The *Saccharomyces cerevisiae* U2 snRNA:pseudouridine-synthase Pus7p is a novel multisite–multisubstrate RNA:Ψ-synthase also acting on tRNAs

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ABSTRACT

The *Saccharomyces cerevisiae* Pus7 protein was recently characterized as a novel RNA:pseudouridine (Ψ)-synthase acting at position 35 in U2 snRNA. However, U2 snRNA was the only potential substrate tested for this enzyme. In this work, we demonstrated that although Pus7p is responsible for the formation of only one of the six Ψ residues present in yeast UsnRNAs, it catalyzes U to Ψ conversion at position 13 in cytoplasmic tRNAs and at position 35 in pre-tRNA^{Tyr}. Sites of RNA modification by Pus7p were identified by analysis of the in vivo RNA modification defects resulting from the absence of active Pus7p production and by in vitro tests using extracts from WT and genetically modified yeast cells. For demonstration of the direct implication of Pus7p in RNA modification, the activity of the WT and mutated Pus7p recombinant proteins was tested on in vitro produced tRNA and pre-tRNA transcripts. Mutation of an aspartic acid residue (D256) that is conserved in all Pus7 homologs abolishes the enzymatic activity both in vivo and in vitro. This suggests the direct involvement of D256 in catalysis. Target sites of Pus7p in RNAs share a common sequence Pu(G/C)UNΨAPu (Pu = purine, N = any nucleotide), which is expected to be important for substrate recognition. Modification of tRNAs by Pus7p explains the presence of Pus7p homologs in archaea and some bacteria species, which do not have U2 snRNA, and in vertebrates, where Ψ34 (equivalent to Ψ35 in yeast) formation in U2 snRNA is an H/ACA snoRNA guided process. Our results increase the number of known RNA modification enzymes acting on different types of cellular RNAs.

Keywords: Yeast; pseudouridine synthase; tRNAs; pre-tRNA^{Tyr}; UsnRNAs; intron-dependent

INTRODUCTION

Most of the metabolically stable RNAs (tRNAs, rRNAs, and UsnRNAs) contain post-transcriptionally modified nucleotides, the most frequent ones are Ψ and 2'-O-methylated residues. These modified residues may be generated in RNA by two distinct mechanisms. The first one is based on a single protein (RNA:Ψ-synthase), which carries both the

RNA recognition capacity and the catalytic activity (for review, see Charette and Gray 2000; Ansmant and Motorin 2001; Ofengand 2002), while in the second one, the modification is performed by an RNP complex that carries the two activities. In this case, a guide RNA (H/ACA sno- or sca-RNA) ensures the recognition of targeted RNA, and one of the proteins bound to the guide RNA has the catalytic activity (Ganot et al. 1997; Ni et al. 1997; and for review, see Lafontaine and Tollervey 1998; Filipowicz et al. 1999; Kiss 2001, 2002). The advantage of this second system is that a single RNA:Ψ-synthase bound to several guide RNAs can catalyze Ψ formation at several positions in a given RNA and also in different RNAs.

Some of the post-transcriptional modifications in tRNAs, rRNAs, and UsnRNAs are highly phylogenetically conserved (Branlant et al. 1981; Veldman et al. 1981; and for review, see Massenet et al. 1998; Charette and Gray 2000;

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

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Decatur and Fournier 2002; Ofengand 2002). This is the case for several pseudouridylation sites in tRNAs (positions 13, 32, 38, 39, and 55; for review, see Sprinzl et al. 1998) and also for six pseudouridylation sites in the spliceosomal UsnRNAs; three of them are located in U2 snRNA (positions 35, 42, 44 in yeast and 34, 41, 43 in human or *Xenopus laevis*; for review, see Massenet et al. 1998). However, such highly phylogenetically conserved modified residues in RNA can be generated by different mechanisms, depending on the organism. This was first demonstrated for Ψ residues in ribosomal RNAs. Their formation is catalyzed by several specific enzymes in bacteria (for review, see Ofengand 2002) and by snoRNPs in eukaryotes (Ganot et al. 1997; for review, see Lafontaine and Tollervey 1998; Kiss 2001, 2002). Recently, it turned out to be the case for two of the conserved Ψ residues in U2 snRNA. Indeed, modifications at positions 34 and 43 (Ψ 45 in *Caenorhabditis elegans*) in vertebrates are likely dependent upon H/ACA guide RNAs (Huttenhofer et al. 2001; Zhao et al. 2002; Patton and Padgett 2003), whereas the corresponding Ψ residues in the *S. cerevisiae* U2 snRNA (Ψ 35 and Ψ 44) are generated by the snoRNA-independent enzymes Pus7p and Pus1p, respectively (Massenet et al. 1999; Ma et al. 2003). Interestingly, Pus1p is a multisubstrate enzyme, which also catalyzes the formation of Ψ residues at eight distinct positions in tRNAs (Simos et al. 1996; Motorin et al. 1998).

From the RNA recognition point of view, RNA: Ψ -synthases, that catalyze uridine (U) to Ψ conversion without assistance of guide RNAs, can be classified into three groups: (1) site-specific enzymes acting on a unique site in a given RNA (like RsuA, Conrad et al. 1999; TruB, Nurse et al. 1995; and Pus4, Becker et al. 1997); (2) region-specific enzymes capable to modify several neighboring positions in a given molecule (e.g., TruA, Kammen et al. 1988; Pus3, Lecoite et al. 1998; RluC, Conrad et al. 1998; and RluD, Huang et al. 1998); and (3) multisite and multisubstrate specific enzymes that modify distinct positions in different classes of RNAs (like *Escherichia coli* RluA, Wrzesinski et al. 1995). The yeast Pus1p enzyme mentioned above belongs to this last group (Motorin et al. 1998; Massenet et al. 1999). Enzymes with broad and dual specificities may be more frequent than currently imagine, because an RNA:methyltransferase with dual specificity was also described (Gu et al. 1994). Together with the guide RNA system, such multisite-specific proteins probably contribute to the generation of a large number of modifications in RNAs with only a limited number of enzymes. Hence, we tested whether the *S. cerevisiae* Pus7 enzyme, which, like Pus1p, modifies U2 snRNA, can also modify tRNAs. Indeed, despite the almost complete characterization of the *S. cerevisiae* genes encoding proteins with RNA: Ψ -synthase signatures (Simos et al. 1996; Lecoite et al. 1998; Becker et al. 1997; Ansmant et al. 2000, 2001), the enzymes catalyzing the formation of the frequent Ψ 13 residue in cytoplasmic tRNAs (7 tRNAs), Ψ 1 residue in cytoplasmic tRNA^{Arg} and tRNA^{Lys}, and Ψ 72 resi-

due in mitochondrial initiator tRNA^{Met} are not discovered yet. Furthermore, the situation concerning Ψ 35 formation, which is restricted to tRNA^{Tyr} in all eukaryotes and takes place on the intron-containing tRNA precursor (Johnson and Abelson 1983), is not clear. Indeed, despite the fact that the yeast multisite-multisubstrate-specific RNA: Ψ -synthase Pus1p modifies this position in pre-tRNA^{Tyr} transcripts in vitro, disruption of the *PUS1* gene in *S. cerevisiae* does not abolish Ψ 35 formation in tRNA^{Tyr}, either in cellular extract or in vivo (Motorin et al. 1998). These data indicated that an additional, yet uncharacterized, yeast RNA: Ψ -synthase is responsible for U35 modification in tRNA^{Tyr}.

In this work, we tested whether the recently identified U2 snRNA: Ψ -synthase Pus7p may display multisubstrate specificity and modify one of the three orphan pseudouridylation sites in cytoplasmic tRNAs (positions 1, 13, and 35), and/or one of the four orphan pseudouridylation sites in yeast UsnRNAs (Ψ 5 and Ψ 6 in U1 snRNA, Ψ 42 in U2 snRNA and Ψ 99 in U5 snRNA) (Massenet et al. 1999). The results reveal that, like Pus1p, Pus7p modifies both U2 snRNA and tRNAs, and that tRNA modification occurs at two distinct positions (13 and 35; Fig. 1). Interestingly, Pus7p does not belong to any of the four identified families of RNA: Ψ -synthases (Ma et al. 2003), explaining why it was not previously identified by computer search based on sequence homology approach (Koonin 1996).

RESULTS

Ψ formation in *S. cerevisiae* U1 and U5 snRNAs is not dependent upon Pus7p

Ma et al. (2003) showed that disruption of the *PUS7* gene in *S. cerevisiae* abolishes U to Ψ conversion at position 35 in U2 snRNA (Fig. 1A), without altering Ψ 42 and Ψ 44 formation. Because the *S. cerevisiae* enzymes responsible for Ψ formation at positions 5 and 6 in U1 snRNA and at position 99 in U5 snRNA are not identified yet, we used the CMCT/RT approach (Bakin and Ofengand 1993) to test for a possible activity of Pus7p at these positions of UsnRNAs. To this end, we analyzed U1 and U5 snRNAs, extracted from the wild-type (WT) and two Δ *PUS7* *S. cerevisiae* BY4742 strains (a and α mating types). The reverse transcriptase stops, indicating the presence of Ψ residues, were identical for RNAs from the WT and both Δ *PUS7* strains. Only the results for the mat-a Δ *PUS7* strain are shown (Fig. 2). Thus, *PUS7* gene disruption does not affect U to Ψ conversion in both U1 and U5 snRNAs. These data strongly indicate that Pus7p forms only one of the six Ψ residues detected in *S. cerevisiae* UsnRNAs.

Disruption of the *PUS7* gene abolishes Ψ 13 formation in tRNAs in vivo

As mentioned in the Introduction, the yeast enzymes responsible for formation of Ψ 1, Ψ 13, and Ψ 35 in cytoplas-

1996; see Fig. 1D). Based on the hypothesis that yeast extract reproduces the tRNA modification pattern found *in vivo*, among the 3' monophosphate nucleotides (3'NMPs) released by T2 RNase digestion of the modified transcripts, only residue Ψ 13 was expected to be labeled and thus detected by chromatography on thin layer plates (TLC). In accordance with Ψ 13 formation with the extract from the WT strain, a U to Ψ conversion occurred

Incubation of in vitro produced tRNA and pre-tRNA transcripts with yeast cell extracts was previously shown to generate specific U to Ψ conversions at almost all positions known to be modified in vivo (Jiang et al. 1997; Motorin et al. 1998). Thus, to complete the analysis of RNA-modification defects in the $\Delta PUS7$ strain, we compared the capacities of WT and $\Delta PUS7$ cell extracts to modify different yeast tRNA transcripts at positions 1 and 13 and an in vitro produced pre-tRNA^{Tyr} at position 35. Formation of Ψ residues at the expected positions was tested by the nearest neighbor approach. The tRNA^{Asp}(GUC) and tRNA^{His}(GUG) transcripts used in the assays were labeled by incorporation of [α -³²P]ATP, because among all Ψ residues described for these two tRNAs, only residue Ψ 13 is followed by an adenosine (A) residue (Sprinzl et al. 1998; see Fig. 1B). Based on the hypoth-

FIGURE 2. Disruption of the *PUS7* gene does not alter formation of Ψ residue in U1 (A) and U5 snRNAs (B). Total RNA was extracted from the WT and mat-a *ΔPUS7* *S. cerevisiae* BY4742 strains and modified by CMCT, for 1, 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. The reverse transcription stops, corresponding to residues Ψ₅ and Ψ₆ (A) and to Ψ₉₉ (B) are indicated by arrows.

5' to an adenosine residue of tRNA^{Asp} (Fig. 4A) and tRNA^{His} (data not shown) upon incubation in this extract. Such conversion was abolished in the extract from the *ΔPUS7* strain (Fig. 4A). Similarly, the activity toward U35 was tested on an in vitro produced precursor of the *S. cerevisiae* tRNA^{Tyr} (Fig. 1C1), that was labeled by incorporation of [α-³²P]ATP. Labeling with this nucleotide was justified by the fact that residue Ψ₃₅ is the only Ψ residue in tRNA^{Tyr} followed by a A residue. Here again, in accordance with the presence of an RNA:Ψ-synthase activity in

the WT extract, T2 RNase digestion followed by 2D TLC demonstrated the formation of a Ψ residue 5' to a A residue upon incubation with this extract (Fig. 4B). No labeled 3'ΨMP was detected in the same conditions with the extract from the *ΔPUS7* strain (Fig. 4B). Moreover, the same result was obtained when the *Arabidopsis thaliana* pre-tRNA^{Tyr} (Fig. 1C2), was used as the substrate. This strongly suggested that Pus7p is responsible for Ψ₃₅ formation in the pre-tRNA^{Tyr}. As a control, we confirmed that deletion of the *PUS1* gene in the BY4742 strain did not alter Ψ₃₅ formation in both *S. cerevisiae* and *A. thaliana* pre-tRNA^{Tyr}.

The tRNA:Ψ1-synthase activity of the extracts was tested on a transcript of the *S. cerevisiae* cytoplasmic tRNA^{Arg}(ACG). Labeling was done with [α-³²P]UTP, because among all the Ψ residues present in tRNA^{Arg}, only residue Ψ₁ is followed by an U residue. As evidenced by TLC, both the WT and the *ΔPUS7* cell extracts were capable to convert the 5' residue of a UU sequence into a Ψ residue in tRNA^{Arg} (Fig. 4C).

Taking into account the previously demonstrated specificity of yeast extracts, we concluded that Pus7p is most probably involved in the formation of residues Ψ₁₃ and Ψ₃₅, but not of residue Ψ₁ of the *S. cerevisiae* cytoplasmic tRNAs. For a more direct demonstration of the implication of Pus7p in Ψ₁₃ and Ψ₃₅ formation, we complemented the *ΔPUS7* strain with the p413GalS-*PUS7* plasmid, bearing a WT copy of the *PUS7* gene under the control of the inducible GalS promoter. As shown by the CMCT/RT analysis of RNAs extracted from the complemented strain (Fig. 3A,B), expression of the *PUS7* gene from plasmid p413GalS restored Ψ₁₃ formation in tRNA^{Asp} and tRNA^{Glu} (lanes 7,8 in Fig. 3A,B, respectively). Restoration of U to Ψ conversion was also observed when extracts from the *ΔPUS7* and *ΔPUS7* complemented strains were used to test for the activities at position 13 of tRNA^{Asp} and 35 of pre-tRNA^{Tyr} (Fig. 4A,B).

Mutation of the conserved aspartic acid residue (D256) in Pus7p abolishes RNA:Ψ-synthase activity

Sequence alignment of bacterial, archaeal, and eukaryotic Pus7p homologs revealed the universal conservation of several aspartic acid residues. However, one of them is present in a conserved GTKD sequence showing some similarity with the conserved sequences at the active site of RNA:Ψ-synthases from other families. Thus, D256 could be the catalytic residue in Pus7p. To test for this possibility, we introduced a mutation in the *PUS7* gene that generated a D256A substitution in Pus7p, and we tested the effect of this mutation on the Pus7p RNA:Ψ-synthase activity in vivo and in vitro. As shown by the CMCT/RT approach (Fig. 3A,B, lanes 9,10), the mutated Pus7 enzyme was no longer capable to form residue Ψ₁₃ in tRNA^{Asp} and tRNA^{Glu} in vivo. As a confirmation, residue Ψ₁₃ was not formed in tRNA^{Asp} upon incubation with an extract of the *ΔPUS7*

FIGURE 3. Ψ 13 formation in cytoplasmic tRNA^{Asp} (A) and tRNA^{Glu} (B) is abolished upon *PUS7* gene disruption and restored by complementation with plasmid p413GalS-*PUS7*. Total RNA was extracted from the WT and mat-a Δ *PUS7* strains and modified by CMCT, for 1, 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. The reverse transcription stops, corresponding to residues Ψ 13, are indicated by arrows. To verify the direct involvement of Pus7p in the U to Ψ conversion at position 13 of tRNAs, the Δ *PUS7* strain was transformed with plasmid p413GalS-*PUS7*, bearing the wild-type (WT) or mutated (D256A) *PUS7* gene. Total RNA was extracted from these two transformed strains, and tRNA^{Asp} (A) and tRNA^{Glu} (B) were analyzed by the CMCT/RT approach (shown in lanes 7, 8, 9, and 10 in A and B).

strain transformed with a plasmid carrying the D256A mutated *PUS7* gene (Fig. 4A).

Similarly, no RNA: Ψ -synthase activity was detected, when the pre-tRNA^{Tyr} labeled with [α -³²P]ATP was incubated with this extract (Fig. 4B). Altogether, these data

strongly suggested that D256 is the catalytic residue of Pus7p.

Recombinant Pus7p has tRNA^{Asp}: Ψ 13- and pre-tRNA^{Tyr}: Ψ 35-synthase activities

To confirm the direct implication of Pus7p in Ψ 13 and Ψ 35 formation in cytoplasmic tRNAs, we produced the recombinant WT His₆-Pus7 and His₆-Pus7D256A proteins in *E. coli*. The RNA: Ψ -synthase activities of the WT and mutant His₆-Pus7 proteins towards the uridine at position 13 in tRNA^{Asp} were tested by both the CMCT/RT and the nearest neighbor approaches. As expected, using the CMCT/RT approach (Fig. 5A), a reverse transcriptase stop corresponding to Ψ 13 formation was detected in the transcript incubated with the WT (lanes 5,6), but not with the variant recombinant enzyme (data not shown). In addition, no other stop indicative of U to Ψ conversion at other positions in tRNA^{Asp} was detected. Similarly, UA to Ψ A conversion in tRNA^{Asp} labeled with [α -³²P]ATP was detected for the WT, but not for the variant recombinant enzyme (Fig. 5B). To confirm that the U to Ψ conversion detected occurred exclusively at position 13, a U13C base substitution was generated in tRNA^{Asp}. As expected, no UA to Ψ A conversion was detected in this variant tRNA^{Asp} (Fig. 5C). Having shown by this experiment the specificity of the U to Ψ conversion detected by TLC, we then used this method for a time-course study of the modification reaction (Fig. 5D). The kinetic curve showed that, in the experimental conditions used, a plateau level of about 0.9 mole of Ψ residue per mole of tRNA^{Asp} was reached after 40 min of incubation. Taken together, these data unambiguously map the site of action of the recombinant Pus7p enzyme at position 13 in tRNA^{Asp} and exclude its possible action at other U residues in this tRNA.

Similarly, the capacity of the recombinant His₆-Pus7 protein to catalyze UA to Ψ A conversion in both yeast and *A. thaliana* pre-tRNAs^{Tyr} labeled with [α -³²P]ATP was demonstrated by TLC analysis (Fig. 6A, and data not shown). No UA to Ψ A conversion was detected when the experiment was performed with the D256A variant protein (Fig. 6A). As several U residues in yeast pre-tRNA^{Tyr} are present in a UA sequence context and that there was no evidence that the purified enzyme will be as much specific as were the yeast extracts, a precise mapping of the formed Ψ residue was made by combining T1 and T2 or P1 RNase digestions, gel electrophoresis, and TLC. According to T1 RNase specificity, complete digestion of the pre-tRNA^{Tyr} by this enzyme was expected to release several short oligonucleotides ranging in size from 1 to 8 nt, and a longer fragment of 15 nt corresponding to the tRNA anticodon loop and the intron (Fig. 6B1). This fragment contains U35 expected to be the target of the recombinant His₆-Pus7 enzyme. Thus, we analyzed the presence of residue Ψ 35 in this T1 RNase product. First, the T1 RNase digestion prod-

A. tRNA^{Asp} *S. cerevisiae* B. pre-tRNA^{Tyr} *S. cerevisiae* C. tRNA^{Arg} *S. cerevisiae*

FIGURE 4. Tests of the tRNA:Ψ1, Ψ13 and Ψ35-synthase activities in different yeast S10 extracts. In vitro transcribed RNA substrates labeled by incorporation of [α - 32 P]ATP (yeast tRNA^{Asp}, A, yeast pre-tRNA^{Tyr}, B) or [α - 32 P]UTP (yeast tRNA^{Arg}, C) were incubated with different S10 extracts in the conditions described in Materials and Methods. Extracts were prepared from cells of the WT BY4742 strain (WT), the isogenic Δ PUS7 strain, and the Δ PUS7 strain complemented with the WT or D256A variant PUS7 gene. The activity on yeast pre-tRNA^{Tyr} of an extract from the Δ PUS1 BY4742 strain was also tested. The tRNA^{Asp} and tRNA^{Arg} transcripts incubated in the same conditions but in the absence of S10 extract, were used as controls. After incubation, the transcripts were digested with T2 RNase and 3'NMPs were fractionated on TLC as described in Materials and Methods. The autoradiograms of the TLC plates are shown. Positions of the NMPs (Ap, Cp, Up, Gp) and ΨMP nucleotides were identified according to Keith (1995). Quantification of the Ψ residue formation was done by measuring the radioactivity in each spot with a PhosphorImager and the ImageQuant software.

ucts from a pre-tRNA^{Tyr} labeled with [α - 32 P]ATP and incubated in the presence or absence of the recombinant His₆-Pus7 enzyme were purified by electrophoresis on a 20% polyacrylamide gel (Fig. 6B2) and the 15-nt fragment was further analyzed by T2 RNase digestion followed by TLC (Fig. 6B3). As expected, formation of a Ψ residue in a ΨA context was detected in the 15-nt fragment, when the pre-tRNA^{Tyr} was incubated with His₆-Pus7p (Fig. 6B3). However, because the 15-nt T1 RNase fragment contained three UA dinucleotides, a second experiment was performed with a pre-tRNA^{Tyr} labeled with [α - 32 P]UTP and incubated in the presence or absence of His₆-Pus7p. Again, the 15-nt T1 RNase digestion product was purified by gel electrophoresis. In this case, digestion was performed with P1 nuclease before TLC analysis. P1 nuclease releases 5'NMPs; thus, all the released U residues of the 15-nt T1 digestion product contained a labeled 5' phosphate, except residue U35 located at the 5' extremity of the T1 digestion product (T1 RNase releases products with 5'OH at the 5'

extremity). Hence, if the U to Ψ conversion in pre-tRNA^{Tyr} was occurring at position 35, no labeled Ψ residue was expected to be detected upon digestion of the 15-nt fragment with P1 nuclease. This was indeed the case, demonstrating the specific action of the His₆-Pus7 recombinant enzyme at position 35 in the 15-nt fragment (Fig. 6B3). Finally, a time course analysis of Ψ formation in pre-tRNA^{Tyr} labeled with [α - 32 P]ATP (Fig. 6C) revealed a plateau, that was reached after about 50 min in the conditions used, and corresponded to a ratio of 1 mole of Ψ residue per mole of pre-tRNA^{Tyr}. Altogether, these data demonstrated the specific action of the recombinant His₆-Pus7 enzyme at position 35 of pre-tRNA^{Tyr}.

In conclusion, the recombinant His₆-Pus7 enzyme has the same specificity in vitro as the Pus7 enzyme in vivo: it modifies specifically U2 snRNA at position 35 (Ma et al. 2003), cytoplasmic tRNAs at position 13, and the pre-tRNA^{Tyr} at position 35. Furthermore, the D256A substitution abolishes all three activities (the verification was also

FIGURE 5. Recombinant His₆-Pus7p catalyzes specifically U to Ψ conversion at position 13 in yeast cytoplasmic tRNA^{Asp}. (A) CMCT/RT mapping of Ψ residues formed upon incubation of tRNA^{Asp} with His₆-Pus7p. Cold in vitro produced tRNA^{Asp} was incubated in the presence (+) or absence (-) of His₆-Pus7p in the conditions described in Materials and Methods. The modified tRNA^{Asp} was analyzed by the CMCT/RT approach (same legend as in Fig. 3). (B) Analysis of Ψ₁₃ formation in tRNA^{Asp} by the nearest neighbor approach. tRNA^{Asp} transcript labeled with [α -³²P]ATP was incubated with the recombinant His₆-Pus7p or the His₆-Pus7D256Ap mutant in the conditions described in Materials and Methods. A control incubation was performed in the absence of the recombinant protein. The incubated tRNAs^{Asp} were digested with T2 RNase and the released 3'NMPs were fractionated by TLC, as described in Materials and Methods. The autoradiograms of the TLC plates are shown. The molar ratio of Ψ residues formed in tRNAs, as deduced from quantification of the radioactivity of the spots, is given at the bottom of the panels. (C) Same experiment as in (B) with the U13C variant tRNA^{Asp}. (D) Time-course analysis of tRNA^{Asp} modification by the recombinant His₆-Pus7p enzyme. tRNA^{Asp} transcript (50–100 fmoles) was incubated with 750 fmoles of His₆-Pus7p in the conditions described in Materials and Methods. Aliquot fractions were collected at the indicated times after the beginning of the incubation. For each fraction, RNA was digested with T2 RNase and the released products were analyzed by 2D TLC. Confidence intervals are calculated taking into account the relative radioactivity measured for the ΨMP spot and the Ap, Cp, Gp, and Up spots on 2D TLC.

done for U2 snRNA; data not shown). This observation reinforces the idea that D256 is the catalytic amino acid involved in modification of the three types of substrates.

DISCUSSION

Pus7 is a novel Ψ-synthase with multisite and multisubstrate specificity

Here, we demonstrate that like Pus1p, the *S. cerevisiae* RNA:Ψ-synthase Pus7p modifies both U2 snRNA, tRNAs,

and pre-tRNA^{Tyr}, and modification in mature tRNAs takes place at position 13 and in the pre-tRNA^{Tyr} at position 35. This observation increases the number of known RNA modification enzymes that act on several RNA substrates, which reinforces the idea that this strategy is rather frequently used to reduce the number of enzymes required for complete post-transcriptional modification of cellular RNAs.

One important question is to know whether the various target sites of Pus7p share some common features (secondary structure or sequence). Clearly, RNA secondary structures around the various modification sites are different (see

Fig. 1). U13 to Ψ 13 conversion takes place in a terminal loop of tRNAs, whereas U35 to Ψ 35 conversion in U2 snRNA occurs in a single-stranded region linking two stem-loop structures, and the target site in pre-tRNA^{Tyr} is located in a short helix. This diversity suggests that RNA secondary structure is probably not an important parameter in target site recognition by Pus7p. Sequence alignment of all the natural substrates of Pus7p (7 tRNAs, the pre-tRNA^{Tyr}, and U2 snRNA) shows that modification occurs in a highly conserved 7-nt long sequence: Pu(G/C)UN Ψ APu (Pu = purine, N = any nucleotide). Importance of this conserved sequence for Pus7p recognition and/or modification is supported by the fact that *S. cerevisiae* tRNAs with unmodified U13 have this residue located in a sequence that does not fit exactly the consensus sequence. For example, the yeast tRNA^{Ala}(ICG) and tRNA^{Arg} (mcm⁵UCU), that both have a U to C substitution at position 11 in the conserved sequence, have an unmodified U13. Thus, the presence of a U residue two nucleotides upstream of the modified site seems to be crucial for recognition and/or modification by Pus7p. Inspection of the sequences of all tRNAs from any kind of species, which are known to contain a Ψ 13 residue (tRNA database Sprinzl et al. 1998; see also Internet site <http://www.unibayreuth.de/departments/biochemie/sprinzl/trna/>), revealed a strong evolutionary conservation of the Pu₉(G/C)₁₀U₁₁N₁₂ Ψ ₁₃A₁₄Pu₁₅ sequence in tRNAs from all organisms, except archaea. Thus, the tRNA: Ψ 13-synthase recognition sequence seems to be highly phylogenetically conserved. Interestingly, the formation of Ψ 35 in the *A. thaliana* pre-tRNA^{Tyr} by plant nuclear extract was also found to require an U₃₃N₃₄U₃₅A₃₆Pu₃₇ sequence (Choffat et al. 1988; Szwedkowska-Kulinska and Beier 1992; Szwedkowska-Kulinska et al. 1995). In this case, apart from this sequence, only the length of the intron seems to be important for the U to Ψ conversion (Choffat et al. 1988; Szwedkowska-Kulinska and Beier 1992; Szwedkowska-Kulinska et al. 1995). In this work we confirmed that the recombinant Pus1p enzyme is also capable to modify yeast pre-tRNA^{Tyr} at position 35 (data not shown), as previously reported (Motorin et al. 1998). However, in yeast cells, only *PUS7* but not *PUS1* gene disruption leads to the loss of Ψ 35-formation activity in pre-tRNA^{Tyr}. This demonstrates that only Pus7p is implicated in U35 modification in pre-tRNA^{Tyr} in vivo. Therefore, the modification of yeast pre-tRNA^{Tyr} transcript by the recombinant Pus1 enzyme probably represents an in vitro artifact.

It should be noted that yeast U2 snRNA modification at position 35 seems to depend not only on the consensus sequence described above, but also on a structural motif located downstream from the modification site (Ma et al. 2003).

Another interesting question, which remains to be

A. pre-tRNA^{Tyr}

B1.

B2.

B3.

C. pre-tRNA^{Tyr}

FIGURE 6. (Legend on next page)

solved, is the site(s) of action of Pus7p in terms of cell compartments. The internal modifications in U2 snRNA, as well as, in pre-tRNA^{Tyr}, are expected to occur in the nucleus. In contrast, Ψ 13 formation in cytoplasmic tRNAs, which is not intron-dependent, may take place either in the nucleus or in the cytoplasm.

Homologs of the Pus7 RNA: Ψ -synthase are present in all three life kingdoms

The multisite-specific RNA: Ψ -synthase Pus7p belongs to a novel, until recently uncharacterized family of RNA: Ψ -synthases. Based on BLAST search, Pus7p has one homolog protein in all Archaea and Eukarya, whose genome has been completely sequenced. In contrast, Pus7 homologs are present only in 17 out of 77 sequenced bacterial genomes. Archaea and bacteria do not have UsnRNAs; nevertheless, the implication of Pus7p in tRNA modification at position 13 likely explains the presence of a Pus7p homolog in these organisms. Indeed, Ψ 13 was detected in one tRNA of *E. coli* and *Thermus thermophilus* and several tRNAs of *Haloferax volcanii* (tRNA database; Sprinzl et al. 1998; see also Internet site <http://www.uni-bayreuth.de/departments/biochemie/sprinzl/trna/>). Only very recently, the enzyme responsible for Ψ 13 formation in *E. coli* was identified (Kaya and Ofengand 2003). Interestingly, the N-terminal domain of this enzyme presents 45% of similarity with the central region of Pus7p.

In contrast to residue Ψ 13, which is rather frequent in living organisms, Ψ 35 in tRNA^{Tyr} seems to be an exclusively eukaryotic feature. Implication of Pus7p in pre-tRNA modification in eukaryotes suggests a gain of function of Pus7p in the course of evolution. However, we cannot rule out the possibility that Pus7p homologs also act on other RNA substrates in bacteria and archaea.

The role of the vertebrate homologs of Pus7p will have to be defined. The most probable target sites are positions 13 and 35 in tRNAs, because Ψ 34 formation in U2 snRNA (equivalent to Ψ 35 in yeast) is dependent upon an H/ACA guide RNA. However, according to recent observation

(Zhao et al. 2002), the *X. laevis* Pus7p homolog may have conserved its capacity to modify U2 snRNA at position 34, because, after depletion of the pugU2–34/44 H/ACA snoRNA, a slow recovery of Ψ 34 formation was observed in *X. laevis* oocytes. In agreement with this observation, we noticed that the U34 pseudouridylation site in *X. laevis* U2 snRNA fits the consensus sequence required for modification by the Pus7p RNA: Ψ -synthase. The existence of two redundant modification systems acting at position 34 in vertebrate U2 snRNA may be related to the high importance of this pseudouridylation site (Newby and Greenbaum 2001; Newby and Greenbaum 2002). Residue Ψ 34 is located in the sequence that base pairs with the intron branch-site sequence. More precisely, Ψ 34 participates to the formation of the base pair that precedes the A residue involved in the first step of the splicing reaction. Residue Ψ 34 in U2 snRNA was shown to be responsible for a peculiar spatial arrangement of the U2 snRNA/branch-point sequence interaction, that places the reactive A residue in a conformation favorable for the first nucleophilic attack of the splicing reaction (Massenet et al. 1998; Newby and Greenbaum 2001; Newby and Greenbaum 2002).

MATERIALS AND METHODS

Yeast strains

The haploid *S. cerevisiae* BY4742 strain (denoted WT in this article) and the two isogenic haploid strains carrying a disruption of the *PUS7* gene (mat-a and mat- α) were obtained from EUROSCARF collection (Germany). As RNA modification were found to be identical in the two mutant strains, only the WT and mat-a Δ *PUS7* strains were used for further experiments. Transformation of yeast *S. cerevisiae* strains was done with the standard lithium acetate procedure (Adams et al. 1997).

Construction of plasmids for yeast cells complementation

For construction of the p413GalS–*PUS7* shuttle vector, the YOR243 (*PUS7*) ORF was PCR amplified from the genomic DNA

FIGURE 6. Recombinant His₆–Pus7p catalyzes specifically the U to Ψ conversion at position 35 in yeast pre-tRNA^{Tyr}. (A) Analysis of Ψ 35 formation in pre-tRNA^{Tyr} by the nearest neighbor approach. Yeast pre-tRNA^{Tyr} uniformly labeled with [α -³²P]ATP was incubated with the recombinant His₆–Pus7p or His₆–Pus7D256A variant in the conditions described in Materials and Methods. A control experiment was performed in the absence of recombinant protein. Modified and unmodified pre-tRNAs^{Tyr} were digested with T2 RNase and the 3'NMPs were fractionated by TLC. The autoradiograms of the TLC plates are shown. The molar ratio of Ψ residues formed in tRNAs, as deduced from quantification of the radioactivity of the spots, is given at the bottom of the panels. (B) Mapping of the Ψ residue formed in the pre-tRNA^{Tyr} by recombinant His₆–Pus7p enzyme. (B1) Cloverleaf representation of the pre-tRNA^{Tyr} with indication of the T1 RNase cleavage sites. The U residues located at the 5' position of an A residue are circled. (B2) Fractionation by gel electrophoresis of the T1 RNase products of the pre-tRNA^{Tyr} labeled by [α -³²P]ATP incorporation. Electrophoresis conditions are given in Materials and Methods. (B3) The 15-nt fragment obtained for a pre-tRNA^{Tyr} incubated in the absence (– His₆–Pus7p) or presence (+ His₆–Pus7p) of the recombinant enzyme were digested with T2 RNase, and the resulting 3'NMPs were fractionated by 2D TLC. A similar experiment was performed with a pre-tRNA^{Tyr} labeled by incorporation of [α -³²P]UTP. In this case, the 15-nt T1 RNase digestion product was hydrolyzed with P1 nuclease and the resulting 5'NMPs were fractionated by 2D TLC. (C) Analysis of time-course formation of Ψ residue in the pre-tRNA^{Tyr} upon incubation with the His₆–Pus7p enzyme. pre-tRNA^{Tyr} labeled by incorporation of [α -³²P]ATP was incubated with His₆–Pus7p enzyme in the conditions described in Materials and Methods. Aliquot fractions were collected at the indicated time after the beginning of the incubation. The pre-tRNA^{Tyr} of each aliquot was digested with T2 RNase. The released 3'NMPs were fractionated by 2D TLC and the radioactivity of each 3'NMP was estimated by measurement with a PhosphorImager. (C) Represents the deduced ratio of Ψ residue moles formed per pre-tRNA^{Tyr} moles as a function of the incubation times. Error bars were calculated as described in Materials and Methods.

of strain BY4742 using an oligonucleotide corresponding to the 5' end of the ORF (AAACTAGCTAGCatgtctgactctca) and an oligonucleotide complementary to the 3' end of the ORF (AAACGC GGATCCcttagatatttcctt). Sense and antisense sequences of the YOR243 ORF are shown in lower case. The restriction sites (*NheI* and *BamHI*) generated by the 5'- and 3'-oligonucleotides are underlined. The amplified PCR product was subcloned into the *SmaI* site of the pUC18 cloning vector and entirely sequenced (pUC18–*PUS7* plasmid). To build the p413GalS–*PUS7* recombinant plasmid the pUC18–*PUS7* was cleaved by *NheI* and *BamHI* and the fragment containing the YOR243 ORF was inserted, downstream from the GalS promoter, between the *XbaI* and *BamHI* sites of the *E. coli/S. cerevisiae* shuttle vector p413GalS (Mumberg et al. 1995). A point mutation (GAT→GCT) generating the D256A substitution in the Pus7p enzyme was introduced in the cloned ORF by PCR-mediated site-directed mutagenesis using the Quick Change Kit (Stratagene).

Preparation of DNA matrices for tRNA production

The template for in vitro transcription of the *S. cerevisiae* pre-tRNA^{Tyr} was generated by PCR amplification of the yeast genomic DNA. The sense primer (AAAAATAATACGACTCACTATAGtctc gtagccaagt) contained the sequence required for addition of a T7 RNA polymerase promoter upstream of the coding sequence (underlined) and the 17 nucleotides at the 5' end of the pre-tRNA^{Tyr} (lower case). The antisense primer (tggtgtccggggcgga) was complementary to the 17 nucleotides at the 3' end of the pre-tRNA^{Tyr}. The nucleotides corresponding to the first base pair of the pre-tRNA^{Tyr} acceptor stem (C1–G72) were converted into a G1–C72 base pair to increase the yield of the transcription reaction. The amplified PCR product was cloned into the *SmaI* site of plasmid pUC19. The same approach was used for amplification and cloning of DNA encoding the WT cytoplasmic tRNA^{Arg}(ACG). The plasmids, pTFM–Asp and pTFM–His, used for in vitro transcription of tRNA^{Asp} and tRNA^{His} were generously provided by C. Florentz (IBMC). The U13C variant of the pTFM–Asp plasmid was obtained by PCR-mediated site-directed mutagenesis using the Quick Change Kit (Stratagene). The DNA template for in vitro transcription of the *A. thaliana* pre-tRNA^{Tyr} was constructed with synthetic oligonucleotides, by matrix-independent filling-in reaction catalyzed by the Pfu DNA polymerase, followed by PCR amplification. First, the central region of the pre-tRNA was produced by the filling-in reaction using partially overlapping oligonucleotides. Then, a second step of amplification was performed using this amplified central region as the matrix and two primers: one bearing a T7 RNA polymerase promoter and the 5' extremity of the pre-tRNA and an antisense primer complementary to the 3' extremity of the pre-tRNA^{Tyr}. The resulting PCR product was cloned into the *SmaI* site of plasmid pUC19. The identity of all the generated recombinant plasmids was verified by DNA sequencing.

Construction of plasmids for protein production and protein production

To generate the pET28b–*PUS7* recombinant plasmid the pUC18–*PUS7* plasmid was cleaved by *NheI* and *BamHI*, and the fragment containing the *PUS7* ORF was inserted, downstream from the T7

promoter and the His₆ sequence, between the *NheI* and *BamHI* sites of the *E. coli* expression vector pET28b (Novagen). A point mutation (GAT→GCT) generating the D256A substitution in the Pus7p enzyme was introduced in the ORF of the recombinant pET28 plasmid as described above for the p314GalS–*PUS7* plasmid.

The recombinant His₆–Pus1p, WT, and D256A His₆–Pus7p enzymes were produced in *E. coli* and purified to homogeneity using Ni-NTA agarose column in the previously described conditions (Simos et al. 1996).

In vitro transcription

Prior to in vitro transcription, the pUC19–pre-tRNAs^{Tyr}, pUC19–tRNA^{Arg}, pTFM–Asp, and the variant construct U13C of the pTFM–Asp plasmid were cleaved with the *BstNI* nuclease; the pTFM–His plasmid was linearized with the *BamHI* nuclease. In vitro T7 RNA-polymerase transcription with or without incorporation of the appropriate [α -³²P]-ribonucleotide triphosphate and purification of the resulting RNA transcripts by electrophoresis on denaturing gel were performed as previously described (Jiang et al. 1997).

Prior to activity tests, in vitro transcribed RNAs were subjected to the following renaturation procedure: 50–100 fmoles (5–10 nM) of ³²P-labeled tRNA or pre-tRNA substrate for nearest neighbor approach or 4 pmoles of cold transcript for CMCT/RT analysis were dissolved in 10 μ L of 100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA, and incubated 5 min at 80°C, followed by a 20-min incubation at room temperature for transcript renaturation.

Analysis of Ψ residues by the CMCT/RT approach

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). This CMCT/RT method is based on the alkaline-resistant modification of Ψ residues by the water-soluble carbodiimide CMCT, followed by detection of modification positions by primer extension analysis. The oligonucleotides, used for analysis of yeast UsnRNAs, were described previously (Massenet et al. 1999). The presence of Ψ 13 residue in cytoplasmic tRNA^{Asp} (anticodon GUC) and tRNA^{Glu} (anticodon UUC) and of Ψ 35 residue in cytoplasmic tRNA^{Tyr} (anticodon G Ψ A) was tested with oligonucleotides complementary to residues 24–45 of tRNA^{Asp}, 37–58 of tRNA^{Glu} and 58–76 of the tRNA^{Tyr}, respectively.

For activity tests of the recombinant His₆–Pus7 enzyme, incubation of 4 pmoles of transcript was performed for 2 h in the presence of 1.25 pmoles of the recombinant Pus7p in 100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA. After incubation, RNA was extracted by phenol-chloroform and used for Ψ localization using the CMCT/RT approach.

Analysis of Ψ residues by the nearest neighbor approach

The RNA: Ψ -synthase activity at position 13 in yeast cytoplasmic tRNA^{Asp} (anticodon GUC) and tRNA^{His} (anticodon GUG) and at

position 35 in the yeast pre-tRNA^{Tyr} (anticodon GUA, containing the intron) and the *A. thaliana* pre-tRNA^{Tyr} (anticodon GUA, containing the intron) were tested by the nearest neighbor approach. Transcripts corresponding to these tRNAs were produced by T7 RNA polymerase in the presence [α -³²P]ATP. The tRNA:Ψ1-synthase activity was tested on a transcript of the yeast cytoplasmic tRNA^{Arg} (anticodon ACG) produced in the presence of [α -³²P]UTP. Cell-free S10 extracts from yeast were prepared as previously (Auxilien et al. 1996). After transcripts renaturation, the reaction was initiated by addition of 10 μ L of S10 yeast extract (final concentration of about 0.5–1.0 mg of total protein/mL) or 10 μ L of buffer containing 750 fmoles of WT or D256A His₆-Pus7 protein. After 1 h incubation at 37°C, the modified RNAs were phenol-extracted, ethanol-precipitated, and digested overnight with T2 RNase (0.01 U/ μ L), in 50 mM ammonium acetate buffer pH 4.6. The resulting 3'NMPs were fractionated by 2D TLC on cellulose plates (Polygram CEL 400, Macherey-Nagel). An isobutyric acid: ammonia 25%:water (66:1:33/v:v:v) mixture was used for the first dimension, while the second dimension was done in 2-propanol:HCl 37%:water (68:17.6:14.4/v:v:v). Assignment of 3'NMPs was based on previously published maps (Keith 1995). Radioactive spots were quantified on a PhosphorImager instrument (Molecular Dynamics) using the ImageQuant software. Calculations of molar amount of the Ψ per mole of tRNA substrate were done taking into account the nucleotide composition of tRNA transcript. The experimental error of quantification was estimated to be of ± 0.05 mole of Ψ residue/mole of tRNA. Error bars on time-course studies of Ψ formation were calculated using the Student's factor for probability 95% and the estimated radioactivities of the ΨMP and NMPs as deduced from PhosphorImager measurement using the ImageQuant software.

Identification of Ψ residues in T1 pre-tRNA^{Tyr} digestion products

Complete cleavage of pre-tRNA^{Tyr} was achieved by incubation with 1 U of T1 RNase for 12 h at 37°C. The resulting oligonucleotides were separated by 20% urea-PAGE, and their position was localized by autoradiography. The band corresponding to the U35-containing oligonucleotide (15-nt) was cut from the gel and was recovered by passive elution. The eluted oligonucleotide was then digested by either T2 RNase or P1 nuclease, and the resulting NMPs were analyzed by 2D TLC as described above. For P1 nuclease, overnight digestion at 37°C was carried out in 50 mM ammonium acetate buffer pH 5.3 in the presence of 0.3 U of enzyme.

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