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Cleavage-induced termination in U2 snRNA gene expression

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ABSTRACT

The maturation of many small nuclear RNAs is dependent on RNase III-like endonuclease mediated cleavage, which generates a loading site for the exosome complex that trims the precursor at its 3′ end. Using a temperature sensitive Pac1 nuclease, here we show that the endonuclease cleavage is equally important in terminating the transcription of the U2 snRNA in *Schizosaccharomyces pombe*. Using a temperature sensitive Dhp1p 5′ → 3′ exonuclease, we demonstrate that it also is an essential component of the termination pathway. Taken together the results support a "reversed torpedoes" model for the termination and maturation of the U2 snRNA; the Pac1 endonuclease cleavage provides entry sites for the 3′ and 5′ exonuclease activities, leading to RNA maturation in one direction and transcript termination in the other.

In the protein encoding genes of eukaryotes, the poly(A) element, which signals 3′ end processing, is also required for the termination [1]. The "torpedo" model for the termination of polyadenylated Pol II transcripts proposes that the poly(A)-mediated cleavage event generates a loading site for a 5′ → 3′ exonuclease, which in turn "torpedoes" the transcribing RNA polymerase [2–4].

In addition to protein encoding genes, Pol II transcribes a large number of small nuclear and nucleolar RNAs (snRNAs and snoRNAs). This group of RNAs, which are not polyadenylated but are derived from longer precursors after being trimmed by exonuclease. In majority of the snoRNAs the entry site for the exonuclease is thought to be generated by the termination event [5], whereas in the U3 snoRNA and U1–U5 snRNAs an endonuclease cleavage has been shown to initiate the trimming process [6–8]. In snoRNAs, the termination event itself has been linked to two RNA sequence elements, GUA[AG] and UCUU within the nascent transcript [9,10]. Further studies suggest that the Nrd1/Nab3 protein complex binds to these elements, which then recruits Sen1 helicase and the exosome complex [5,11]. Sen1 protein, through its helicase activity, is thought to remove the RNA polymerase from the DNA template and provide an entry site for exosome trimming. The processing pathway in snRNAs has been the subject of considerable investigation. In vertebrates, a sequence element immediately downstream of the mature 3′ end directs transcript cleavage by an endonuclease that has not been identified [12,13]. In yeast cells, the 3′ end processing signal is an extended hairpin structure, downstream of the mature RNA sequence, which provides a cleavage site for the RNase III-like endonuclease (Rnt1 in *S. cerevisiae*). The 3′ end which is generated, again serves as the entry site for the exosome complex [7,8]. The termination mechanism, however, is not well understood. In their studies on the termination elements of nonpolyadenylated RNAs, Steinmetz and coworkers demonstrated that, similar to the snoRNAs, the U4 snRNA gene transcription can be terminated by Nrd1-binding factor, downstream of the Rnt1 cleavage site [14].

Recently, we showed that with U3 snoRNA transcripts in *Schizosaccharomyces pombe*, a Pac1 endonuclease cleaved downstream hairpin structure is required for termination without a need for additional sequence [15,16]. Since RNA maturation in the U2 snRNA, as with the U3 snoRNA, is initiated by Pac1 endonuclease cleavage [17], we have examined the possibility that termination is again triggered by Pac1 endonuclease cleavage. Using temperature sensitive proteins we now provide direct evidence that both the RNase III homolog and a 5′ → 3′ exonuclease are required for transcript termination.

Materials and methods

Strains and vectors. Escherichia coli, strain C490 (rec A-, rk-, mk-, thr-, leu-, met-) was used as a host for the pTZ19R [18] and pFL20 [19] cloning vectors. *Schizosaccharomyces pombe*, strain GP969 (h-, leu1-32, ura4-D18) was used as a host to express the pFL20 yeast recombinants. Strains TS138 (h- ade6-M616 ura4-D18 pac1-A3425) and JP44 (h-JP44 (h- leu1-32 snm1-1ts) or KP38 (h+ ade6-M216 leu1 his7-lacI-GFP-his7+ lys1-lacO-lys1+ ura4-D18 dhp1-1<<ura4+) were temperature sensitive for the Pac1 endonuclease or Dhp1p exonuclease, respectively.

Preparation and expression of mutant U2 snRNA genes. Schizosaccharomyces pombe genomic DNA was extracted from logarithmically growing cells essentially as described by Hoffman and Winston [20]. Cultures (10 ml) with absorbancies of 0.4–0.6 at

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550 nm were harvested by centrifugation, washed with water and resuspended in 200 μl breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0), 200 μl 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 next and the DNA was again precipitated with ethanol.

For gene expression analyses, the appropriate regions in the U2 snRNA gene were prepared by PCR amplification cloned directly into the pFL20 yeast shuttle vector using its unique PvuII site (Fig. 1). These included 77 bp upstream and 9 bp downstream or 48 bp downstream as indicated. The 3′ ETS region from *S. pombe* rDNA was prepared in a similar manner with primers including BamHI site adapters and cloned in the unique BamHI site. Inserts and orientations were confirmed by PCR-based assay and restriction enzyme digestion. Plasmid DNA preparations were based on the methods of Birnboim and Doly [21] and the pFL20 recombinants were used to transform *S. pombe* cells by the method of Okazaki and coworkers [22].

Preparation and analysis of cellular U2 snRNAs transcripts. To assay nascent U2 snRNA transcripts, logarithmically growing cells were disrupted by vortex with an equal volume of glass beads [23] and cellular RNA was prepared using the sodium dodecyl sulfate/phenol protocol of Steele and coworkers [24]. Read-through transcription was assayed by RT-PCR after treatment with 0.1 U per μl of RNase-free DNase [25]. The RT reactions (20 μl total volume) were performed using 200 ng of DNase-treated RNA, 0.2 mM dNTPs, 30 pmol of primer and 40 U of MmuLV reverse transcriptase (Fermentas, Inc., Hanover, MD), incubated for 2 h at 37 °C. For PCRs (50 μl total volume) 0.1–10% of the RT reaction product was used with 0.2 mM dNTPs, 30 pmol of each primer and 1 U of Taq polymerase for 30 cycles. Downstream-specific primers, 5′-GGTTTAGGATGTGAGG-3′ and 5′-GCTAATGTCAACACAAC-3′ were used for all assays.

Results

To examine the possibility that precursor cleavage can induce transcript termination in U2 snRNA transcripts, initially a transformation-based termination assay was used to detect read-through transcription. In the past studies on the U3 snoRNA [16] the disruption of a downstream *Ura*3 gene by read through was demonstrated to result in transformation failure. As indicated in Fig. 1, the U2 snRNA promoter region and coding gene sequence were PCR amplified together with or without the downstream extended hairpin sequence and cloned in the unique PvuII site in the pFL20 yeast shuttle vector with a conflicting orientation to the *Ura*3 gene that serves as a selectable marker during yeast cell transformation. As also shown in Fig. 1, many transformants were evident when the hairpin was present (upper panel) but very few transformants were observed when the hairpin structure was removed (middle panel). Furthermore, when a Pac1 cleaved hairpin sequence from the 3′ ETS of an rDNA transcription unit in *S. pombe* [26,27] was inserted into the unique BamHI site between the truncated U2 snRNA gene and the selection marker (*Ura*3), a high transformation efficiency again was evident (lower panel). Clearly this unrelated but cleaved sequence can effectively substitute for the normal hairpin.

To confirm directly that termination failure had occurred, read-through transcription was assayed by RT-PCR. As shown in Fig. 2, identical U2 snRNA gene constructs were prepared but with the opposite orientation to ensure a high level of yeast transformation (see plates). Representative transformants were assayed using

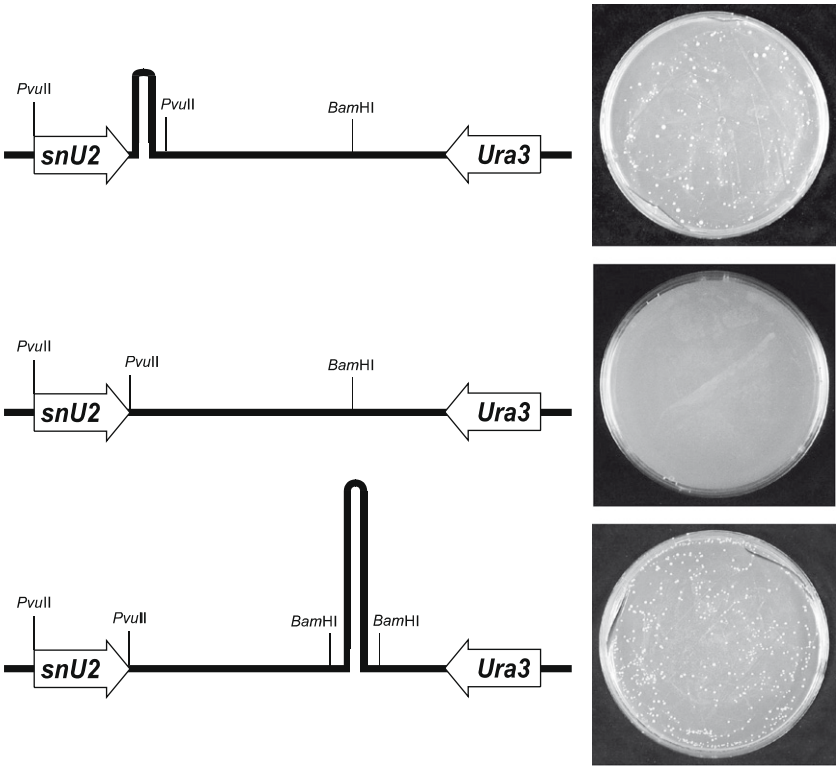

Fig. 1. Transformation failure with a U2 snRNA gene lacking a Pac1 endonuclease cleavage site. The upstream and coding sequences for the U2 snRNA gene was PCR amplified together with (upper panel) or without (middle panel) the downstream Pac1 nuclease-cleaved hairpin sequence and cloned into the PvuII site of the pFL20 yeast shuttle vector [19]. Recombinants with a conflicting orientation to the *Ura*3 gene marker were used to transform *S. pombe* cells (see Petri plates). A construct with the Pac1 nuclease-cleaved 3′ ETS sequence of a *S. pombe* rDNA transcription unit was also examined (lower panel).

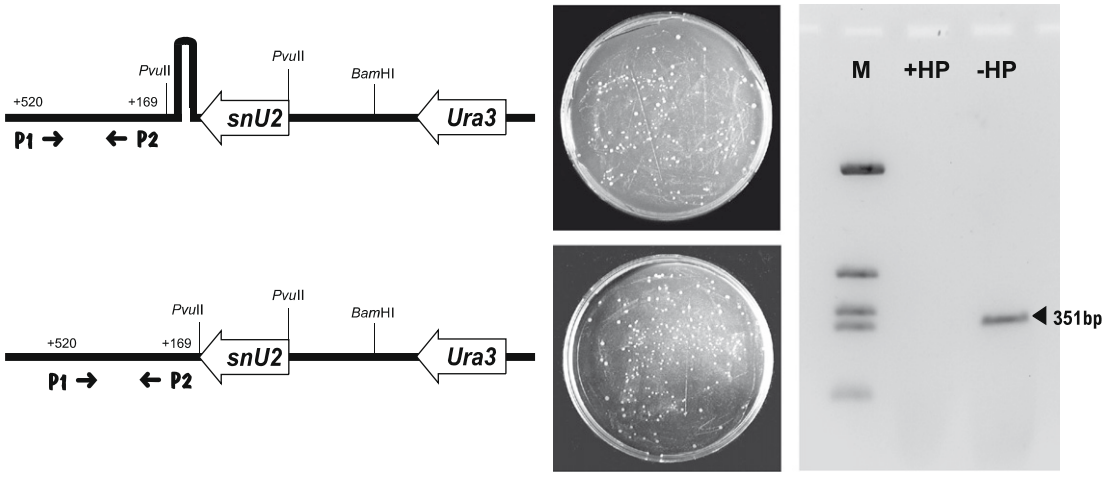

Fig. 2. Read-through transcription in a transformation defective construct. Constructs U2 snRNA gene with (upper panel) or without (lower panel) the downstream hairpin sequence were prepared as described in Fig. 1, and recombinants in the same orientation as the *Ura*3 gene were used to transform *S. pombe* cells (see Petri plates). RNA was prepared from representative transformants assayed for read-through transcription by RT-PCR using primers (P1/P2) specific for downstream region. Any PCR amplified DNA from transcripts with (+HP) and without (−HP) the hairpin sequence was fractionated on a 2% agarose gel (right panel) together with length markers (M); the anticipated 351 bp fragment is indicated on the right.

primers specific for the downstream region (P1/P2). Once again the results were strikingly different depending on whether the hairpin was present or absent. As anticipated a 351 bp read-through fragment clearly was evident when the hairpin was absent but was completely gone when the hairpin was present, confirming termination failure in the absence of the hairpin structure.

In the past studies [17,28], a mutant strain JP44 (h- leu1-32 snm1-1ts), temperature sensitive for the Pac1 RNase III-like endonuclease, was reported and used to examine 3′ end processing of the snRNAs of *S. pombe* cells. To demonstrate further the role of the Pac1 endonuclease in the termination process, in the present study this strain again was examined at both permissive and non permissive temperatures. The cells were grown to mid-log phase at 23 °C and then moved to the restrictive temperature (37 °C) for 5 h. Total RNA was extracted at both temperatures and, as shown in Fig. 3, together with transcript specific primers was used to assess the presence of read-through transcripts downstream of the hairpin sequence. After RT-PCR amplification, no transcript was detected downstream of this sequence at the lower temperatures (23 °C) whereas read-through transcription clearly was evi-

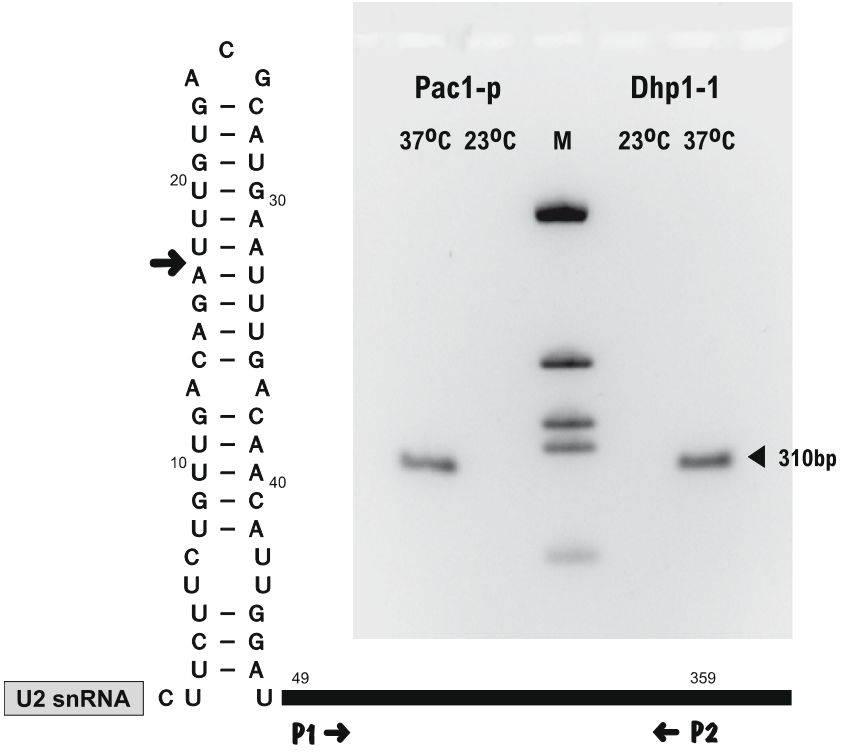

Fig. 3. Read-through transcription in *S. pombe* with defective Pac1 or Dhp1p nuclease. Total RNA was extracted from *S. pombe* strain TS138, temperature sensitive for Pac1 or strain KP38, temperatures sensitive for *dhp1-1* after growth at 23 or 37 °C for 5 h and the extent of transcription was determined by RT-PCR assay using primers (P1/P2) complementary to sequence downstream of the Pac1 cleavage site (arrow) as indicated in the schematic. Any PCR amplified DNA was fractionated on a 2% agarose gel (right panel) together with length markers (M); the anticipated 310 bp fragment is indicated on the right.

dent at the higher temperature (37 °C) even more than 330 bp downstream of the cleavage site. Whether caused by deletion of the hairpin (Figs. 1 and 2) or protein mutation (Fig. 3), in both instances transcript termination failure always was observed without Pac1 RNase cleavage.

In budding yeast and humans, transcript cleavage within pre-mRNA transcripts generates an entry site for the nuclear 5′ → 3′ exonuclease (Rat1 and Xrn2, respectively), which leads to transcription termination [3,4]. A similar mechanism has been speculated to function in the termination of U3 snoRNA gene transcripts [15]. To examine this possibility directly with U2 snRNA transcripts, a *S. pombe* strain KP38 (h+ ade6-M216 leu1 his7-lacI-GFP-his7+ lys1-lacO-lys1+ ura4-D18 dhp1-1<<ura4+) which has been reported to carry a temperature sensitive mutation in the dhp1^{+} locus [29], was used to assess the role of the nuclear 5′ → 3′ exonuclease (Dhp1p) in the termination of U2 snRNA transcription. Again, the temperature sensitive strain was grown at 23 °C to mid-log phase and then shifted to the restrictive temperature (37 °C) for 5 h. Total RNA was isolated at both temperatures and the presence of read-through transcripts downstream of the Pac1 cleavage hairpin sequence was assessed using RT-PCR. The anticipated 310 bp fragment was present only when the Dhp1p 5′ → 3′ exonuclease was inactive and RNA (37 °C) transcript clearly was present 330 nucleotides downstream of the Pac1 cleaved hairpin sequence.

Discussion

Endonuclease-mediated 3′ end processing has been a commonly reported feature of the U1–U5 snRNAs [17,30,31]. Similarly, in a strain depleted of Pac1 activity, U2 and U4 snRNA transcripts have been shown to be extended as far as 300 nucleotides downstream [28] and, although transcript termination never was considered, the impairment of Pac1 endonuclease cleavage in nascent U2 snRNA transcripts was reported to result in larger unprocessed precursor molecules [17]. The present results show these extended transcripts were not simply ending at termination sites, rather, they represent termination failure. The results also provide experimental evidence for the "reversed torpedoes" model, demonstrating that both the Pac1 and Dhp1p activities are critical to U2 snRNA transcript termination. As previously noted in studies of the U3 snoRNA, [16], the results also provide evidence that Pol II termination mechanisms can be more similar than previously believed. In all cases, transcript cleavage appears to initiate the termination process and, at least in the case of the U2 snRNA, the nuclear 5′ → 3′ exonuclease activity is essential, as demonstrated previously for polyadenylated transcripts [3,4].

As noted earlier, transcript termination of the U4 snRNA in *S. cerevisiae* appears dependent on the Nrd1-binding region, downstream of the Rnt1 cleavage site and Sen1 helicase activity has been implicated in the removal of the RNA polymerase from the DNA template [14]. This may reflect a difference in termination pathways among the species but, since the fact of cleavage has never been tested, the difference may be more in terms of detail rather than in the basic mechanism. For example, Nrd1-binding could represent a pause element while Sen1 could represent a different type of torpedo. Morlando and coworkers [32] actually have demonstrated an Rnt1-independent processing pathway for snRNAs which also relies on a Nrd1-binding element, leaving still another possibility. Since a sequence specific endonuclease cleavage event in snRNA processing has been demonstrated in higher eukaryotic [12,13], a basic "reversed torpedoes" model could still apply in all instances with a cleavage event initiating termination and alternate torpedoes whether exonuclease or helicase activities, ultimately leading to the termination of transcription, in some instances perhaps aided by pause elements. Whatever the case, the present study supports clearly the model for the U2 snRNA in *S. pombe* and further demonstrates some basic similarities in Pol II transcript termination.

Acknowledgments

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