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Yeast pre-rRNA is processed at the A' site

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Abstract

Maturation of the pre-ribosomal RNA (pre-rRNA) in eukaryotes involves a series of processing steps that remove transcribed spacer sequences to produce mature ribosomes. In many organisms two processing sites are located in the 5’external transcribed spacer (5’ETS), along with the site that defines the 5’-end of the 18S rRNA. However, the pre-rRNA processing site near the start site of transcription, known as A’, has long been believed to be absent in the single-celled yeast, *Saccharomyces cerevisiae*. Here we provide evidence that the A’ pre-rRNA processing site is also present in the yeast 5’ETS, confirming conservation among single-celled and multicellular eukaryotes.

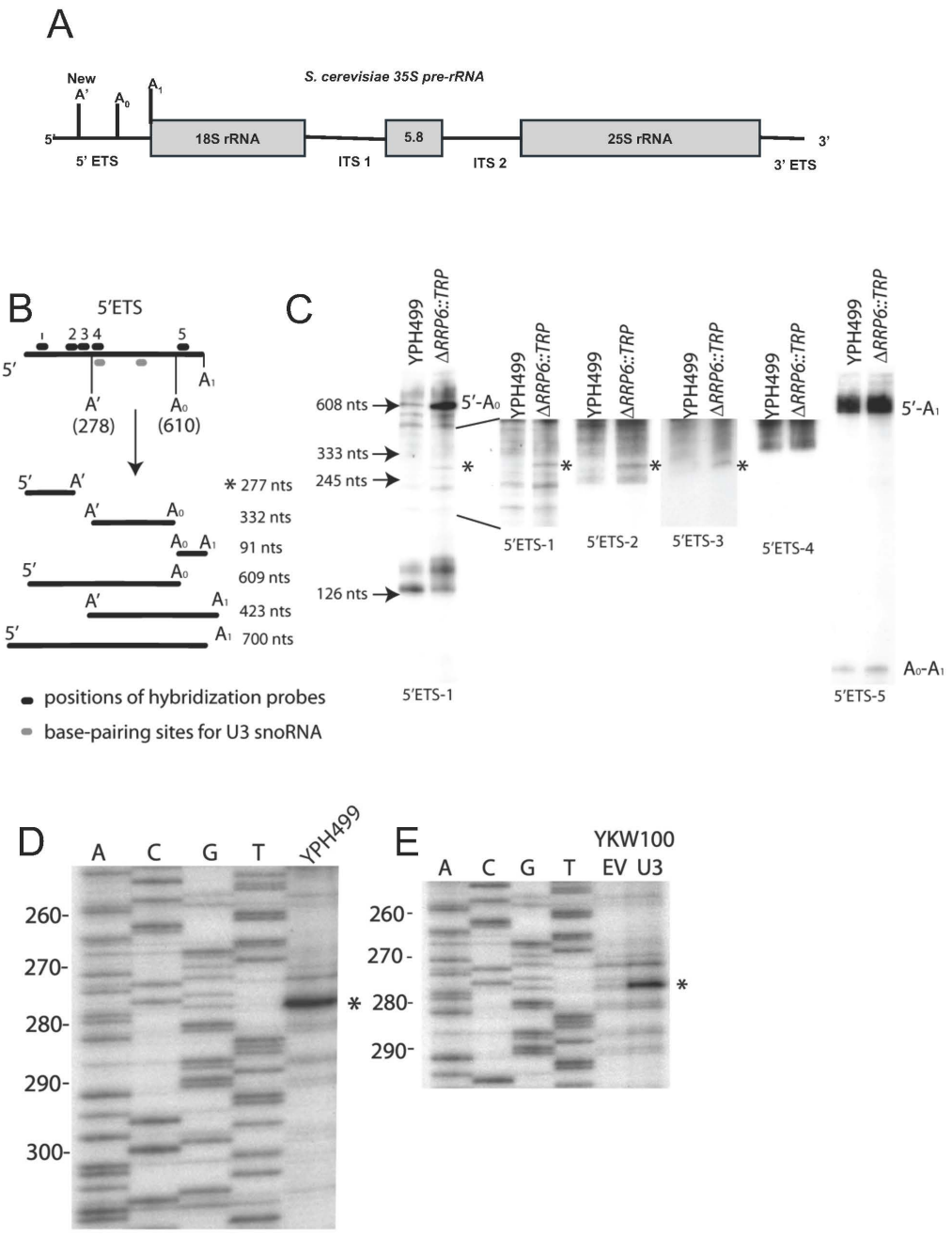

Figure 1. Yeast pre-rRNA is processed at the A' site:

Figure 1A. Schematic representation of the 35S pre-rRNA in *S. cerevisiae*. The 35S pre-rRNA encodes external and internal transcribed spacers (5'ETS, 3'ETS and ITS1, ITS2), which are processed to make the mature 18S, 5.8S and 25S rRNAs. Only the pre-rRNA processing sites in the 5'ETS are indicated, with A' specified as "New".

Figure 1B. Predicted pre-rRNA cleavage fragments from the yeast 5'ETS. Schematic representation of the 5'ETS with the three processing sites (A', A_0, A_1) and the predicted RNA fragments generated by processing at these sites. The annealing positions of the oligonucleotide probes (5'ETS-1 to 5) are depicted above the pre-rRNA. The U3 snoRNA binding sites are shown in gray below the pre-rRNA. The asterisk indicates the 5'-A' rRNA fragment resulting from processing at A'.

Figure 1C. The A' processing site in *S. cerevisiae* is detectable by northern blotting of pre-rRNA processing intermediates in the Δ*rrp6::TRP* strain. Northern blot analysis of pre-rRNA fragments from the 5' ETS with the indicated oligonucleotides probes (5'ETS-1 to 5) from Fig. 1B. Total RNA was extracted from the parental YPH499 and the Δ*rrp6::TRP* strains and analyzed on 8% polyacrylamide gels. As markers, the U14 snoRNA (126 nts), snR10 (245 nts), U3 snoRNA (333 nts) and snR30 (608 nts) were also probed for with complementary oligonucleotides (arrows). The asterisk indicates the 5'-A' rRNA fragment resulting from processing at A'. The pre-RNA fragments of length about 130 nts visible with the 5'ETS-1 probe are consistently present in northern blots, but their identity has not been established.

Figure 1D. Processing at the A' site can be detected by primer extension. Total RNA was extracted from the parental YPH499 strain and analyzed by primer extension with an oligonucleotide complementary to nts 401-423 (lane YPH499). The A' cleavage site is indicated by an asterisk. Lanes A,C,G,T represent a sequencing ladder.

Figure 1E. Processing at the A' site is U3 snoRNA-dependent. Strain YKW100, either bearing empty vector (EV) or a plasmid constitutively expressing the U3 snoRNA (U3), was grown in glucose to repress expression of the genomic U3 snoRNA. Total RNA was extracted and analyzed by primer extension using an oligonucleotide complementary to nts 401-423. The A' cleavage site is indicated by an asterisk. Lanes A,C,G,T represent a sequencing ladder.

Description

Ribosome biogenesis, the process of making ribosomes, occurs in the nucleolus in all eukaryotes. It is a complex and energy-intensive process (Warner, 1999) that requires the activity of all 3 RNA polymerases (Aubert et al., 2018; Bohnsack & Bohnsack, 2019; Dörner et al., 2023; Tomecki et al., 2017). The process begins with the transcription of the primary transcript, the 35S pre-rRNA in *Saccharomyces cerevisiae* (*S. cerevisiae*; Fig. 1A), by RNA polymerase I. Pre-rRNA transcription is followed by a series of pre-rRNA processing steps that produce the 18S rRNA (small ribosomal subunit) and the 5.8S and 25S rRNAs (large ribosomal subunit) which are assembled with ribosomal proteins to make mature ribosomes (Vanden Broeck & Klinge, 2024). The U3 small ribonucleoprotein (U3 snoRNP), a key component of the SSU processome (Dragon et al., 2002), is essential for the pre-rRNA cleavages that produce the mature 18S rRNA (Hughes & Ares, 1991; Samarsky & Fournier, 1998; Wehner et al., 2002; Wormsley et al., 2001)

Among the many pre-18S rRNA processing sites identified in eukaryotes, the A' site in the 5'ETS (Fig. 1A) is not thought to be found in the yeast *S. cerevisiae*. This site is referred to as A' or 01 in humans, A' or 0 in mouse, A' in trypanosomes or P in *Arabidopsis thaliana* (Craig et al., 1987; Hartshorne & Toyofuku, 1999; Kass et al., 1987; Kent et al., 2009; Mullineux & Lafontaine, 2012; Rouquette et al., 2005; Sikorska et al., 2017; Tomecki et al., 2017). In yeast the presence of this site, referred to here as A', has not been detected and is generally believed to be absent in this organism (Bohnsack & Bohnsack, 2019; Mullineux & Lafontaine, 2012; Tomecki et al., 2017; Venema & Tollervey, 1999;Woolford & Baserga, 2013).

To determine whether the A' processing site exists in yeast, we analyzed pre-rRNA isolated from a strain in which the 3' to 5' exonuclease component of the exosome, *RRP6*, was disrupted (*Δrrp6*). Rrp6 plays a role in degrading pre-rRNA fragments and its absence allows the detection of previously unstable pre-rRNAs (Allmang et al., 1999; Kent et al., 2009; Kobyłecki et al., 2018; Sloan et al., 2014). Using northern blotting and primer extension, we confirm the presence of the A' processing site in the 5'ETS of *S. cerevisiae* pre-rRNA when Rrp6 is absent. We further confirm the presence of A' processing by primer extension in the parent strain (YPH499) and demonstrate that the presence of the U3 snoRNA is required for optimal processing at A'. These findings provide evidence for the conservation of this pre-rRNA processing site from the yeast, *S. cerevisiae*, to humans.

Northern blots of pre-rRNA processing intermediates reveal A' site cleavage during yeast ribosome biogenesis. Disruption of *RRP6* (*Δrrp6)*, a gene encoding a nuclear exosome component, has previously been used to detect short-lived pre-rRNA processing intermediates (Allmang et al., 1999; Kent et al., 2009; Kobyłecki et al., 2018; Sloan et al., 2014). In the *Δrrp6* strain, pre-rRNA processing intermediates or fragments accumulate, which facilitates their detection. To enhance the identification of A' site processing, we generated the *Δrrp6*::*TRP* strain in which the *RRP6* gene is disrupted. We analyzed the pre-rRNA fragments resulting from 5'ETS processing in this strain compared to the parent strain, YPH499, by northern blotting using oligonucleotide probes complementary to the 5' ETS.

Northern blot analysis yields a pre-rRNA fragment that extends from the transcription start site (5') to a new cleavage site (A') when *RRP6* is disrupted. Figure 1B shows the predicted lengths of potential processing products of the 5'ETS if cleavage were also to occur at A', as well as at the known A0 and A1 sites. We focused on fragments smaller than the 5'ETS (700 nts), since A' processing is predicted to occur near the 5' end of the 5'ETS. We used a series of oligonucleotide probes that anneal to different sequences in the 5'ETS to identify the pre-rRNAs (5' ETS1-5 in Fig. 1B).

Hybridization to snoRNAs of known lengths was used to size the pre-rRNA fragments (Figure 1C, numbered arrows). In the *Δrrp6* strain, changes in the levels of many of the pre-rRNA fragments are detectable (Fig. 1C). As expected, the 5'-A_0 pre-rRNA fragment (608 nt) is the most prominent fragment to accumulate to a higher level in the *Δrrp6* strain when compared to the parent strain (Fig. 1C, 5'ETS-1 left). The 5'-A_1 pre-rRNA fragment (about 700 nts) is also visible (Fig. 1C, 5'ETS-5). There is a new pre-rRNA fragment (5'-A') in the *Δrrp6* strain, about 300 nt long, that hybridizes to probes 5' to the A' pre-rRNA cleavage site (Fig. 1C, 5'ETS-1 right, 5'ETS-2, 5'ETS-3, asterisks), but not to probes 3' to the A' cleavage site (Fig. 1C, 5'ETS-4, 5'ETS-5). This pre-rRNA fragment results from processing at a new site, A', in the pre-rRNA, generating the 5'-A' pre-rRNA fragment.

The A' pre-rRNA processing site is detectable by primer extension. To precisely localize the A' cleavage site in the YPH499 parent yeast strain, we performed primer extension on the pre-rRNA using a primer that anneals between the proposed A' processing site and A_0 at nucleotides (nts) 401-423 (see 400-5'ETS in Table 1). To precisely map the cleavage site, a sequencing ladder was run at the same time (Fig. 1D, lanes A,C,G,T). The primer extension revealed a few weak stops and a strong stop at nt 278, which we interpret as processing between nts 277/278 (Figure 1D lane YPH499). Thus, the A' processing site is detectable at nt 278 by primer extension in *S. cerevisiae*.

A' processing depends on the presence of the U3 snoRNA. Since the A_1 and A_0 cleavages in the 5'ETS are dependent on the presence of the U3 snoRNA (Hughes & Ares, 1991; Samarsky & Fournier, 1998; Wehner et al., 2002; Wormsley et al., 2001), we investigated whether cleavage at A' is similarly dependent. We compared cleavage at nts 277/278 in the presence and absence of the U3 snoRNA by primer extension. RNA was extracted from the YKW100 strain, in which the only remaining U3 snoRNA gene is under a galactose inducible/ glucose repressible promoter (Wehner et al., 2002). In glucose, yeast can grow and process pre-18S rRNA when the U3 snoRNA is expressed from a plasmid, but not when carrying an empty vector (Hughes, 1996). We observed that the primer extension stop at nt 278 is severely reduced in yeast cells carrying the empty vector (EV) when grown in glucose, where the U3 snoRNA is not expressed (Figure 1E lane EV). However, when the U3 snoRNA is expressed from a plasmid under the same conditions, the stop at nt 278 remains strong (Figure 1E compare lanes EV and U3). These results demonstrate that efficient processing at the A' site requires the presence of the U3 snoRNA.

Here we have shown that pre-rRNA processing in *S. cerevisiae* pre-rRNA also occurs at the A' site in the 5'ETS, as reported for other eukaryotes such as humans, mice, plants and trypanosomes (Craig et al., 1987; Hartshorne & Toyofuku, 1999; Kass et al., 1987; Kent et al., 2009; Mullineux & Lafontaine, 2012; Rouquette et al., 2005; Sikorska et al., 2017; Tomecki et al., 2017). We have confirmed the presence of the cleavage site at nt 277/278 in the 5'ETS by northern blotting after gene disruption of the 3' to 5' exonuclease, *RRP6*, and by primer extension. Using genetic depletion, we show that processing at the A' site depends on the presence of the U3 snoRNA, which base pairs at nt 282-292 (Dutca et al., 2011; Marmier-Gourrier et al., 2011). Thus, pre-rRNA processing and the role of the U3 snoRNA in mediating cleavage in the 5' ETS are similar among many of the studied eukaryotes, including in unicellular organisms like *S. cerevisiae* and *T. brucei*.

Rrp6 is a 3' to 5' exoribonuclease that is present mainly in the nucleus and is associated with the RNA exosome (Fox & Mosley, 2016; Januszyk & Lima, 2014; Stuparević et al., 2021; Zinder & Lima, 2017). Our results show that in *S. cerevisiae* the protein Rrp6 participates in the degradation of the pre-rRNA fragments, 5'-A' and 5'-A_0, resulting from the processing that takes place in the 5'ETS. These pre-rRNAs are more abundant or detectable only in the *Δrrp6* strain. Accumulation of the 5'-A_0 fragment has also been observed in earlier studies in *S. cerevisiae* when components of the exosome or exosome associated factors were depleted (Allmang et al., 1999; de la Cruz et al., 1998). The Rrp6 homologs from humans and mice (EXOSC10) and *Arabidopsis thaliana* (RRP6L2) also participate in the degradation of 5'ETS rRNA fragments (Kent et al., 2009; Kobyłecki et al., 2018; Lange et al., 2008). Likewise, the role of the Rrp6 protein in ribosome biogenesis is similar in diverse eukaryotes.

Methods

Strains and media. The *Δrrp6::TRP* strain was generated from the strain YPH499 (*MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) using the pFA6a-TRP1 plasmid as described (Longtine et al., 1998). The YKW100 (*MAT**a** ura3-52 his3-Δleu2 lys2-801^{amber} trp1-Δ63 u3aΔ UAS_{GAL}:U3A::URA3 u3bΔ::LEU2*) (Wehner et al., 2002) strain was used in primer extension experiments. Yeast were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose), YPG/R (1% yeast extract, 2% peptone, 2% galactose and 2% raffinose) or yeast selective media (SC-TRP, Clontech) supplemented with either 2% dextrose or 2% galactose and 2% raffinose. The solid medium contained 2% Bactoagar. Yeast transformations were performed using the standard lithium acetate protocol (Gietz et al., 1995). For depletion experiments, the YKW100 strain with the appropriate plasmid was grown in selective media (SC-TRP, Clontech) with galactose and raffinose to an optical density at 600 nm of 0.4-0.8 and shifted to YPD for 16 hours.

Plasmids. The plasmid pRS314 U3 WT contains a copy of the U3 snoRNA gene in the yeast expression vector pRS314 (*AMP^R, TRP, CEN/ARS*) (Wehner et al., 2002). After transforming the plasmids into YKW100, the strains were

maintained on SC-TRP.

RNA manipulations: primer extension and northern blots. Total RNA was obtained by hot phenol extraction (Ausubel, 1995). For analyzing small RNAs, 7 mg of total RNA were separated on 8% denaturing polyacrylamide gels and transferred to Hybond XL membranes (GE Healthcare), as described (Samarsky & Fournier, 1998; Wormsley et al., 2001). A series of oligonucleotides were used to detect the desired pre-rRNAs and snoRNAs (see Figure 1 and Table 1).

The primer extension protocol was performed as described (Dutca et al., 2011) with the following modifications: 2 mg of total RNA were used with 1 pmol of a 5'end- ^{32}P labeled oligonucleotide. The oligonucleotides used are in Table 1. The primer extension reaction was performed at 45 ^{o}C.

Reagents

Table 1. List of oligonucleotides used for generating the delta *rrp6* strain, northern blotting and primer extension.

Oligonucleotide	Sequence
RRP6.F1	5'-TAGACGAAATAGGAACAACAAACAGCTTATAAGCAC CCAATAAGTGCGTTCGGATCCCCGGGTTAATTAA-3'
RRP6.R1	5'-ATGAAAATTACCATAATTTATAAATAAAAAAATACG CTTGTTTTACATAAGAATTCGAGCTCGTTTAA AC -3'
5'-ETS1	5'-GTCTTCAACTGCTTTCGCAT C-3'
400-5'ETS	5'-GGAATGGTACGTTTGATATCGCT-3'
5'-ETS2	5'- CCC ACG ATG AGACTGTTCAG-3'
5'-ETS3	5'-GCTCACCAATGGAATCGCAAG-3'
5'-ETS4	5'-GCTAGTAATCCACCAAATCCTTC-3'
5'ETS-5	5'-CCACCTATTCCCTCTTGCTAGAAG-3'

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