

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

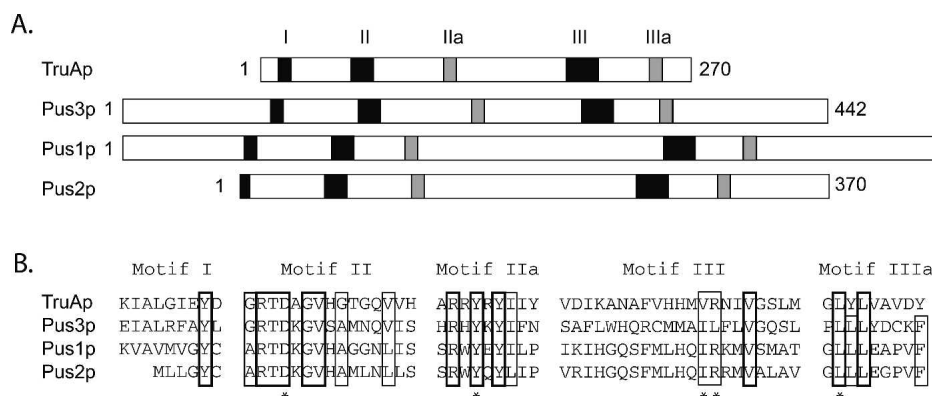


FIGURE 1. Alignment of the amino acid sequences of the *Escherichia coli* TruA and *S. cerevisiae* Pus1p, Pus2p, and Pus3p tRNA: Ψ -synthases of the TruA family. (A) Proteins are schematically represented as bars, whose lengths are proportional to lengths of the polypeptide chains. Motifs I, II, IIa, 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.

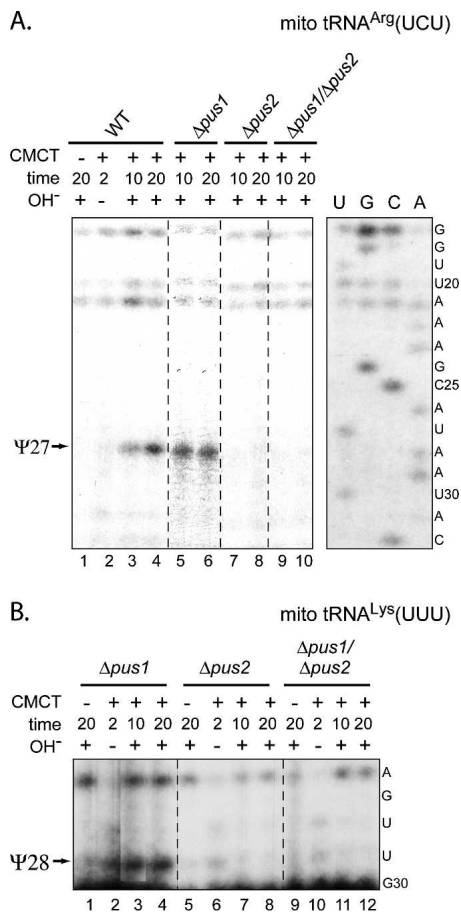


FIGURE 2. Disruption of the *PUS2* gene leads to the absence of Ψ 27 residues in the mitochondrial tRNA^{Arg}(UCU) (A) and Ψ 28 in the mitochondrial tRNA^{Lys}(UUU) (B). Total RNAs extracted from the WT, Δ *pus1*, Δ *pus2*, and Δ *pus1*/ Δ *pus2* strain were modified by CMCT, for 2, 10, and 20 min with (+) 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

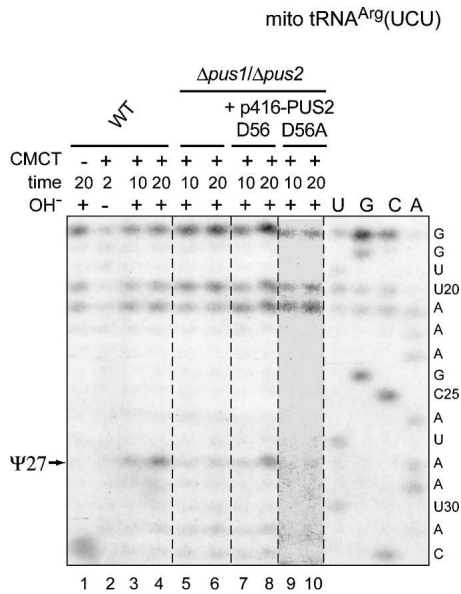


FIGURE 3. The aspartic acid residue D56 in the Pus2p active site is essential for the RNA: Ψ -synthase activity. The yeast *S. cerevisiae* $\Delta pus1/\Delta pus2$ strain was transformed with recombinant p416GalS plasmids, expressing the WT (D56) or mutated (D56A) PUS2 ORF. Total RNAs were extracted from the WT, the untransformed $\Delta pus1/\Delta pus2$, and the two transformed $\Delta pus1/\Delta pus2$ strains. The mitochondrial tRNA^{Arg}(UCU) was analyzed by the CMCT/RT approach (same legend as in Fig. 2). The reverse transcription stop, corresponding to residue Ψ 27 is indicated by an arrow.

tRNA known to be modified at position 27 (tRNA^{His}[GUG]) and on a cytoplasmic tRNA that has Ψ residues at both positions 27 and 55 (tRNA^{Val}[UAC]). The analysis was performed using the nearest neighbor approach. To this end, the mitochondrial tRNA^{His}(GUG) and the cytoplasmic tRNA^{Val}(UAC) were labeled by [α -³²P]ATP and [α -³²P]CTP incorporation, respectively. The results obtained are given in Table 1. Formation of Ψ residues were detected at positions 27 and 55 in the cytoplasmic tRNA^{Val}(UAC) and at position 27 in the mitochondrial tRNA^{His}(GUG), after incubation with the WT and the $\Delta pus2$ cell extracts. In contrast, residue Ψ 27 was not formed in any of these two tRNAs after incubation in both the $\Delta pus1$ and the $\Delta pus1/\Delta pus2$ cell extracts. The following interpretation can be proposed: (1) Pus1p in the extract has

an intrinsic capability of modifying mitochondrial as well as cytoplasmic tRNAs, and (2) the absence of tRNA: Ψ 27-synthase activity on both the cytoplasmic and the mitochondrial tRNA transcripts in the $\Delta pus1$ extract is due to the absence of Pus2p in the cellular extract.

The tight association of Pus2p with the insoluble fraction of disrupted yeast cells, which probably contain the mitochondria, can explain its almost complete loss during preparation of cell-free extract and the observed absence of Pus2p activity in these extracts. Indeed, by expression of an HA-tagged Pus2p in the *S. cerevisiae* strain 26-G-7 (Open BioSystems), we showed by Western Blot analysis that the S10 cell-free soluble extract is devoid of HA-tagged Pus2p (Fig. 4B).

Taken together, our results show that Pus1p is able to modify both the cytoplasmic and mitochondrial tRNAs in vitro. However, it has a strict cytoplasmic/nuclear localization and therefore only modifies cytoplasmic tRNAs in vivo. In contrast, Pus2p is very rapidly targeted to mitochondria and is not present in a yeast soluble cell fraction. This rapid transport to mitochondria likely explains its absence of activity on cytoplasmic tRNAs.

Nonconventional mitochondrial localization signal in Pus2p

Taken together, our data suggested that Pus2p is efficiently targeted to mitochondria after its synthesis. This was rather surprising since no clear mitochondrial signal peptide was found at the N-terminus of Pus2p. Moreover, among the *S. cerevisiae* RNA: Ψ -synthases acting in mitochondria (Pus3p, Pus4p, Pus5p, and Pus9p), only Ydl036p (Pus9p) has a characteristic N-terminal targeting signal (Mason 1998; Behm-Ansmant et al. 2004). All other mitochondrial yeast tRNA: Ψ -synthases, although efficiently transported to this compartment, have no characteristic N-terminal signals (Becker et al. 1997; Lecointe et al. 1998; Ansmant et al. 2000, 2001). Nevertheless, based on their amino acid sequences, all of them are predicted to have a predominant mitochondrial localization by the PSORTII software.

On the other hand, a new mechanism was recently found to allow the efficient targeting of proteins without characteristic N-terminal signals to mitochondria. It is based on the presence of a sequence element in the 3'-UTR of

TABLE 1. Pseudouridine formation in selected yeast tRNA transcripts catalyzed by extracts of the $\Delta pus1$, $\Delta pus2$, and $\Delta pus1/\Delta pus2$ yeast strains

Position tested in tRNA	tRNA transcript/labeling	WT strain	$\Delta pus1$ strain	$\Delta pus2$ strain	$\Delta pus1/\Delta pus2$ strain
27 + 55	Yeast cytoplasmic tRNA ^{Val} (UAC)/CTP	1.36	0.71	1.42	0.80
27	Yeast mitochondrial tRNA ^{His} (GUG)/ATP	0.61	0.01	0.57	0.02
72	Yeast mitochondrial tRNA ^{Met} (CAU)/ATP	0.78	0.83	0.72	0.78

Radiolabeled transcripts were incubated with yeast extract for 60 min at 37°C, and the Ψ content was analyzed by 2D TLC after RNase T2 digestion.

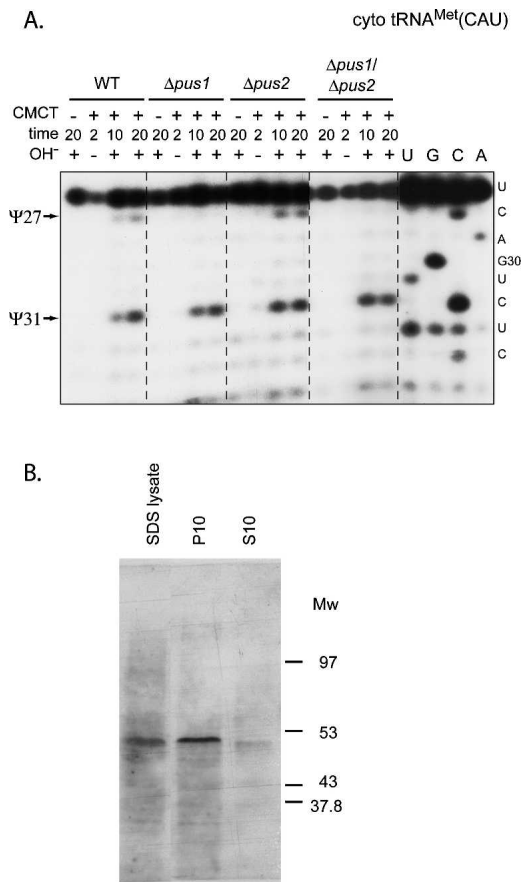


FIGURE 4. Formation of residue Ψ 27 in the cytoplasmic tRNA^{Met} (CAU) is not affected by disruption of the *PUS2* ORF. (A) Total RNAs extracted from the WT, $\Delta pus1$, $\Delta pus2$, and $\Delta pus1/\Delta pus2$ strains were subjected to CMCT modification (same legend as in Fig. 2). The presence of residues Ψ 27 and Ψ 31 in tRNA^{Met}(CAU) was tested by primer extension using the oligonucleotide described in Materials and Methods. A sequence ladder was made with the same oligonucleotide. Arrows indicate the reverse transcription stops, corresponding to residues Ψ 27 and Ψ 31. (B) Western blot analysis of HA-tagged Pus2p in yeast *S. cerevisiae* strain 26-G-7 (Open BioSystems). Yeast cells lysed by SDS (SDS lysate), insoluble fraction (P10), and S10 extract (S10) were loaded onto 10% PAAG, and separated proteins were transferred to nitrocellulose membrane. Staining was done using monoclonal anti-HA antibodies (12CA5, Roche) and ECL Plus kit (GE Healthcare). Molecular weights of marker proteins are indicated on the left of the panel.

mRNAs that allows mRNA concentration and translation in close vicinity of mitochondria (for review, see Jensen and Johnson 2001; Tekotte and Davis 2002). We can exclude the utilization of this mechanism for rapid transport of Pus2p to mitochondria. Indeed, the *PUS2* ORF that we expressed in the $\Delta pus2$ cells was followed by a foreign 260-base pair (bp) DNA sequence (the CYC1-terminator of p416GalS plasmid), instead of the authentic 3'-UTR of the *PUS2* gene. Despite this, the expressed Pus2p efficiently complemented the mitochondrial tRNA: Ψ 27-synthase activity in $\Delta pus2$ strain. This indicates that Pus2p probably uses another mechanism for efficient transport into mitochondria.

Two distinct RNA: Ψ -synthases act at positions 27 and 28 in tRNAs

Based on several previous studies, up to now it has been accepted that the modification of a given position in tRNAs is ensured by the product(s) of a unique gene in both the cytoplasmic and mitochondrial compartments (Martin and Hopper 1994; Rose et al. 1995; Becker et al. 1997; Lecointe et al. 1998; Ansmant et al. 2001). Either a unique protein is expressed from the gene and it has a dual cytoplasmic and mitochondrial localization (Becker et al. 1997; Lecointe et al. 1998; Ansmant et al. 2001), or two distinct enzymes differing by the presence of an N-terminal mitochondrial targeting signal in one of them are produced from the unique gene by the alternative utilization of AUG initiation codons (Martin and Hopper 1994; Rose et al. 1995). The only reported exception is the Rib2p/Pus8p and Pus9p pair of tRNA: Ψ -synthases: Rib2p/Pus8p is a cytoplasmic tRNA: Ψ 32-synthase, while Pus9p can act both in the cytoplasm and mitochondrial compartments with a predominant activity in mitochondria (Behm-Ansmant et al. 2004). Here we show that another pair of RNA: Ψ -synthases is involved in the modification of tRNA positions 27 and 28. Nevertheless, the situation is quite different from the Rib2p/Pus8p-Pus9p pair, since the Pus1p activity is strictly cytoplasmic, while Pus2p activity is strictly mitochondrial.

One possible explanation for the presence of a specific mitochondrial enzyme in addition to Pus1p may be a preferential localization of Pus1p in the nucleus, where it modifies the tRNA^{Ile}(UUA) precursor (Simos et al. 1996; Motorin et al. 1998) and U2 snRNA (Massenet et al. 1999). In addition, Pus1p participates in tRNA export from the nucleus in the cytoplasm (Simos et al. 1996; Grosshans et al. 2001). These various functions of Pus1p may be incompatible with its additional mitochondrial localization and may explain the necessity of a dedicated mitochondrial enzyme with a similar substrate specificity (Pus2p).

Although conserved in yeast, the *PUS1* and *PUS2* genes may be deleted without marked growth phenotype

Another question is to know whether the existence of two redundant tRNA: Ψ -synthases with different sites of action is evolutionarily conserved. A search in genomic databanks of genes that may encode Pus1p and Pus2p homologs revealed the occurrence of genes encoding these two proteins only in yeasts that belong to the *Saccharomycetoidae* and the *Schizosaccharomycetoidae* families. They have a strong similarity with both Pus1p and Pus2p. However, only one gene that may encode a Pus1p/Pus2p homolog was found in vertebrates and other eukaryotes (Chen and Patton 1999, 2000). In relation to this observation, it must be pointed out that in yeasts, modification at position 44 in U2 snRNA is catalyzed by Pus1p (Massenet et al. 1999), whereas

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 (*pus2Δ::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 (*pus1Δ::HIS3*) in the genomic DNA of the RS453 *S. cerevisiae* strain (Simos et al. 1996). Yeast *S. cerevisiae* strain 26-G-7 expressing 3× 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 *pus2Δ::Kan^r* and the *pus1Δ::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 Ψ27 in mitochondrial tRNA^{Arg}(UCU), Ψ28 in mitochondrial tRNA^{Lys}(UUU), and Ψ27 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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