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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