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The *Saccharomyces cerevisiae* Pus2 protein encoded by YGL063w ORF is a mitochondrial tRNA: Ψ 27/28-synthase

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ABSTRACT

The RNA:pseudouridine (Ψ)-synthase family is one of the most complex families of RNA modification enzymes. Ten genes encoding putative RNA: Ψ -synthases have been identified in *S. cerevisiae*. Most of the encoded enzymes have been characterized experimentally. Only the putative RNA: Ψ -synthase Pus2p (encoded by the YGL063w ORF) had no identified substrate. Here, we analyzed Ψ residues in cytoplasmic and mitochondrial tRNAs extracted from *S. cerevisiae* strains, carrying disruptions in the PUS1 and/or PUS2 ORFs. Our results demonstrate that Pus2p is a mitochondrial-specific tRNA: Ψ -synthase acting at positions 27 and 28 in tRNAs. The importance of the Asp56 residue in the conserved ARTD motif of the Pus2p catalytic site is demonstrated *in vivo*. Interestingly, in spite of the absence of a characteristic N-terminal targeting signal, our data strongly suggest an efficient and rapid targeting of Pus2p in yeast mitochondria. In contradiction with the commonly held idea that a unique nuclear gene encodes the enzyme required for both cytoplasmic and mitochondrial tRNA modifications, here we show the existence of an enzyme specifically dedicated to mitochondrial tRNA modification (Pus2p), the corresponding modification in cytoplasmic tRNAs being catalyzed by another protein (Pus1p).

Keywords: *S. cerevisiae*; pseudouridine; RNA:pseudouridine-synthase; tRNAs; mitochondria

INTRODUCTION

In all organisms, pseudouridine (Ψ) and 2'-O-methylated residues are the most frequent modifications in RNAs (see The RNA Modification Database, URL <http://medstat.med.utah.edu/RNAmods/>). The Ψ residues are formed by base isomerization of the transcriptionally incorporated uridine residues (Kammen et al. 1988; Huang et al. 1997). This reaction is catalyzed by RNA: Ψ -synthases, which do not require cofactors or energy for catalysis. RNA: Ψ -synthases represent a broad group of proteins that are divided into five distinct families (TruA, TruB, RluA, RsuA, and TruD) on the basis of amino acid sequence homology (see Koonin 1996; Conrad et al. 1998; Kaya and Ofengand 2003). Based

on computer analysis of bacterial and yeast genomes, nine genes encoding putative RNA: Ψ -synthases have been identified in *Saccharomyces cerevisiae* (Koonin 1996). The RNA: Ψ -synthase activity of the products of eight of them has been identified. Only the substrate specificity of the PUS2 gene product has not yet been characterized. RNA: Ψ -synthases Pus3p, Pus4p, and Pus6p are tRNA-specific enzymes acting on both cytoplasmic and mitochondrial tRNAs (Becker et al. 1997; Lecoite et al. 1998; Ansmant et al. 2001). Pus5p is responsible for the modification of the mitochondrial 21S rRNA (Ansmant et al. 2000), and Cbf5p is associated with the H/ACA snoRNA guides and catalyzes Ψ formation in cytoplasmic rRNAs and U2 snRNA (Lafontaine et al. 1998; Kiss et al. 2004; Ma et al. 2005). Pus7p was not identified using sequence homology but by genome-wide screening of GST-tagged yeast ORFs. It was initially characterized as a U2 snRNA-specific Ψ -synthase (Ma et al. 2003), but it also catalyzes tRNA modification at positions 13 and 35 (Behm-Ansmant et al. 2003). We found that two distinct enzymes, Rib2p/Pus8p and Pus9p, are required for Ψ 32 formation in tRNAs, depending on their cytoplasmic or mitochondrial localization, and cytoplasmic Rib2p/Pus8p contains both a tRNA: Ψ 32-synthase domain and a DRAP-deaminase domain (Behm-Ansmant et al.

Abbreviations: 2D, bidimensional; CMCT, 1-cyclohexyl-3-[2-morpholinoethyl] carbodiimide metho-*p*-toluenesulfonate; NMP, nucleotide monophosphate; Ψ , pseudouridine; ORF, open reading frame; PCR, polymerase chain reaction; RT, reverse transcriptase; TLC, thin layer chromatography; U, uridine; WT, wild-type.

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2004). Pus1p is an RNA: Ψ -synthase with a multisite specificity (tRNA positions 1, 26, 27, 28, 34, 36, 65, and 67) (Motorin et al. 1998; Behm-Ansmant et al. 2006). In addition, it also modifies U2 snRNA at position 44 (Massenet et al. 1999). In contrast to yeast cytoplasmic tRNAs, their mitochondrial counterparts are significantly less modified; only uridines at positions 27, 28, 31, 32, 38, 39, 55, and 72 may be converted to Ψ s. Based on the assumption that a unique enzyme is generally used to modify both cytoplasmic and mitochondrial tRNAs, Pus1p was also expected to be responsible for the formation of residues Ψ 27 and Ψ 28 commonly present in mitochondrial tRNAs. However, its activity in yeast mitochondria was never tested experimentally. The only remaining RNA: Ψ -synthase without identified substrates (Pus2p) shows strong sequence homology with Pus1p, especially in motifs II and IIa (Fig. 1), hinting at a similar catalytic activity for these two enzymes. Furthermore, in spite of the absence of a characteristic motif for mitochondrial localization in Pus2p, its preferential mitochondrial localization is clearly predicted by the NNPSL software (Reinhardt and Hubbard 1998). Thus, we hypothesized that Pus2p may be the mitochondrial counterpart of Pus1p. Our interest in the discovery of the yeast RNA: Ψ -synthase responsible for modification of mitochondrial tRNAs at positions 27 and 28 was reinforced by the discovery of a direct link between Ψ 27 formation in human mitochondrial tRNA and a rare mitochondrial disorder called mitochondrial myopathy and sideroblastic anemia (MLASA) (Patton et al. 2005).

To test this hypothesis, we analyzed the effects of individual or simultaneous deletions of *PUS1* and *PUS2* ORFs on the in vivo tRNA pseudouridylation pattern. The loss of *PUS1* did not affect pseudouridylation at positions 27 and 28 in mitochondrial tRNAs, while the deletion of *PUS2* abolished Ψ 27 and Ψ 28 formation in these species. Since the enzyme responsible for Ψ 72 formation in the

mitochondrial tRNA^{Met}_i(CAU) has not yet been identified, we also tested the possible activity of Pus2p at position 72 of this tRNA. The data presented demonstrate that Pus2p is a yeast mitochondrial RNA: Ψ -synthase acting at positions 27 and 28, but not at position 72.

RESULTS AND DISCUSSION

Mitochondrial tRNA modifications are affected by *PUS2* ORF disruption

To investigate whether Ψ formation at positions 27 and 28 in mitochondrial tRNAs is catalyzed by Pus1p or Pus2p, we tested the effect of individual or simultaneous *PUS1* and *PUS2* ORF deletions on mitochondrial tRNA pseudouridylation. The haploid *S. cerevisiae* strain RS453, whose *PUS1* ORF was disrupted by insertion of the *HIS3* gene (*pus1 Δ ::HIS3*, denoted Δ *pus1*) (Simos et al. 1996), was used to test for the effect of the absence of Pus1p expression. To analyze the consequences of the absence of Pus2p expression, we used the haploid *S. cerevisiae* strain BY4742, whose *PUS2* ORF is disrupted by a *Kan^r* gene replacement (*pus2 Δ ::Kan^r*, denoted Δ *pus2*; EUROSCARF). The effects of the simultaneous *PUS1* and *PUS2* ORF disruptions were tested in the haploid Δ *pus1*/ Δ *pus2* strain, which we isolated by mating of the *pus2 Δ ::Kan^r* and the *pus1 Δ ::HIS3* strains and dissecting the resulting diploids.

Six out of the 17 sequenced mitochondrial tRNAs in *S. cerevisiae* bear a Ψ residue at position 27. However, four of them are not suitable for analysis by the CMCT/RT approach because of the presence of a m²G26 or/and m¹G37 residue(s), which block(s) primer extension analysis. To avoid this difficulty when testing the Pus1p and Pus2p activity at position 27 we used tRNA^{Arg}(UCU), which does not contain these modifications at positions 26 and 37. Pseudouridine at position 28 is found in only

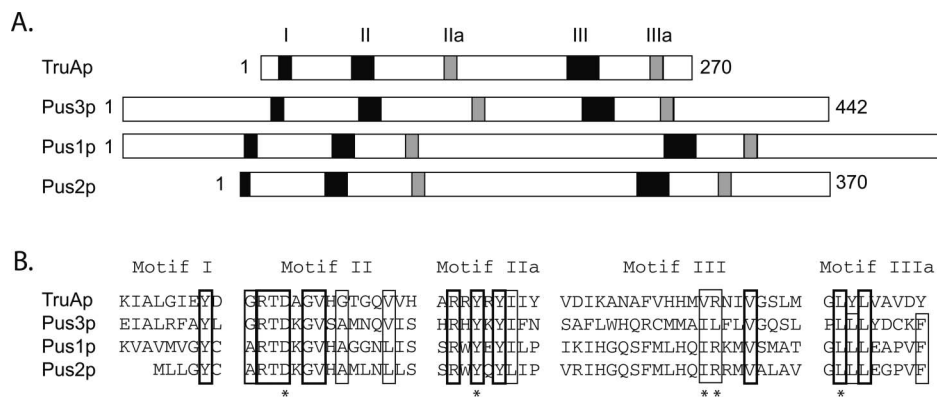


FIGURE 1. Alignment of the amino acid sequences of the *Escherichia coli* TruA and *S. cerevisiae* Pus1p, Pus2p, and Pus3p tRNA: Ψ -synthases of the TruA family. (A) Proteins are schematically represented as bars, whose lengths are proportional to lengths of the polypeptide chains. Motifs I, II, IIa, III, and IIIa of RNA: Ψ -synthases (Del Campo et al. 2004) are represented by black or gray rectangles. (B) The alignments of the amino acid sequences of the conserved blocks I to IIIa are shown. Identical and semiconserved residues are boxed. The thickness of the line is an indication of the level of sequence conservation. The conserved residues (D, Y, I, R, L) in the tRNA: Ψ -synthases active sites are indicated by asterisks.

one mitochondrial tRNA, the tRNA^{Lys}(UUU), and fortunately, Ψ 28 formation in this tRNA could also be analyzed by the CMCT/RT approach. Disruption of the *PUS1* ORF affected neither Ψ 27 formation in the mitochondrial tRNA^{Arg}(UCU) (Fig. 2A, lanes 5,6) nor Ψ 28 formation in mitochondrial tRNA^{Lys}(UUU) (Fig. 2B, lanes 3,4). In contrast, after deletion of *PUS2* ORF, both of these pseudouridylations were abolished (Fig. 2A,B, lanes 7, 8 in both panels). Accordingly, similar results were obtained with the Δ *pus1*/ Δ *pus2* strain carrying both deletions (Fig. 2A,B, panels A and B, lanes 9,10 and 11,12, respectively). Transformation of this strain with a plasmid expressing the WT protein restored Ψ 27 formation in tRNA^{Arg}(UCU),

while no modification was detected in the strain expressing a variant protein with a D56A substitution in Motif II (Fig. 3, lanes 7–10).

Since Ψ 72 in tRNA^{Met}₁(CAU) is located too close to the tRNA 3'-extremity to be analyzed by the CMCT/RT approach, the possible implication of *Pus2p* in Ψ 72 formation was only tested in vitro by the nearest-neighbor approach. To this end, the tRNA: Ψ 72-synthase activity of cell-free extracts prepared from different strains was tested on a transcript of the mitochondrial tRNA^{Met}₁(CAU) labeled by [α -³²P]ATP incorporation. As evidenced by thin layer chromatography (TLC) analysis of the labeled RNase T2 digestion products, none of the gene disruptions affected the activity of the extracts (Table 1, see also Behm-Ansmant et al. 2006). Thus, *Pus1p* and *Pus2p* are probably not involved in Ψ formation at position 72.

Other pseudouridines in cytoplasmic yeast tRNA normally formed by *Pus1p* (positions 1, 34, 36, 65, 67) are absent in the mitochondrial compartment; thus, positions 27 and 28 are the only possible targets for *Pus2p* in mitochondria.

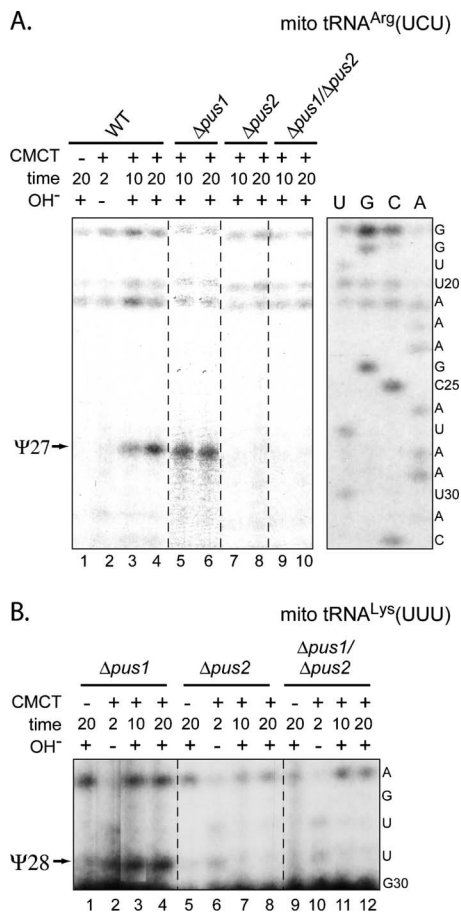


FIGURE 2. Disruption of the *PUS2* gene leads to the absence of Ψ 27 residues in the mitochondrial tRNA^{Arg}(UCU) (A) and Ψ 28 in the mitochondrial tRNA^{Lys}(UUU) (B). Total RNAs extracted from the WT, Δ *pus1*, Δ *pus2*, and Δ *pus1*/ Δ *pus2* strain were modified by CMCT, for 2, 10, and 20 min with (+) or without (-) subsequent alkaline treatment (OH⁻). A control experiment was performed in the absence of CMCT treatment. Lanes U, G, C, and A correspond to the sequencing ladders obtained with the same oligonucleotide. Primer extension analyses were performed on the mitochondrial tRNA^{Arg}(UCU) (A) and tRNA^{Lys}(UUU) (B) using specific oligonucleotide primers. The reverse transcription stops, corresponding to residue Ψ 27 in tRNA^{Arg}(UCU) and Ψ 28 in tRNA^{Lys}(UUU), are indicated by arrows.

Pus2p is unable to insure cytoplasmic tRNA modification

Since *Pus1p* and *Pus2p* have redundant activity toward Ψ 27 and Ψ 28, we tested if *Pus2p* could complement the absence of active *Pus1p* for cytoplasmic tRNA modification. Previous studies have already demonstrated the absence of tRNA: Ψ 27-synthase activity in extracts from the Δ *pus1* strain (Motorin et al. 1998). However, this apparent absence of in vitro *Pus2p* activity could result from protein inactivation or from inappropriate reaction conditions. We therefore verified the absence of in vivo *Pus2p* activity on cytoplasmic tRNAs by CMCT/RT analysis of the cytoplasmic tRNA^{Met}(CAU) extracted from the Δ *pus1* strain. As shown in Figure 4, lanes 7, 8, 15, and 16, no Ψ residue was detected at position 27 in this tRNA, whereas it was detected in tRNA^{Met}(CAU) extracted from the Δ *pus2* strain (Fig. 4A, lanes 11,12). Formation of Ψ 31, which was used as a control, remained unaffected. Thus, *Pus2p* is not able to complement the absence of *Pus1p* activity in vivo.

How to explain the inability of *Pus2p* to modify cytoplasmic tRNAs? Either *Pus2p* cannot modify cytoplasmic tRNAs due to the absence of the required recognition determinants in these tRNAs or, immediately after protein synthesis, *Pus2p* is rapidly and predominantly targeted to mitochondria and thus its potential substrates in the cytoplasm escape modification. The first hypothesis seems unlikely since in contrast to their vertebrates counterparts, yeast cytoplasmic and mitochondrial tRNAs have rather similar 2D structures (Wesolowski and Fukuhara 1979; Leehey et al. 1995).

To validate the second hypothesis, we compared the in vitro tRNA: Ψ 27-synthase activities of extracts from the WT, Δ *pus1*, Δ *pus2*, and Δ *pus1*/ Δ *pus2* cells on a mitochondrial

a scaRNA guided system is used in vertebrates (Ma et al. 2005). The emergence of the scaRNA guided modification system for UsnRNAs probably had a strong influence on the activity of members of the RNA: Ψ -synthase families. The striking similarity between Pus1p and Pus2p suggests that the presence of two distinct enzymes may have arisen from gene duplication, followed by acquisition of the appropriate subcellular targeting signals.

Despite our observation of nonoverlapping functions of Pus1p and Pus2p, individual and even simultaneous disruptions of their genes had no marked effect on cell viability and growth, even in growth conditions allowing detection of mitochondrial respiratory defects (medium with glycerol as the only carbon source, data not shown). The absence of marked growth phenotype is a rather general property of strains carrying disruption in a gene encoding an RNA modification enzyme. When growth phenotypes were detected, they were almost always due to the lack of an additional protein function distinct from the RNA modification activity.

MATERIALS AND METHODS

Yeast strains

The haploid *S. cerevisiae* BY4742 strain (denoted WT in this article) and the haploid strain carrying a disruption of the *PUS2* gene (*pus2 Δ ::Kan^r*, denoted Δ *pus2*), were obtained from the EUROSCARF collection (Germany). The haploid Δ *pus1* yeast strain was a generous gift of G. Simos (University of Larissa, Larissa, Greece). It was obtained by interruption of the *PUS1* gene by the *HIS3* auxotrophic marker (*pus1 Δ ::HIS3*) in the genomic DNA of the RS453 *S. cerevisiae* strain (Simos et al. 1996). Yeast *S. cerevisiae* strain 26-G-7 expressing 3 \times HA-tagged Pus2p (insertion position 312) was obtained from Open BioSystems. A haploid strain with simultaneous deletions of the *PUS1* and *PUS2* genes was generated by mating of the *pus2 Δ ::Kan^r* and the *pus1 Δ ::HIS3* strains. After sporulation of the obtained diploid mutants, asci were separated by microdissection.

Complementation of the disrupted strains

To generate a *S. cerevisiae* plasmid expressing Pus2p, the coding region of the *PUS2* ORF together with the upstream 300-bp region were amplified from the genomic DNA of the *S. cerevisiae* BY4742 strain. The oligonucleotides used generated a NheI and an EcoRI restriction site at the 5'- and 3'-extremities, respectively. The amplified DNA fragment was inserted at the SmaI site of plasmid pUC18. The resulting construct (pUC18-PUS2) was cleaved by the NheI and EcoRI restriction endonucleases, and the fragment encoding Pus2p was inserted between the XbaI and EcoRI sites of plasmid p416GalS (Mumberg et al. 1994). A point mutation in the putative RNA: Ψ -synthase active site of Pus2p was introduced by PCR-mediated site-directed mutagenesis (D56A, GAC->GCC). All the recombinant genes produced were entirely sequenced. Yeast transformation was performed with the standard lithium acetate procedure (Gietz et al. 1992; Adams et al. 1997).

CMCT/RT mapping of Ψ residues in RNA

Preparation of total RNA from yeast strains and CMCT-RT mapping of Ψ residues were performed as described previously (Bakin and Ofengand 1993; Massenet et al. 1999; Ansmant et al. 2000). The presence of residue Ψ 27 in mitochondrial tRNA^{Arg}(UCU), Ψ 28 in mitochondrial tRNA^{Lys}(UUU), and Ψ 27 in cytoplasmic tRNA^{Met}(CAU) was analyzed using oligonucleotides complementary to residues 46–62, residues 43–72, and residues 40–57, respectively.

Analysis of Ψ residue formation by the nearest neighbor approach

The coding sequences of the mitochondrial tRNA^{His}(GUG) and tRNA^{Met}_i(CAU) were PCR amplified from the genomic DNA of the BY4742 *S. cerevisiae* strain and inserted downstream from a T7 RNA polymerase promoter at the SmaI site of plasmid pUC18. The pTFM-Val plasmid used for in vitro transcription of cytoplasmic tRNA^{Val}(UAC) was kindly provided by C. Florentz (IBMC, Strasbourg, France). Cell-free S10 extracts preparation and tests of their in vitro RNA: Ψ -synthase activities were performed as previously described (Auxilien et al. 1996; Behm-Ansmant et al. 2003).

Sequence analysis

The NNPSL software (Reinhardt and Hubbard 1998, <http://predict.sanger.ac.uk/nnpsl/>, Web site) was used for prediction of the Pus2p subcellular localization. This program takes into account the amino acid composition of the whole protein, its predicted secondary structure, and various characteristic features, such as the presence of a mitochondrial targeting sequence or of a potential signal peptidase cleavage site.

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