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Pac1 endonuclease and Dhp1p 5′ → 3′ exonuclease are required for U3 snoRNA termination in *Schizosaccharomyces pombe*

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ABSTRACT

Maturation of some snoRNAs is dependent on RNase III-like endonuclease-mediated transcript cleavage, which serves as an entry for the nuclear exosome complex that trims the transcript at the 3′-end. Sequence deletions suggest this cleavage in the U3 snoRNA transcripts of *Schizosaccharomyces pombe* can induce transcript termination. Using mutational analyses, we demonstrate that the degree of cleavage correlates closely with both RNA maturation and transcript termination. We also show that the RNase III-like endonuclease, Pac1, and the nuclear 5′-exonuclease, Dhp1p, are essential for RNA production and transcript termination, supporting a "reversed torpedoes" model in which the endonuclease cut allows 5′- and 3′-exonuclease activities access to the transcript, leading simultaneously to transcript termination in one direction and RNA maturation in the other.

1. Introduction

In the majority of bacterial genes the mature end of RNA molecules is produced simply by transcript termination. In eukaryotic cells, endonuclease cleavage operationally replaces the termination in generating the 3′ ends of most RNA molecules. Some have speculated that termination remains a critical event to prevent RNA polymerase from interfering with the expression of the downstream gene and to free the enzyme to maintain an adequate pool in the cell.

Although studied intensely, many aspects of the RNA polymerase II (Pol II) transcript termination mechanism(s), particularly as it applies to the non-coding RNA transcripts, are not well understood. In the case of pre-mRNA transcripts, the polyadenylation signal on the nascent transcript triggers both 3′ end processing and transcript termination [1]. Two models have been put forward to explain this observation. The first model, referred to as anti-terminator, proposes that the polyadenylation sequence modifies the polymerase-associated factors, resulting in a less processive RNA polymerase [2]. The second, often referred to as the "torpedo" model, suggests that the polyadenylation-mediated cleavage generates a loading site for a 5′ → 3′ exonuclease, which in turn "torpedoes" the transcribing RNA polymerase [3–5].

In addition to polyadenylated RNAs, Pol II transcribes a large and diverse family of other RNAs often referred to as non-polyadenylated RNAs. In *Saccharomyces cerevisiae*, the termination of the snR13 RNA and a number of other snoRNA transcripts has been reported to require two RNA binding proteins, Nrd1 and Nab3, which bind specifically to two common RNA sequence motifs, GUA[AG] and UCUU [6,7]. Genetic and physical data indicate that binding of the Nrd1/Nab3 complex recruits both the Sen1 helicase and exosome complex [8,9]. Sen1 through its helicase activity is thought to remove the RNA polymerase from the DNA template, with the exosome complex then trimming the transcript from the free 3′ end terminal to produce the mature RNA [9,10]. On the other hand, with other Pol II non-polyadenylated transcripts (e.g., U1–U5 snRNAs and the U3 snoRNA) the RNase III-like endonuclease, Rnt1, has been demonstrated to introduce a cut within a hairpin structure immediately downstream of the coding region which acts as the entry site for the exosome complex [11,12]. More recent studies on U3 snoRNA expression in *Schizosaccharomyces pombe* [13,14] now indicate that this downstream endonuclease cleavage event also can initiate efficient transcript termination without other sequence elements but the mechanism has remained speculative.

In this study the relationships between the nuclease activities and RNA sequence/structure were examined as a further step to define the termination mechanism. The results support a "reversed torpedoes" model for the termination and maturation of the U3 snoRNA in which the RNase III-like Pac1 endonuclease induces 5′ → 3′ Dhp1p exoribonuclease cleavage in the downstream direction, resulting in transcript termination, and 3′ → 5′ exosome cleavage in the opposite direction, leading to mature U3 snoRNA.

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2. Materials and methods

2.1. Strains and vectors

Escherichia coli, strain C490 (rec A-, rk-, mk-, thr-, leu-, met-) was used as a host for the pTZ19R [15] and pFL20 [16] cloning vectors. *S. 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- A342T) and 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 their Pac1 endonuclease or Dhp1p exonuclease, respectively.

2.2. Preparation and expression of mutant U3B snoRNA genes

An efficiently expressed U3B snoRNA gene expression system containing a "tagged" *S. pombe* snU32 locus [17] was used to study the effects of nucleotide changes, in vivo. Base substitutions were introduced into the downstream region of the "tagged" gene by PCR amplification using a "megaprimer" strategy [18]. Upstream and downstream specific primers with BamHI adapter extensions were used to prepare 0.5 kilobase (kb) mutant fragments containing 69 bp of upstream and 198 bp of downstream sequence together with the "tagged" gene coding sequence and cloned in the pFL20 yeast shuttle vector using its unique BamHI site. Mutations were confirmed by DNA sequencing and the pFL20 recombinants were used to transform *S. pombe* cells [19]. Three transformants were examined for each mutant sequence.

2.3. Preparation and analysis of cellular U3 snoRNAs and transcripts

To assay the total cellular U3 snoRNA or nascent transcripts, logarithmically growing cells were disrupted by vortex with glass beads [20] and cellular RNA was prepared using a SDS/phenol protocol [21]. The relative amounts of U3B snoRNA or pre-snoRNA were determined by RT-PCR after treatment with 0.1U per ul of RNase-free DNase I [22]. RT reactions (20 μl) were performed using 400 ng of DNase-treated RNA, 0.2 mM dNTPs, 30 pmoles of primer and 40 units of MmuLV reverse transcriptase (Fermentas Inc., Hanover, MD), incubated for 2 h at 37 °C. For PCR 0.1–10% of the RT reaction product was used with 0.2 mM dNTPs, 30 pmoles of each primer and 1 unit of Taq polymerase for 30 cycles.

To assay individual U3 snoRNA components [16], U3 snoRNA was purified on 8% denaturing polyacrylamide gels before conversion to DNA by RT-PCR. [α-^{32}P] dCTP (2–5 μCi) was added to the PCR reaction to label the PCR amplified DNA. The labeled DNA was recovered by precipitation, digested with MboI endonuclease (Invitrogen Corp., Carlsbad, CA) and fragments were fractionated on 12% non-denaturing polyacrylamide gels. Images of autoradiographs were captured with a Umax Astra 600P scanner (Umax Technologies, CA) and quantified using Molecular Analyst PC software (BioRad Laboratories, CA).

2.4. Pac1 ribonuclease digestion analyses

RNA substrates for digestion analyses were prepared in vitro using T7 RNA polymerase [23,24]. Templates for RNA synthesis were prepared first by PCR amplification and cloned in pTZ19R [25]. Transcription reactions were performed with 0.4–2 μg of a DNA template; the RNA was purified on an 8% denaturing polyacrylamide gel and labeled at the 5′-end using bacteriophage T4 polynucleotide kinase and [γ-^{32}P] ATP, after dephosphorylation with calf intestinal phosphatase [26]. The labeled RNAs were purified again on a denaturing 8% polyacrylamide gel.

For digestion, Pac1 RNase was prepared and cleavage reactions were performed essentially as described by Rotondo and Frendewey [27]. The labeled RNAs were digested for 60 min at 30 °C in 30 μl of buffer containing 5 mM $MgCl_2$, 1 mM DTT and 30 mM Tris–HCl, pH 8.1. Reactions were stopped with the addition of an equal volume of gel loading buffer (formamide containing 0.05% xylene cyanol and 0.05% bromo-phenol blue), the solution was heated for two minutes at 65 °C, and 5 μl aliquots were fractionated on 8% polyacrylamide gels containing 8.3 M urea. RNA partially digested with T1 ribonuclease [28] also was applied as length markers.

2.5. Chromatin immunoprecipitation (ChIP) assay

The ChIP analyses were conducted essentially as described by Keogh and Buratowski [29]. Cross linking was carried out for 20 min at 24 °C and chromatin was sheared by sonication using a Heat Systems Inc. sonicator (model XL2020). Immunoprecipitation was performed with Protein G-Sepharose 4B Fast Flow (Sigma–Aldrich Canada Ltd.) and elution was performed for 15 min at 65 °C. Cross links were reversed with proteinase K (20 mg/ml) by incubation at 42 °C for 1 h and 65 °C for 4 h. For the PCR reactions (50 μl), aliquots of the antibody precipitated DNA were used with 0.2 mM dNTPs, 30 pmol of each primer and one unit of Taq polymerase for 30 cycles.

3. Results

As observed with polyadenylated Pol II transcripts, for at least some non-polyadenylated transcripts, we have speculated that RNase III-like nuclease cleavage provides a loading site for a 5′ → 3′ exonuclease to " torpedo" the transcribing polymerase molecule [13]. To more directly evaluate the need for the cleavage event, in this study the relationship has been examined further with a more detailed mutational analysis and the use of temperature sensitive enzymes. Mutations were introduced by a PCR-based megaprimer strategy [17] and expressed in vivo using an efficiently expressed plasmid-associated gene system [16]. The "tagged" snU32 locus encoding the U3B snoRNA was expressed under its own promoter and the dominant plasmid-derived U3B snoRNA could readily and specifically be detected by restriction fragment length polymorphism after RT-PCR amplification (see bottom panels in Fig. 1).

As indicated in the RNA schematic, also shown in Fig. 1, nucleotide changes were systematically introduced into the Pac1 RNase-cleaved downstream hairpin structure [13], a three nucleotides substitution in the cleavage site itself, centered at +50, a 2 nucleotide substitution on each side of the cleavage site, the first beginning at +46 and the second ending at +56 as well as a further three nucleotide change more distal to the cleavage site centered at +41. All the changes were designed to locally disrupt the secondary structure for evaluation both in vitro and in vivo. Initially, mutant RNA was prepared in vitro using T7 RNA polymerase and subjected to Pac1 nuclease digestion. As shown in Fig. 1 (upper panels), the degree of cleavage varied dramatically depending on the introduced nucleotide changes. Very little or essentially no cleavage was observed with the changes in the cleavage site (+50) itself, but partial and more complete digestion was evident with changes adjacent to the site (+46 and +56, respectively). Somewhat surprising was the lack of cleavage with the more distal changes at +41. This was similar to previously studies on the substrate specificity of eukaryotic RNase III-like enzymes, suggesting a separate protein binding domain [30,31]. Whatever the reason for the changes in cleavage efficiency, the differences provided an opportunity to better examine the relationships between RNA

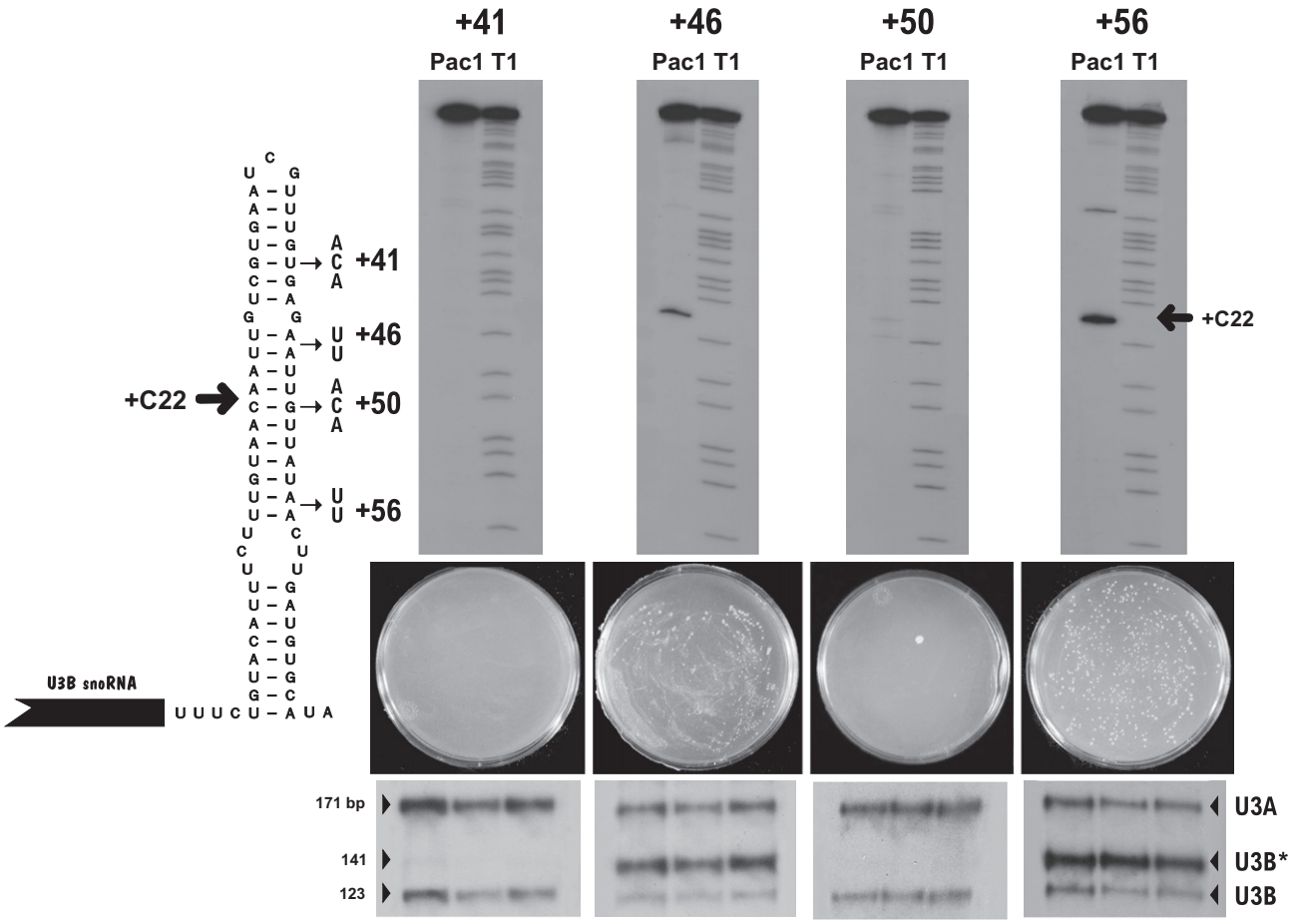

Fig. 1. Pac1 endonuclease cleavage is required for *S. pombe* U3 snoRNA gene transcription termination and RNA maturation. Nucleotide substitutions were introduced into different regions of the terminal hairpin structure (+41 to +56) as indicated on the left and mutant 5′ end-labeled RNA substrates were prepared in vitro using T7 RNA polymerase as described in Section 2. The mutant RNAs were digested with *S. pombe* RNase III-like Pac1 endonuclease and the fragments (Pac1) were fractionated on denaturing 8% polyacrylamide gels (upper panels); T1 RNase digests were included as fragment length markers (T1). U3 snoRNA genes containing the same mutations were cloned in the unique BamH1 site of the pFL20 yeast shuttle vector in a direction opposite to the *Ura*3 selection marker and used to transform *S. pombe* cells (middle panels). Total RNA was extracted from cells also transformed with equivalent mutants cloned in the same orientation as the *Ura*3 gene, to permit efficient transformation in each case. Purified U3 snoRNA was prepared from each transformant by gel electrophoresis and cDNA was prepared from each RNA by RT-PCR for RNA assay by restriction fragment length polymorphism using MboI digestion [16]. To differentiate the U3 snoRNAs, the digests were fractionated on 12% non-denaturing polyacrylamide gels as shown in the lower panels. The positions of diagnostic fragments for the host cell U3A and U3B snoRNAs and the plasmid-derived "tagged" U3B snoRNA are indicated on the right.

cleavage, transcript termination and U3 snoRNA maturation. As shown in the middle and lower panels, both transcript termination, as measured by the transformation frequency [13] and "tagged" U3 snoRNA levels, as measured by the restriction fragment length polymorphism [16], strongly reflected the cleavage data. As previously shown [13], termination can be assayed using the transformation frequency because plasmid-associated U3 snoRNA genes lacking downstream termination elements can severely inhibit transformation by disrupting the expression of the selectable marker (*Ura*3) which follows (e.g., Fig. 1, middle panels). This only occurs when the U3 snoRNA gene is inserted and expressed in a conflicting direction. Equally, any tagged plasmid-derived U3 snoRNA is detected as a distinct band migrating between fragments of U3A snoRNA and U3B snoRNA (U3B* in Fig. 1, lower panel). When little or no cleavage RNA was observed in vitro (+41 and +50), read-through clearly occurred as essentially no transformants were evident on the petri plates (middle panels) due to *Ura*3 gene (selective marker) interference [13]. Equally (lower panels), when a significant degree of cleavage occurred, mature "tagged" RNA was clearly evident (+46 and +56) but was essentially absent when the Pac 1 RNase was unable to cleave the RNA (+41 and +50). As recently described [16], uncleaved RNA transcripts are subject to "quality control" in vivo, and degraded rapidly.

The effect of Pac1 nuclease cleavage was evaluated further, in vivo, when a previously described [32,33] temperature sensitive (ts) Pac1 nucleases containing strains of *S. pombe* was examined for transcript accumulation at a restrictive temperature. Two strains, JP44 and ts138, which have been reported to be equivalent were examined with comparable results. Normal *S. pombe* and cells with the ts mutation were grown to mid log phase at a permissive temperature (23 °C) and then at 36 °C (restrictive) for 5 h. As indicated in Fig. 2 for the JP44 strain, transcript-specific primers were used to assess the presence of transcript within the downstream hairpin region as well as further downstream for read-through. Using RT-PCR amplification, transcript was readily detected in the hairpin region in both normal and temperature sensitive cells (left panel) but no transcript was detected downstream of this region in normal cells where read-through transcription was clearly evident with the temperature sensitive mutant, even more than 300 bp downstream of the cleavage site. Whether caused by sequence change (Fig. 1) or protein mutation (Fig. 2), in both instances transcribed termination failure was always observed without Pac1 RNase cleavage.

Based on this and previous study, it was attractive to speculate that the "torpedo" model can be applied to non-polyadenylated Pol II transcript termination, in that an exonuclease could gain access

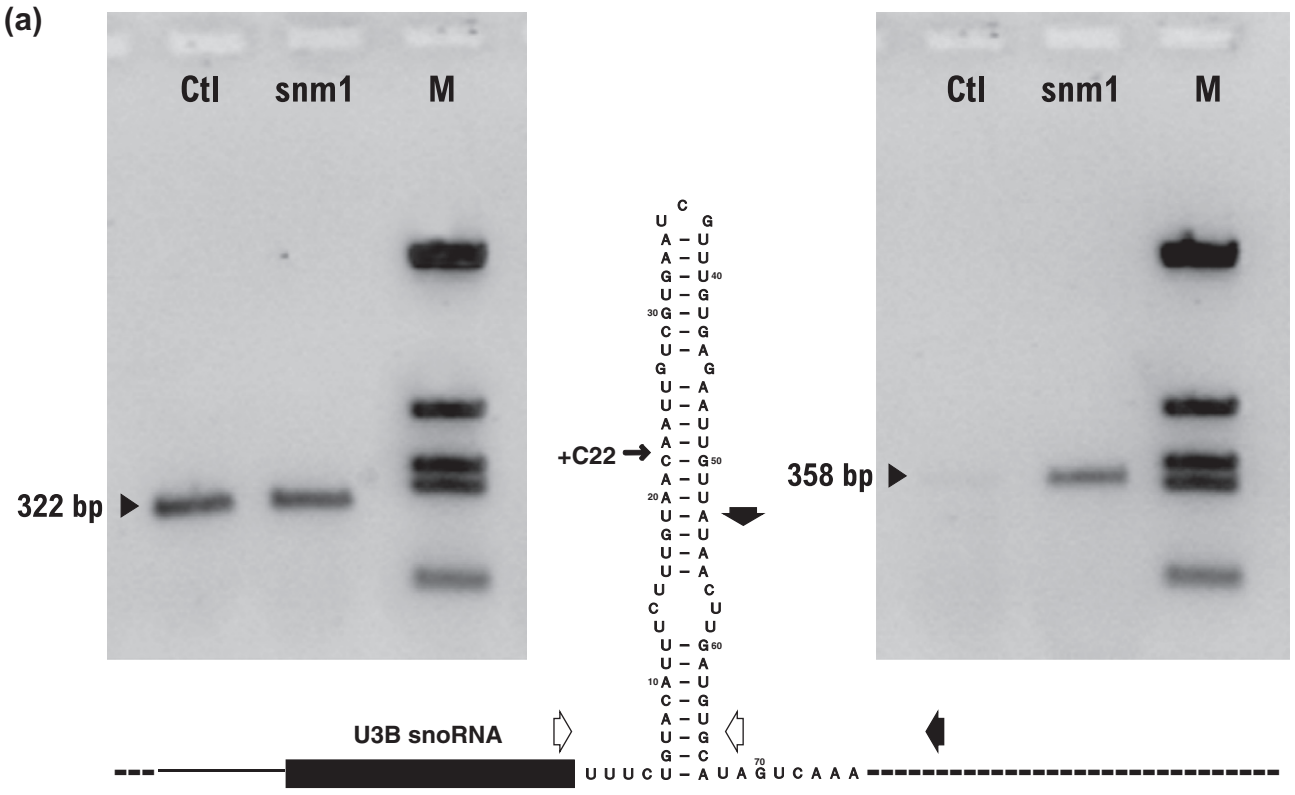

Fig. 2. U3 snoRNA read-through transcription in *S. pombe* with a defective Pac1 or Dhp1p nuclease. (a) Total RNA was extracted from normal *S. pombe* cells (Ctl) or the ts Pac1 mutant, JP44 (snm1) after growth at 37 °C for 5 h; the extent of transcription was determined using RT-PCR and primers (open and closed arrowheads) complementary to the hairpin structure region (left panel) or the region downstream of it (right panel) as described in Section 2. The PCR amplified DNA was fractionated on 2% agarose gels together with a fragment length marker (M); deduced fragment lengths were as indicated. (b) Total RNA was extracted from normal *S. pombe* cells (Ctl) or the KP38 ts *dhp*1-1 mutant after growth at 37 °C for 4 h and the extent of transcription was determined using RT-PCR as described above.

to the growing transcript after Pac1 nuclease cleavage. To examine this possibility more directly, a strain of *S. pombe* KP38 containing a temperature sensitive, nuclear 5′ → 3′ exonuclease (Dhp1-1) was obtained from Tatebayashi and co-workers [34]. The new strain was grown at 25 °C to mid log phase and then shifted to the restrictive temperature (36 °C) for 4 h. RNA extracts from cells growing at permissive and restrictive temperatures were again examined for downstream transcripts using RT-PCR amplification. As also shown in Fig. 2, when transcript-specific primers were used again to detect transcripts in both the hairpin region (left panel) and downstream region (right panel) the results were very different at the permissive vs restrictive temperature. In both cases, transcript was readily detected in the hairpin region but, once more, at the restrictive temperature, it was evident only when the 5′ → 3′

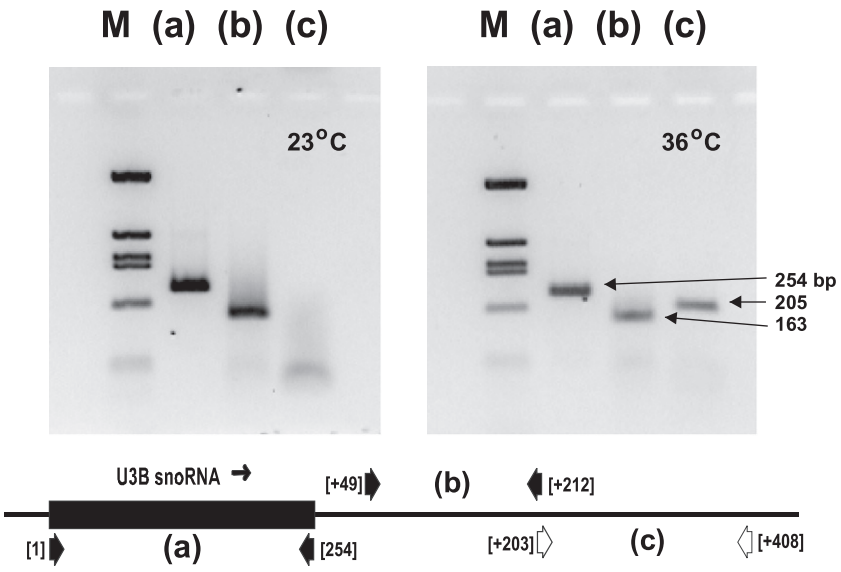

Fig. 3. Chromatin immunnoprecipitation maps of U3 snoRNA transcription in *S. pombe* with a defective Dhp1p exonuclease. Chromatin was prepared from *S. pombe* KP38 cells grown at 23 and 36 °C for 4 h and the localization of U3 snoRNA-transcribing pol II was mapped by ChIP analysis essentially as described by Keogh and Buratowski [29]. The presence of the Pol II CTD subunit was detected by PCR using primers complementary to the U3B snoRNA gene (a), immediately downstream of the RNA sequence (b) or beyond the normal region of termination (c). The sizes of the PCR products, fractionated on 2% agarose gels and positions of primers were as indicated.

exonuclease activity was absent, consistent with the proposed role in transcript termination. Read-through again was evident more than 400 bp downstream of the mature U3 snoRNA sequence.

The use of a temperature sensitive exonuclease clearly demonstrates read-through but does not distinguish between termination and RNA instability as the reason for the absence of downstream transcripts in normal cells. Our transformation assay (Fig. 1) based on the disruption of *Ura*3 gene expression strongly suggested transcript termination was the reason. To further unequivocally demonstrate this, ChIP analysis [29] was conducted using Anti-polII CTD antibody. As shown in Fig. 3, when *S. pombe* KP38 containing the temperature sensitive, nuclear 5′ → 3′ exonuclease (Dhp1-1) was compared at 23 and 36 °C, no downstream RNA polymerase again was evident 23 °C but the polymerase could be readily detected downstream (lane c) in the exonuclease sensitive strain at the non-permissive temperature (36 °C). These results again indicate that the Dhp1p 5′ → 3′ exonuclease is required for U3 snoRNA termination.

4. Discussion

The "reversed torpedoes" model for the termination and maturation of a non-polyadenylated Pol II transcript [13] proposes that RNase III-like nuclease cleavage of a downstream hairpin structure in the nascent transcript acts to initiate 5′ → 3′ exonuclease cleavage in the forward direction which results in transcript termination while simultaneously allowing exosomal trimming in the 3′ → 5′ direction leading to RNA maturation. The present study provides direct evidence for this, demonstrating that impaired transcript cleavage due to either RNA or protein mutation (see Figs. 1 and 2) leads to read-through transcription and a failure in RNA maturation. The results also show that defective 5′ → 3′ exonuclease degradation (Figs. 2 and 3) similarly leads to termination failure with greatly extended downstream RNA transcripts.

Whether polyadenylated or not, it appears that any Pol II transcript can be terminated by this basic mechanism, the difference being only the cleavage activity (the polyA cleavage factors or an RNase III-like endonuclease). In fact, a similar mechanism appears to apply to RNA polymerase I as recently reported in *S. cerevisiae* [35,36]. The initial observation that a Pac1-cleaved hairpin from the 3'ETS of *S. pombe* can induce Pol II termination [13] has been extended to rDNA with the more recent studies indicating that equivalent enzymology appears to be used in both cases. While not interpreted in a similar fashion, a number of earlier studies also support or confirm the present observations. Studies by Rupert and co-workers [32] on the U2 snRNA have demonstrated that the impairment of Pac1 cleavage also resulted in an extended transcript and no RNA maturation. Similarly, in their studies on both the U2 and U4 snRNAs under depleted Pac1 nuclease conditions, Frendeway and co-workers [33] have reported reduced mature RNA levels and extended transcripts as far as 300 nucleotides downstream. In both studies, transcript termination was not considered but clearly the observations are consistent with the "reversed torpedoes" model.

While a similar basic mechanism appears to apply to coding and many non-coding Pol II transcripts, it appears not to apply to others. Buratowski and co-workers [9] have shown that RNase III-like Rnt1 endonuclease depletion in *S. cerevisiae* cells had no effect on termination in their studies of snR13 and snR33 gene transcription. An extenuating factor, however, appears to be the lack of a downstream hairpin in the genes that were examined; Chanfreau and co-workers [37] previously had indicated that in these instances, cellular inactivation of the endonuclease also does not impair RNA maturation. It appears that another mechanism may function in these cases. In other instances such as the U4 snRNA (snR14 locus in *S. cerevisiae*) transcript termination appears to rely on a Nrd1-binding factor, downstream of the Rnt1 cleavage site [38] and Sen1 helicase activity has been implicated in the removal of the RNA polymerase from the DNA template. Unfortunately, the effect of cleavage appears not to have been tested in this case, raising the possibility of a pause element that enhances termination efficiency or even a fail-safe mechanism. More recent studies [39,40] have demonstrated actual instances of endonuclease-mediated cleavage as an alternative termination pathway in both coding and non-coding RNAs. Clearly, when a cleavage site is present such a mechanism can operate for sn and snoRNAs in both yeasts. Whatever the case and despite the similarities between polyadenylated Pol II transcripts and the U3 snoRNA, other mechanisms cannot be

excluded. Perhaps this attests further to the critical need for an efficient termination mechanism in all circumstances, to avoid the type of downstream interference that we observed with the plasmid-associated *Ura*3 gene and have exploited to assay U3snoRNA transcript termination in some of our studies.

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