or transcription relative (same *versus* opposite) to the selectable marker (*Ura* 3). Because read-through transcription appeared to be interfering with *Ura* 3 gene expression, in each case, the extent of transcription was evaluated using RT-PCR as illustrated in the examples shown in Fig. 2. Indeed, transcripts of mutant genes with normal transformation rates were found to extend only to the end of the 3′ end hairpin structure (+68, *upper panels*), whereas the others (when evaluated in the same orientation) were observed to extend downstream (+135, *upper panels*). Run-on transcription equally was evident when only downstream-specific primers were used (*lower panel*); transcription of the more distal and extended region (+523 to +1166) and the 643-bp product only was present with the abbreviated hairpin (+45). Clearly, termination failure or read-through was interfering with *Ura* 3 gene expression, in turn, preventing or greatly impeding transformant growth.

Using transformation frequency as an initial assay for termination failure, a study of factors affecting transcript termination was initiated with systematic mutations being introduced into the downstream region. As indicated in Table 1, none of the deletions beyond the hairpin (U3B_68+ to U3B_2135+) had any significant effect on termination, but a deletion of only a few residues forming the hairpin (U3B_59+) had a dramatic effect. Similarly, all other significant changes to the hairpin (U3B_18+ to U3B_45+) resulted in very low transformation frequencies when transcription occurred in the conflicting (opposite) direction. In strong contrast, when equivalent constructs were prepared in a reverse orientation so transcripts did not conflict (same), the transformation frequency returned to normal (U3B_18− to U3B_59−), but run-on transcription could be readily detected by RT-PCR as illustrated in Fig. 2.

To establish the relationship of this hairpin to any extended termini, ligation-mediated RT-PCR (28) was used to detect and identify the normal termini in *S. pombe*. As shown in Fig. 3, only a single major terminal other than that of the mature U3 snoRNA was detected. Subsequent DNA cloning and sequence analyses (Fig. 3, *lower panel*) identified the end as nucleotide $+C^{22}$, located approximately halfway up the 3′ end hairpin structure.

In view of the fact that the hairpin seemed to resemble an RNase III substrate and there have been past reports of RNase

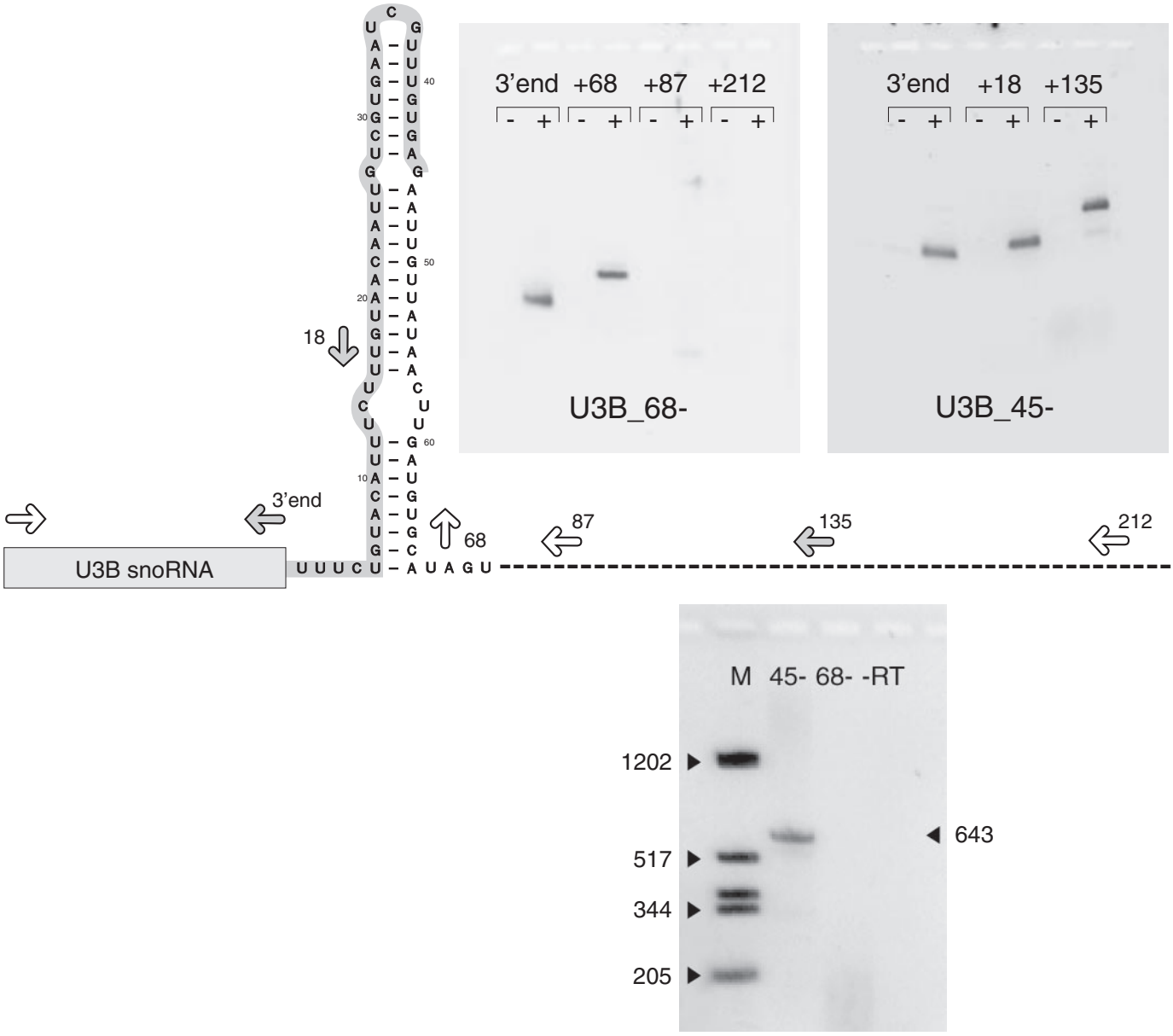

FIGURE 2. **Read through transcription in transformation defective constructs.** The cells were transformed with U3B snoRNA genes containing a complete normal (U3B_68−) or an abbreviated (*gray shading*) mutant (U3B_45−) 3′ end stem cloned in the same orientation as the selective marker (*Ura* 3), and the extent of transcription was determined using RT-PCR and primers (*arrows*) complementary to the mature RNA and various positions (*upper panels*) in the downstream region. The distal position of each primer is identified numerically; the reaction products with and without reverse transcriptase (+ or −) were fractionated on agarose gels. Equivalent hairpin constructs containing identical downstream regions were examined further (*lower panel*) using primers specific for a more distal downstream region beginning at +523 and ending at +1166. The reaction products for U3B_45− (*45*−) and U3B_68− (*68*−) together with the control for the U3B_45− reaction lacking reverse transcriptase (−RT) and a fragment length marker (*M*) were fractionated on an agarose gel; fragment lengths are indicated by the *arrowheads*.

III-like cleavages in the 3′ ends of snRNA transcripts (35–40), the susceptibility of the U3 snoRNA gene-associated structure to Pac1 ribonuclease cleavage (a RNase III-like nuclease in *S. pombe*) was examined. As shown in Fig. 4, when hairpin RNA was prepared *in vitro* using T7 RNA polymerase, labeled at the 5′ end with [γ-^{32}P]ATP and incubated with Pac1 nuclease (33), the hairpin was efficiently cleaved at nucleotide $+C^{22}$, fully consistent with the prior terminal analyses (Fig. 3). As a control, RNA also was prepared from the opposite strand (*i.e.* the complementary sequence). As also shown in Fig. 4, no cleavage was observed with this RNA, and when this hairpin sequence was substituted in the plasmid-associated gene, the transformation frequency was observed to drop dramatically (*left plate*), again consistent with termination failure.

Because sequence downstream of the hairpin was not essential for transformation failure and a comparison of the entire downstream sequences in the two U3 snoRNA genes of *S. pombe* revealed little sequence conservation other than the extended hairpin structure, an attempt was made to determine whether hairpin cleavage was sufficient, in itself, to induce termination by using an unrelated Pac1 nuclease cleavage site. The best characterized site in *S. pombe* is found in an extended hairpin within the 3′ external transcribed spacer of the rRNA transcripts. Previously examined in several laboratories including

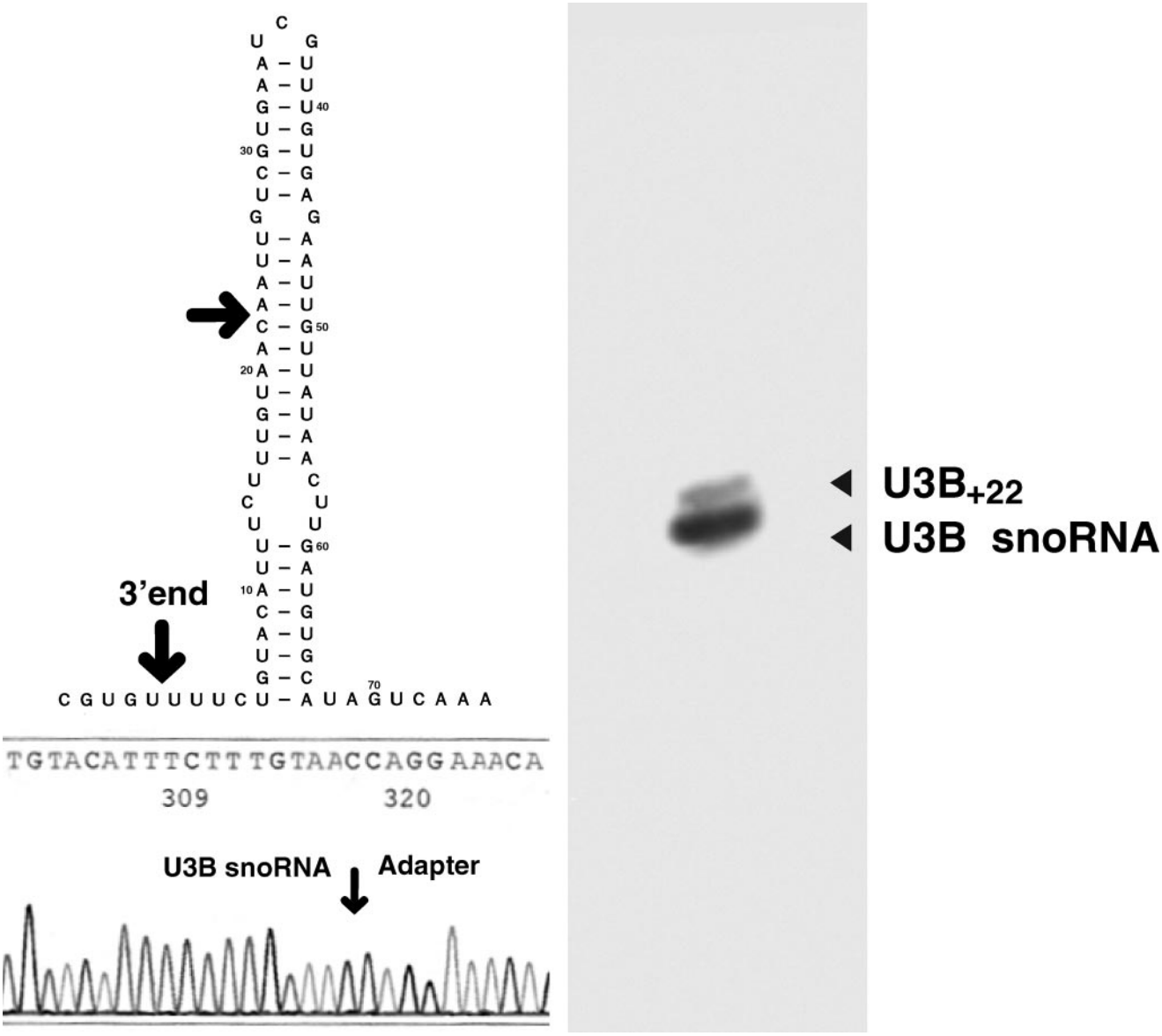

FIGURE 3. **Ligation-mediated RT-PCR indicates one major extended terminal.** Whole cell RNA was prepared and ligated to an adapter RNA oligonucleotide using T4 RNA ligase; adapter-bound U3B snoRNA sequences were amplified specifically by RT-PCR and cloned for sequence analyses. The gel-fractionated RT-PCR products used for sequence analyses are shown on the *right*; the single major nucleotide $+C^{22}$ terminal extension is identified on the *left* (sequence printout shown in *lower panel*).

our own (41–43), this hairpin is readily cleaved *in vitro*. As shown in Fig. 5, when this hairpin structure was substituted in the U3 snoRNA gene, the effect was dramatic. Even though there was no sequence homology, and it was a much more extended structure, this substitution (rDNA hairpin) was able to efficiently induce termination (Fig. 5*B*) and a production of mature plasmid-derived RNA (Fig. 5*C*). Clearly this cleavage was sufficient to induce Pol II transcript termination in *S. pombe*. In strong contrast to the rDNA-derived hairpin sequence, when the complementary sequence was substituted (*Complementary*), the transformation frequency was again very low (Fig. 5*B*) when the U3 snoRNA gene was expressed in the conflicting orientation, and plasmid-derived RNA was not detected (Fig. 5*C*), when this alternate gene construct was inserted so transcription occurred in the same direction as the *Ura* 3 gene, and transformation was possible.

Because many of our analyses (Table 1) indicated that hairpin mutations affected both transcript termination and RNA maturation in a similar fashion, an attempt was made to separate these events for independent study. As diagrammed in Fig. 6, a new construct was prepared in which a slightly shortened, termination defective hairpin remained at the 3′ end of the U3B snoRNA sequence, and a new hairpin was added 1.7-kilobase pairs downstream of it (U3B_59TH+). As anticipated, a control construct with only the defective hairpin (U3B_59+ or U3B_59−) resulted in little or no plasmid-derived U3B snoRNA (Fig. 6, *left panel*), whereas the new construct with both hairpins produced high levels of U3 snoRNA (Fig. 6, *middle panel*). Surprisingly, a construct with only a downstream hairpin (U3B_0TH+) also terminated normally and resulted in high levels of U3B snoRNA (Fig. 6, *right panel*). Three separate transformants were examined in

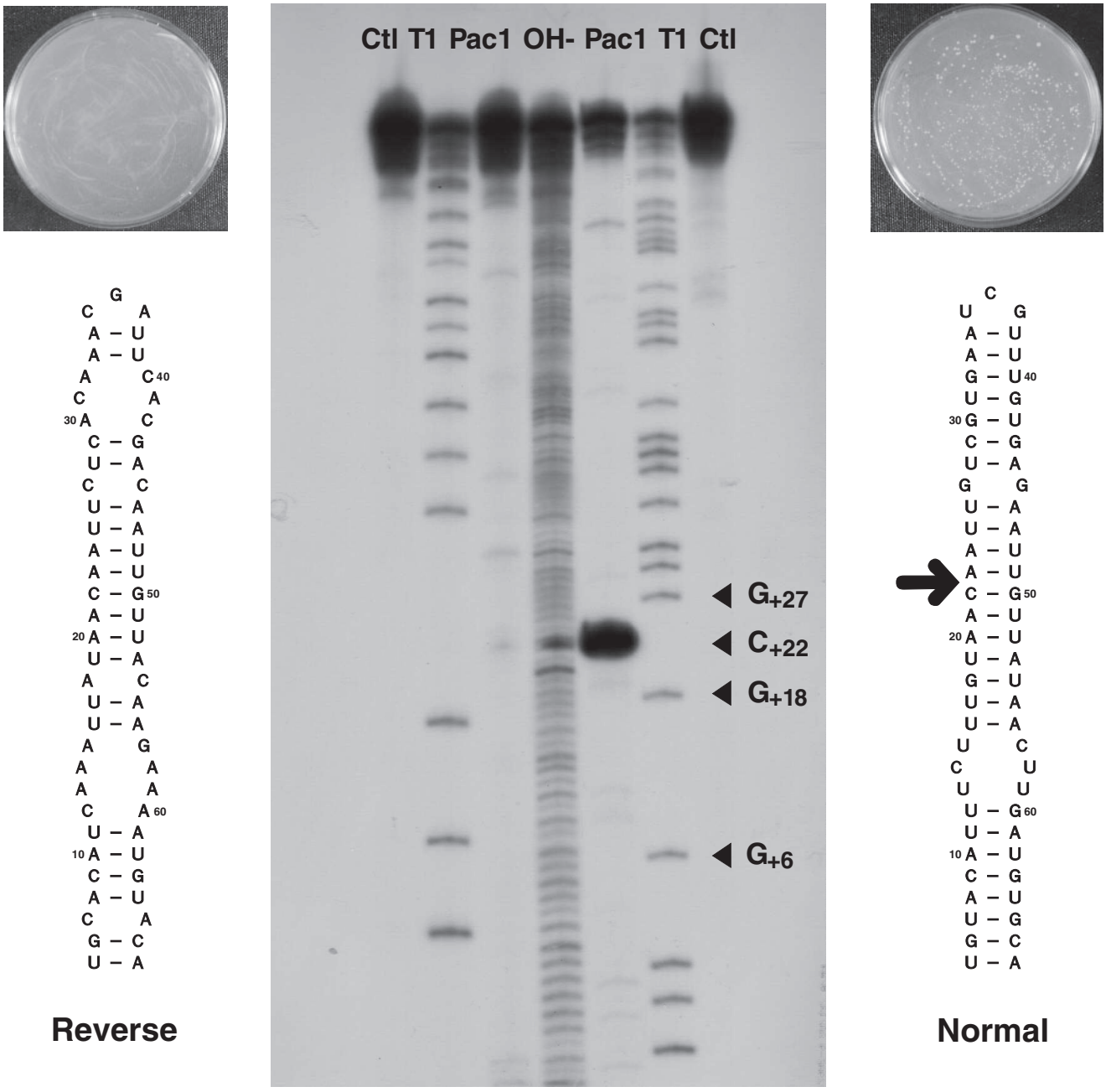

FIGURE 4. **A Pac1 cleavage site in the 3′ end hairpin corresponds with the extended terminal.** Hairpin RNA was prepared in both orientations using T7 RNA polymerase and 5′ end labeled using polynucleotide kinase (*Ctl*). Both were incubated with RNase III-like Pac1 nuclease from *S. pombe* (*Pac1*), and the products were fractionated on an 8% denaturing polyacrylamide gel (*center panel*). Partial base (*OH*−) or T1 RNase (*T1*) RNA digests were included as markers. Significant cleavage only occurred at nucleotide $+C^{22}$ in the normal hairpin (*arrow*). Transformation plates for U3 snoRNA gene constructs encoding equivalent hairpin structures are included in the *left* and *right panels*.

each case to ensure a reproducible result. This observation was further supported by a systematic series of hairpin mutations. As summarized in Table 2, with an intact downstream hairpin, all of the constructs containing a shortened hairpin sequence at the 3′ end of the plasmid-associated U3 snoRNA gene (U3B_59TH+ to U3B_0TH+) resulted in normally high transformation frequencies as well as high levels of plasmid-derived RNA. Constructs with defective downstream hairpins (U3B_59TH-18+ and U3B_59TH-36+) continued to reflect a failure in transcript termination, as did a construct with a complete hairpin sequence inserted in the reverse orientation (U3B_59revTH+) to encode a complementary RNA sequence.

DISCUSSION

The present studies indicate that, like polyadenylated transcripts, termination of the U3 snoRNA transcripts and 3′ end processing in *S. pombe* are coupled; cleavage by a RNase III-like Pac1 nuclease is sufficient to simultaneously induce both events. Based on the "torpedo" model (1, 44, 45) for the cleavage and termination of polyadenylated protein-encoding gene transcripts, we suggest that a "reversed torpedoes" model may

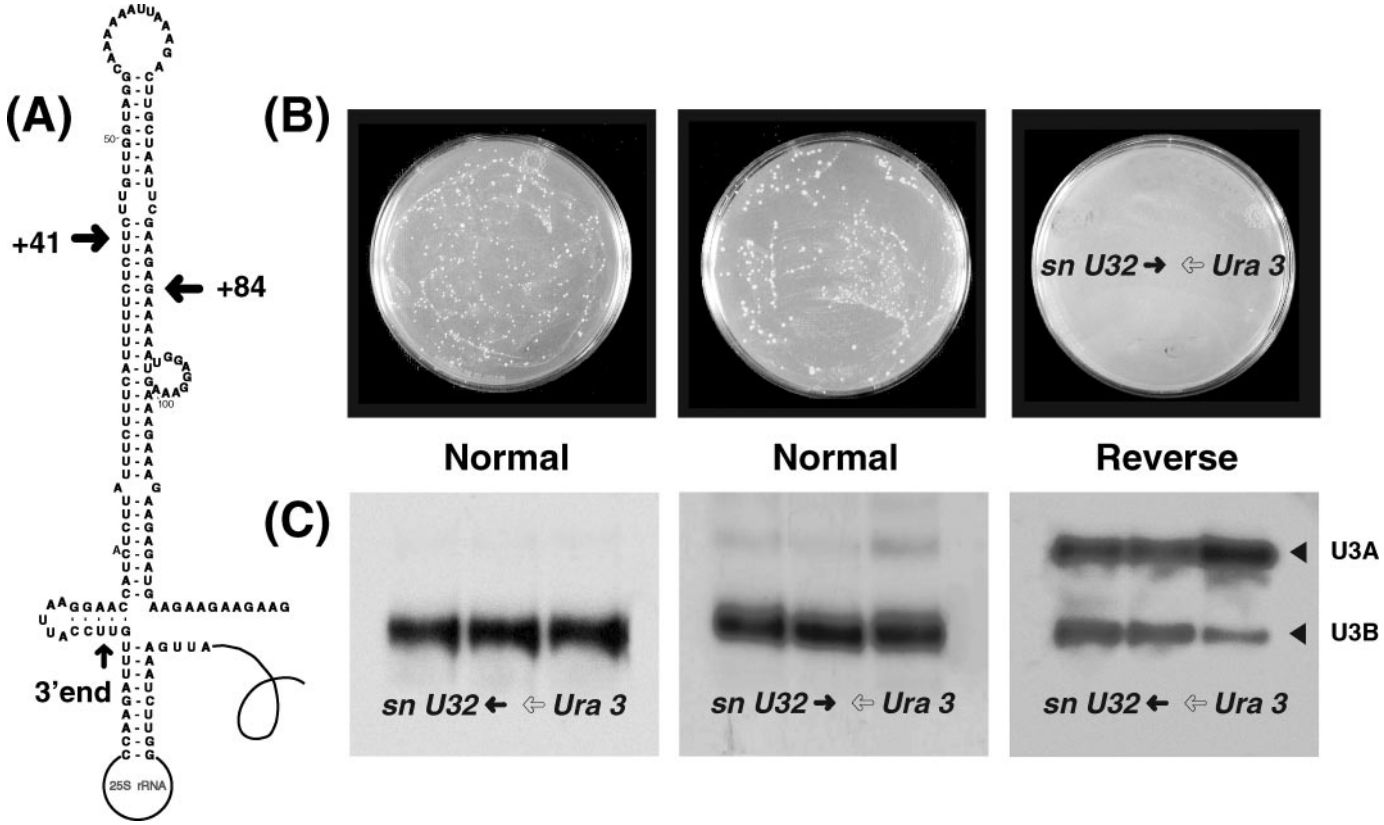

FIGURE 5. **The Pac1 cleaved 3′ end hairpin in *S. pombe* rDNA effectively substitutes for the U3 snoRNA hairpin.** The Pac1 cleaved (*arrows*) hairpin or complementary sequence from the 3′ external transcribed spacer of *S. pombe* rDNA (*A*) was substituted for the U3 snoRNA hairpin, and the pFL20 constructs containing these recombinants and expressing RNA in both the same and opposite direction as the *Ura* 3 gene were used to transform *S. pombe* cells. The plates (*B*) indicate a high transformation frequency with the U3 snoRNA/rDNA hairpin recombinants inserted in either gene orientation but very little transformation with the complementary sequence replacing the normal hairpin structure. RNA was prepared from three representative transformants for each construct, and the relative amounts of U3 snoRNAs were determined by restriction fragment length polymorphism after RT-PCR amplification. U3 snoRNA was efficiently synthesized with the normal rDNA hairpin sequence, but no plasmid-derived RNA was detected when the complementary sequence was present (*C*).

be a useful way to explain this observed coupling between U3 snoRNA maturation and transcript termination. A single cleavage event allows exonucleases or other effector molecules to gain access to termini in the nascent transcript leading to RNA maturation in one direction (3′ → 5′) and transcript termination in the other direction (5′ → 3′). The transcribing RNA polymerase is again " torpedoed," resulting in termination, whereas in the opposite direction, an extended terminal is trimmed until some feature associated with the mature RNA protects the nascent RNA from further digestion. Because the downstream sequence can be completely and effectively replaced with a totally unrelated Pac1 nuclease-susceptible sequence, other features in the extended 3′ end or downstream of it are not required for either process, although factors may still act to facilitate termination (*e.g.* a pause element). This observation is supported further by the fact that the position of the cleavage site also is not critical, and even when the hairpin sequence is moved almost 2 kilobase pairs downstream of the U3 snoRNA-encoding sequence, both transcript termination and RNA maturation occur efficiently (Fig. 6). The only critical feature is the cleavage site where hairpins that did not contain such a site failed to induce termination Figs. 4 and 5). Because 5′ → 3′ trimming actually has not been demonstrated, termination may be based on an "anti-terminator" or allosteric mechanism, but cleavage remains the activating event.

U3 snoRNA expression, in general, has been studied most extensively in *S. cerevisiae*. Kufel *et al.* (35, 46) have described a complex pathway for 3′ end processing that involves cleavage by the endonuclease Rnt1p, like Pac1 in *S. pombe* also a homologue of RNase III, followed by 3′ → 5′ exonuclease cleavage involving the exosome complex and perhaps Rex1–3p proteins. A Lsm2–8p complex functions as a chaperone in conjunction with Lhp1p protein to stabilize the pre-U3 snoRNA during 3′ end processing. All of these observations are consistent with the present results in *S. pombe* cells. In fact, the observed 3′ → 5′ exonuclease cleavage involving the exosome complex provides direct evidence for a second "torpedo" in the reverse direction.

Different observations have been reported with other sn/snoRNA genes. A polyadenylation cleavage factor, CFIA, has been implicated in 3′ end formation of several other box C/D and H/ACA snoRNAs and for the U2 and U5 snRNAs (47, 48). Because some (35–40) but not all (*e.g.* 47–49) snRNAs or snoRNAs contain RNase III-like cleavage sites in their downstream regions, this observation may indicate that alternative types of cleavage are utilized by different genes to simultaneously initiate transcript termination and RNA maturation. In fact, autocatalytic RNA cleavage in human pre-mRNA has been reported to promote transcription termination with polyadenylated RNA (50), again raising the possibility that the mechanism of cleavage itself is not critical.

Little attention actually has been given to U3 snoRNA transcript termination in *S. cerevisiae*, but again other nonpolyadenylated transcripts have been examined. Brow and co-workers (8) have reported that the essential RNA-binding protein Nrd1 directs poly(A)-independent 3′ end formation and that the termination signal may be transmitted through Ssu72 protein, an essential Pol II-binding protein (11, 12). On the other hand, Carroll *et al.* (2) have identified *cis*-sequence elements that appear to direct the termination of nonpolyadenylated snoRNA transcripts in *S. cerevisiae*. These short sequence motifs, GUA[AG] and UCUU, are known binding sites for Nrd1 and Nab3 proteins, respectively, proteins that appear to play a significant role in defining nonpoly(A) terminators (3, 4). Both of the sequence elements are commonly mapped downstream of the 3′ end and hairpin structure. Mutations in a helicase (Sen1) also have been observed (4, 36) to cause defective termination at multiple snoRNA genes, providing further alternatives.

At first glance many of these reports appear to conflict with the current observations in *S. pombe*, but with more careful analyses, they may not be inconsistent with a "reversed torpedoes" model. A RNase III-like cleavage does occur in U3 snoRNA gene transcripts of *S. cerevisiae*, followed with exonuclease trimming and RNA stabilization at the mature 3′ end. It is true that a number of box H/ACA and box C/D snoRNA transcripts do not have a downstream RNase III-like cleavage site, but both the Ssu72 protein and the CFIA subunit, which appear to influence termination and/or 3′ end maturation with these genes, also are known to function in poly(A) site cleavage (51–53). This raises the possibility that the cleavage process may be redundant to ensure that termination does occur or represents an alternative method of cleavage in nonpolyadenylated transcripts that do not contain RNase III-like cleavage sites. Likewise, a requirement for downstream sequence elements, as has been reported in *S. cerevisiae*, appears not to apply in *S. pombe*, but if these elements serve as part of a pause function to slow the polymerase and facilitate termination, the "torpedo" simply may be more efficient in *S. pombe* for other reasons. Equally, the fact that a helicase (Sen1) affects termination in some genes (8, 14) raises the possibility that alternative types of "torpedo" function with different genes. Whether the processes differ somewhat, either between genes or phylogenetically or, per-

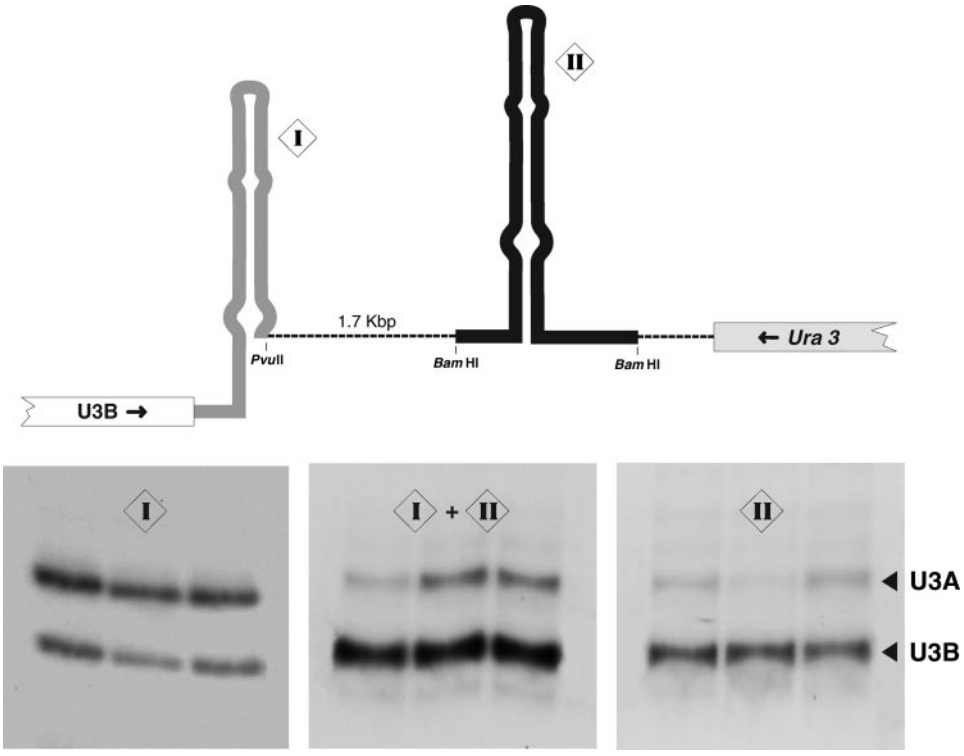

FIGURE 6. **Location of the 3′ end hairpin is not critical for U3 snoRNA synthesis.** U3B snoRNA gene constructs were prepared and expressed in both orientations (relative to the *Ura* 3 gene) with a single abbreviated hairpin in the normal position (*I*), an intact hairpin located 1.7 kilobase pairs downstream of the coding sequence (*II*), or both features together (*I* + *II*). The relative amounts of U3 snoRNAs were determined in three representative transformant colonies for each construct by restriction fragment length polymorphism after RT-PCR amplification. The subsequent gel analyses (*lower panels*) indicate efficiently expressed plasmid derived RNA is only observed when an intact hairpin is present (*middle* and *right panels*).

TABLE 2
U3 snoRNA genes with alternative hairpin sequences

Plasmid[a]	Downstream sequence[b]	Orientation[a]	Transformation efficiency[c]	Growth rate[d]	U3 snoRNA[e]
U3B_59TH+	59 + TH	Opposite	4275	4.6 ± 0.2	96.7 ± 2.5
U3B_45TH+	45 + TH	Opposite	3900	4.3 ± 0.1	94.7 ± 1.2
U3B_32TH+	32 + TH	Opposite	6900	4.7 ± 0.1	91.7 ± 5.1
U3B_18TH+	18 + TH	Opposite	8700	4.7 ± 0.2	98.4 ± 0.6
U3B_0TH+	0 + TH	Opposite	7200	4.7 ± 0.2	84.0 ± 7.0
U3B_59TH-18+	59 + THΔ18	Opposite	150	NA[f]	NA
U3B_59TH-36+	59 + THΔ36	Opposite	120	NA	NA
U3B_59revTH+	59 + revTH	Opposite	90	NA	NA

[a] Direction of U3 snoRNA transcription is opposite (+) to the *Ura3* gene.
[b] TH indicates a normal hairpin sequence 1.7 kilobase pairs downstream of the mature 3′ end.
[c] Number of transformants per μg of plasmid DNA.
[d] Doubling time in hours.
[e] Amount of U3B snoRNA as a percentage of the total U3 snoRNA.
[f] NA, not applicable.

haps, are redundant in one or both yeasts, will have to be clarified through further comparative analyses. In the interim, it is clear that, as observed with polyadenylated transcripts, non-poly(A) Pol II termination can be induced by transcript cleavage, a step that actually may be characteristic of all Pol II termination events.

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