RNA Sequence and Two-dimensional Structure Features Required for Efficient Substrate Modification by the Saccharomyces cerevisiae RNA:Ψ-Synthase Pus7p*

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Alan Urban1, Isabelle Behm-Ansmant, Christiane Branlant, and Yuri Motorin2

From the Laboratoire Maturation des ARN et Enzymologie Moléculaire, UMR 7567, CNRS-UHP Nancy I, Nancy Université, 54506 Vandoeuvre-les-Nancy Cedex, France

The RNA:pseudouridine (Ψ) synthase Pus7p of Saccharomyces cerevisiae is a multisite-specific enzyme that is able to modify U13 in several yeast tRNAs, U35 in the pre-tRNA1Tyr (GΨA), U35 in U2 small nuclear RNA, and U55 in 5 S rRNA. Pus7p belongs to the universally conserved TruD-like family of RNA:Ψ-synthases found in bacteria, archaea, and eukarya. Although several RNA substrates for yeast Pus7p have been identified, specificity of their recognition and modification has not been studied. However, conservation of a 7-nt-long sequence, including the modified U residue, in all natural Pus7p substrates suggested the importance of these nucleotides for Pus7p recognition and/or catalysis. Using site-directed mutagenesis, we designed a set of RNA variants derived from the yeast tRNA1GUC(pseudouridine synthase Pus7p), the pre-tRNA1Tyr(GΨA), and U2 small nuclear RNA and tested their ability to be modified by Pus7p in vitro. We demonstrated that the highly conserved U12 and A13 residues (nucleotide numbers refer to target U0) are crucial identity elements for efficient modification by Pus7p. Nucleotide substitutions at other surrounding positions (−4, −3, +2, +3) have only a moderate effect. Surprisingly, the identity of the nucleotide immediately 5′ to the target U0 residue (position −1) is not important for efficient modification. Alteration of tRNA three-dimensional structure had no detectable effect on Pus7p activity at position 13. However, our results suggest that the presence of at least one stem-loop structure including or close to the target U nucleotide is required for Pus7p-catalyzed modification.

RNA:pseudouridine (Ψ)-synthases catalyze the post-transcriptional U to Ψ conversion in RNAs. Extensive studies during the last 10 years allowed the discovery and characterization of almost the complete set of bacterial and yeast enzymes responsible for this modification in tRNAs, rRNAs, and small nuclear RNAs (snRNAs).

All known RNA:Ψ-synthases share a set of conserved amino acid sequence motifs and can be grouped into six distinct families based on the degree of amino acid sequence homology: families related to TruA, TruB, RluA, RsuA, TruD, and PsuX, respectively (1–3). The Saccharomyces cerevisiae RNA:Ψ-synthase Pus7p belongs to the recently described TruD-related family of proteins whose members are conserved in all kingdoms of life but have no apparent sequence homology with the other RNA:Ψ-synthases. However, strong homologies were found at the level of the three-dimensional structure of the catalytic site (2). Pus7p was initially fished out in a high throughput screening aiming to characterize U2 snRNA-specific pseudouridylation activity. By this approach, Ψ35-forming activity in U2 snRNA was attributed to the YOR243c open reading frame, designed as the PUS7 (pseudouridine synthase 7) gene (4). Later, by analysis of in vivo RNA modification defects resulting from deletion of the PUS7 gene and by in vitro tests using the recombinant protein, we identified other targets of the Pus7p enzyme (U13 in several yeast cytoplasmic tRNAs and U35 in the intron-containing pre-tRNA1Tyr(GΨA)) (5). Recently, the activity of Pus7p at position 50 in 5 S rRNA was also demonstrated (6).

The activity of the Escherichia coli homologue of the yeast Pus7p (TruD protein) (2) was also characterized. Since U35 in the bacterial tRNA1Tyr(GUA) is not converted to Ψ and since snRNAs do not exist in these organisms, the activity of the bacterial TruD enzyme seems to be restricted to U13 conversion in tRNAs (2). TruD-like proteins are also present in archaea. However, their activity and specificity have not been studied up to now.

The rules governing RNA substrate recognition by RNA:Ψ-synthases and RNA:modification enzymes in general have only been elucidated in a few cases (for a review, see Ref. 7). Some of the characterized RNA:Ψ-synthases have a rather strict substrate specificity and are only able to modify one position in only one type of cellular RNA, such as, for instance, tRNAs or even in only one cellular RNA, like rRNA. This is the case for several of the characterized yeast RNA:Ψ-synthases. Pus5p modifies a unique position in the mitochondrial 21 S rRNA (8), Pus6p modifies only position 31 in both cytoplasmic and mito-

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1 Predoctoral Fellow from the “Ministère de la Jeunesse, de l’Education Nationale et de la Recherche.”
2 To whom correspondence should be addressed: Laboratoire Maturation des ARN et Enzymologie Moléculaire, Nancy Université, UMR 7567 CNRS-UHP Nancy I, Faculté des Sciences, BP 239, 54506 Vandoeuvre-les-Nancy Cedex, France. Tel.: 33-3-8368-4316; Fax: 33-3-8368-4307; E-mail: Yuri.Motorin@maem.uhp-nancy.fr.

3 The abbreviations used are: snRNA, small nuclear RNA; WT, wild type; nt, nucleotide(s).
chondrial tRNAs (9), and Pus8p and Pus9p modify position 32 in the cytoplasmic and mitochondrial tRNAs, respectively (10). Finally, Pus4p, which converts the universally conserved U55 residue into a U residue in all elongator tRNAs (11), is the only one well studied example of RNA:Ψ-synthase acting at a unique position in tRNAs. It was demonstrated that this strict substrate specificity depends on the universally conserved G35UUCNANNCC66 sequence in most tRNAs and on the particular three-dimensional structure of the TΨC loop, which is stabilized by a reverse Hoogsteen interaction between residues U54 and A58. Mutations, introduced at any of the conserved positions, completely abolished or considerably reduced the modification efficiency (12). Very similar results were also obtained for bacterial TruB (13). The substrate specificity of other characterized yeast RNA:Ψ-synthases, Pus5p, Pus6p, Pus8p, and Pus9p enzymes, was not studied in detail, so that the sequence and structure requirements for activity of these enzymes are not known.

In contrast to the yeast RNA:Ψ-synthases Pus4p, Pus5p, Pus6p, Pus8p, and Pus9p, both Pus1p and Pus7p are peculiar in acting on different types of substrates: eight positions in tRNAs and position 44 in U2 snRNA for Pus1p (14–16) and position 13 in cytoplasmic tRNAs, 35 in the pre-tRNA^Tyr^ (Ψ'A), and U2 snRNA, and 50 in S S rRNA for Pus7p (4–6). One can ask the question how RNA:Ψ-synthes Pus7p can recognize such different substrates with a high degree of specificity. These substrate RNAs have different size, sequence, and also different two- and three-dimensional structures.

RNA substrate specificity of yeast Pus1p was not systematically studied, but, taken together, the previously published and unpublished observations indicate that Pus1p modifies multiple accessible uridines in a flexible segment between two RNA helices or even at the extremity of the helix (14–16). In some cases, the preference for a purine residue immediately 3' to the modified U was observed, but this tendency seems not be general for all Pus1p substrates.

RNA regions modified by Pus7p also show no obvious common features at the level of the two-dimensional structure: U13 in cytoplasmic tRNAs, U35 in the pre-tRNA^Tyr^ (Ψ'A), and U50 in S S rRNA are located in helical regions according to the two-dimensional structures proposed for these RNAs (Fig. 1) (17–19). In tRNAs, the stability of these helices are quite low. For yeast 5 S rRNA, several alternative two-dimensional structures have been proposed (see Fig. 1A) (20, 21). The base-pairing patterns differ in the region covering Ψ50, and this nucleotide appears to be unpaired in the unified two-dimensional structure proposed for eukaryotic 5 S rRNA (22). In contrast, U35 in U2 snRNA is located in a single-stranded RNA region (23, 24) (Fig. 1A). On the other hand, inspection of the Pus7p RNA targets in S. cerevisiae revealed a high conservation of a 7-nt-long sequence, including the target U residue (5). The newly identified Pus7p target in 5 S rRNA (Ψ50) is also surrounded by the same conserved sequence (6) (Fig. 1B).

Site-directed mutagenesis of the plant Arabidopsis thaliana pre-tRNA^Tyr^ (Ψ'A), which also contains an intron and is pseudouridylated at position 35 had revealed the importance of the U33N34U35A36Pu37 sequence for efficient Ψ35 formation in a wheat germ and HeLa extracts (25). To test whether the sequence recognition is a general feature of the eukaryal Pus7-like enzymes and if the conserved residues have an equal importance in different types of RNA substrates, we produced variants of each of the yeast Pus7p substrates (tRNA^Asp^ (GUC), pre-tRNA^Tyr^ (Ψ'A), and U2 snRNA) with base substitutions at positions −4, −3, −2, −1, +1, +2, or +3 and compared the activity of the recombinant Pus7p on these variant RNAs. Since the cytoplasmic tRNA^Ala^ (ICG), which contains the conserved 7-nt long sequence including position 13, except for a C to U substitution at position −2, is not pseudouridylated at position 13 in vivo, we tested whether it can be modified in vitro after C11U substitution. Several mitochondrial tRNAs also have a sequence around the U13 residue that fits to the consensus sequence of the Pus7p substrates. Therefore, we also tested the in vitro activity of recombinant Pus7p on these tRNAs. Finally, we produced several variants of the tRNA^Asp^ (GUC), pre-tRNA^Tyr^ (Ψ'A) and U2 snRNA substrates of Pus7p that were designed in order to modify their two- and three-dimensional structures. Then we tested the activity of the recombinant Pus7p on these RNA variants. Altogether, the data obtained for all three types of Pus7p RNA substrates tested in this study demonstrate that two highly conserved residues around the target U are most important for Pus7p modification and that other conserved or semiconserved residues have a modulator effect on the activity, depending on the identity of the RNA. Furthermore, the presence of some stable RNA two-dimensional structure elements was found to reinforce the activity, whereas a highly compact three-dimensional structure may decrease the action of Pus7p.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The haploid yeast strain BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 from the EUROSCARF collection was used as a source of genomic DNA for PCR amplification.

The plasmids used for in vitro transcription of tRNA^Asp^ (GUC) and its mutants ΔΨ-SL, MC3, and MC4 were kindly provided by C. Florentz (IBMC, Strasbourg, France). The construct bearing the sequence of S. cerevisiae pre-tRNA^Tyr^ (Ψ'A) under the control of T7 promoter was described previously (5). Plasmid pT7U2Sc, kindly provided by P. Fabrizio, was used for in vitro transcription of S. cerevisiae U2 snRNA fragment 1–139 (14). Other sequences of tRNA genes used in the study were PCR-amplified and inserted into the SmaI site of pUC19, along with the T7 promoter sequence at 5' end of the plasmid.

**DNA Methods**—The haploid yeast strain BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 was used for PCR amplification. All variants of tRNA^Asp^ (GUC), pre-tRNA^Tyr^ (Ψ'A), and U2 snRNA with point mutations were made by site-directed mutagenesis using the QuikChange kit (Stratagene). The

To test whether the sequence recognition is a general feature of the eukaryal Pus7-like enzymes and if the conserved residues have an equal importance in different types of RNA substrates, we produced variants of each of the yeast Pus7p substrates (tRNA^Asp^ (GUC), pre-tRNA^Tyr^ (Ψ'A), and U2 snRNA) with base substitutions at positions −4, −3, −2, −1, +1, +2, or +3 and compared the activity of the recombinant Pus7p on these variant RNAs. Since the cytoplasmic tRNA^Ala^ (ICG), which contains the conserved 7-nt long sequence including position 13, except for a C to U substitution at position −2, is not pseudouridylated at position 13 in vivo, we tested whether it can be modified in vitro after C11U substitution. Several mitochondrial tRNAs also have a sequence around the U13 residue that fits to the consensus sequence of the Pus7p substrates. Therefore, we also tested the in vitro activity of recombinant Pus7p on these tRNAs. Finally, we produced several variants of the tRNA^Asp^ (GUC), pre-tRNA^Tyr^ (Ψ'A) and U2 snRNA substrates of Pus7p that were designed in order to modify their two- and three-dimensional structures. Then we tested the activity of the recombinant Pus7p on these RNA variants. Altogether, the data obtained for all three types of Pus7p RNA substrates tested in this study demonstrate that two highly conserved residues around the target U are most important for Pus7p modification and that other conserved or semiconserved residues have a modulator effect on the activity, depending on the identity of the RNA. Furthermore, the presence of some stable RNA two-dimensional structure elements was found to reinforce the activity, whereas a highly compact three-dimensional structure may decrease the action of Pus7p.
sequences of all the generated recombinant plasmids were verified by DNA sequencing.

*In vitro* T7 RNA polymerase transcription with or without incorporation of the appropriate [$\alpha$-$^{32}$P]NTP and purification of the resulting RNA transcripts by electrophoresis on denaturing gel were performed as previously described (5, 26).

**Cloning, Expression, and Purification of the Recombinant His$_6$-Pus7p**—Preparation of the pET28-PUS7 plasmid used for expression of the recombinant S. cerevisiae Pus7p was described previously (5). N-terminally His$_6$-tagged Pus7p protein was expressed in *E. coli* strain BL21 (DE3)-RII (Strategene). The transformed cells were grown for 5 h in autoinduction ZYM-5052 medium (27) at 37 °C followed by 20 h at 20 °C. The lysate was cleared by centrifugation (20,000 g, 15 min). The supernatant was filtered through a 0.2 µm filter and subjected to sonication (20% duty cycle, Output 6). The enzymatic activity of the recombinant Pus7p was assayed as described previously (5). N-terminally His$_6$-tagged Pus7p protein was purified by Ni$_2$-NTA resin (Amersham Biosciences) charged with Ni$_2$-NTA beads. The rose (Amersham Biosciences), using the Image-Quant software.

**RESULTS**

**Importance of the Conserved Sequence in RNA Substrates for Pus7p Activity in Vitro**—The sequence alignment of the identified *S. cerevisiae* Pus7p substrates (Fig. 1B) revealed the strict conservation of a U residue at position −2 and an A residue at position +1 (as referred to the modified uridine). A G or a C residue is found at position −3, a purine residue at both positions −4 and +2, and a pyrimidine residue at position +3. In contrast, any nucleotide can be found at position −1, suggesting that the identity of this residue is not important for Pus7p activity.

In order to test the influence of the conserved sequence on Pus7p activity, a set of tRNA$^{Asp}$ (GUC), pre-tRNA$^{Tyr}$ (GΨA), and 5′-terminal regions of U2 snRNA variants were produced. The 5′-terminal region of U2 snRNA (positions 1–139), which contains all the functional elements of U2 snRNA, was previously shown to be sufficient for Pus7p activity (4). Versions of these three RNAs with any nucleotide at positions −4, −3, −2, +1, and +2 were produced by *in vitro* transcription. Variant RNAs with a pyrimidine to purine substitution at position +3 were also generated. In addition, one variant with a base substitution at position −1 was used as a control.

The recombinant Pus7p was produced as previously described (5), and its activity on the variant and WT RNAs was tested both in the presence of a large excess of RNA substrate (1 µM of RNA for 10 nM of enzyme) and in conditions where the enzyme was in large excess (about 1 pmol labeled RNA alone for 1 µM enzyme). The level of RNA modification by Pus7p was measured by the nearest neighbor approach after a long incubation period (90 min). This incubation time was selected based on the observation that complete modification of each of the WT RNAs was obtained after about 30 min of incubation under the same conditions. If some of the conserved residues in the RNA substrate were important for Pus7p activity, after their replacement, we expected to detect a decreased level of *Ψ* formation after 90 min of incubation. For each variant RNA, the identity of the [[$\alpha$-$^{32}$P]NTP used for transcription was defined according to the identity of the residue at position +1 as referred to the modified uridine. After incubation with the enzyme, the RNAs were digested with RNase T2, and the released 3′-phosphate mononucleotides were fractionated by two-dimensional thin layer chromatography. The yield of U to *Ψ* conversion was evaluated by measurement of the radioactivity in the fractionated 3′-phosphate mononucleotides, as previously described (5). Quantification of the formed *Ψ* residue is less accurate for U2 snRNA as compared to tRNA$^{Asp}$ (GUC) and pre-tRNA$^{Tyr}$ (GΨA), because of its length and a high occurrence of UA dinucleotides in this RNA. To verify that the observed *Ψ* residue formed in the WT RNAs indeed corresponds to the expected position of U to *Ψ* conversions, control experiments were performed with variants bearing U to C substitution of the target U (U13C tRNA$^{Asp}$ (GUC), U35C pre-tRNA$^{Tyr}$ (GΨA), and U35C snRNA U2 5′-terminal region transcripts). As expected, no significant formation of *Ψ* residue was detected in these mutant RNAs after a 90-min incubation with Pus7p (see supplemental material). These results confirmed the strict specificity of Pus7p for a single position within all of these
TABLE 1
Effect of mutations in tRNA\textsuperscript{Asp}\textsuperscript{GUC} and pre-tRNA\textsuperscript{Tyr}\textsuperscript{G\textsuperscript{YA}} transcripts on their modification by recombinant Pus7p in catalytic reaction conditions

| Position | tRNA\textsuperscript{Asp}\textsuperscript{GUC} \(\Psi\text{ quantification} \) | pre-tRNA\textsuperscript{Tyr}\textsuperscript{G\textsuperscript{YA}} \(\Psi\text{ quantification} \) |
|----------|--------------------------------|--------------------------------|
|          | mol \(\Psi\)/mol tRNA | mol \(\Psi\)/mol tRNA |
| WT       | 0.84 ± 0.03 | WT | 0.83 ± 0.02 |
| −4       | 0.74 ± 0.06 | A9C | 0.46 ± 0.03 |
|          | 0.65 ± 0.05 | A9G | 0.41 ± 0.03 |
|          | 0.70 ± 0.06 | A9U | 0.48 ± 0.02 |
| −3       | 0.56 ± 0.03 | G10A | 0.84 ± 0.04 |
|          | 0.71 ± 0.03 | G10C | 0.69 ± 0.02 |
|          | 0.64 ± 0.03 | G10U | 0.74 ± 0.03 |
| −2       | 0.01 ± 0.05 | U11A | 0.03 ± 0.01 |
|          | 0.04 ± 0.01 | U11C | 0.04 ± 0.01 |
|          | 0.02 ± 0.02 | U11G | 0.06 ± 0.01 |
| U11A/A24U | 0.02 ± 0.03 | U11C/A24G | 0.01 ± 0.02 |
| U11C/A24G | 0.01 ± 0.02 | U11G/A24C | 0.01 ± 0.03 |
| −1       | 0.81 ± 0.03 | U12C | 0.81 ± 0.03 |
|          | 0.00 ± 0.01 | U13C | 0.03 ± 0.01 |
| +1       | 0.05 ± 0.01 | A14C | 0.09 ± 0.05 |
|          | 0.05 ± 0.02 | A14G | 0.03 ± 0.01 |
|          | 0.02 ± 0.01 | A14U | 0.00 ± 0.01 |
| +2       | 0.24 ± 0.02 | A15C | 0.22 ± 0.01 |
|          | 0.38 ± 0.03 | A15G | 0.31 ± 0.02 |
|          | 0.46 ± 0.03 | A15U | 0.39 ± 0.02 |
| +3       | 0.79 ± 0.03 | U16A | 0.76 ± 0.02 |

substrates; thus, we proceeded to definition of the identity elements required for this RNA recognition specificity.

Residues at Positions −2 and +1 Play a Crucial Role for \(U\) to \(\Psi\) Conversion by Pus7p—In vitro modification of the WT and variants of tRNA\textsuperscript{Asp}\textsuperscript{GUC} and pre-tRNA\textsuperscript{Tyr}\textsuperscript{G\textsuperscript{YA}} by recombinant Pus7p was performed both in the excess of RNA substrate (Table 1) and enzyme (Pus7p) (Fig. 2). All tests were done in triplicate, and the mean values of the U to \(\Psi\) conversion level, together with the S.D. value, for each RNA substrate are given in Table 1 and in Fig. 2. The autoradiograms of the representative two-dimensional TLC obtained in one of these three series of experiments are shown as examples in Fig. S1. Under both experimental conditions, a major observation is that the yield of \(\Psi\) formation strongly depended on the position of the base substitution. Second, nearly identical results were obtained when using an excess of substrate or an excess of enzyme (Fig. 2). This might reflect the occurrence of structural heterogeneity of some of the variant RNAs, with only one part of the molecules being able to be modified. Since base substitutions in the tRNA\textsuperscript{Asp}\textsuperscript{GUC} were expected to alter the tRNA two-dimensional structure, we tested whether the presence of compensatory mutations at position 24 in the D-stem could compensate for the strong decrease of \(\Psi\) formation found for base substitutions at position 11 (position −2 in the conserved sequence). As shown in Table 1, restoration of a Watson-Crick base pair between nucleotides 11 and 24 in the D-stem did not increase
RNA Recognition by the Yeast Pus7p

A. Cytoplasmic

| tRNA Ala (IGC) | -GGGCCGUUGCGCUAGCU-GGU--AGCGCGUCUCCCUU |
| tRNA Arg (1CU) | -GCUUGCGCGCGCUAAU--GGC--AAGCGGCUUGACUC |
| tRNA Arg (1CU) | -GCUUUGCGCGCGCUAAU--GGC--AAGCGGCUUGACUC |
| tRNA Phe (GAA) | -GCUUUAUGCUUUGU-GGU--AAAGCGGUAUAAGUG |
| tRNA Gly (NCC) | -AUGAGAUAUAAGUUAAGU-GGU--AAACUGAGGACUN |
| tRNA Lys (NUU) | -GAGAAUAUAAGUUAAGU-GGU--AAACUGAGGACUN |
| tRNA Met (CAU) | -AAGUGCUUGGCUUAAGU-GGU--AACAUGUUGACUN |
| tRNA Arg (ACG) | -AUAUCUUUAAGUUAAGU-GGU--AAAACUGAGGACUN |
| tRNA Arg (NCU) | -GCUUCUUCUGCUUAAU-AGGU--AAAGCGGUAUAAGUG |
| tRNA Thr (UAG) | -GUAAUAUAAAGUUAAGU-GGU--AAACUGAGGACUN |
| tRNA Trp (UCA) | -AAGGAUAUAAGUUAAGU-GGU--AAACUGAGGACUN |
| tRNA Ini (CAU) | -UGCAUGAGGCUAAAGU-GGU--AACAUGUUGACUN |

Conserved sequence: -4 -3 -2 -1 0 +1 +2 +3

B. S. cerevisiae

trNA Ala (IGC)

Control

0.01 ± 0.01

0.04 ± 0.02

trNA Ala (IGC) C11U

0.00 ± 0.01

0.95 ± 0.03

C. S. cerevisiae

trNA Trp (UCA)

Control

0.01 ± 0.01

0.77 ± 0.02

mitochondrial
the Pus7p activity. Therefore, modification of the sequence and not of RNA structure is responsible for the observed effect.

The levels of \( \Psi \) formation in both tRNA transcripts bearing base substitutions at both positions -2 and +1 were nearly identical to those formed in control molecules (U13C substitution in tRNA\(^{\text{Asp}}\)(GUC) and U35C substitution in the pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A)). These data clearly indicated that base substitutions at positions -2 and +1 in both tRNA\(^{\text{Asp}}\)(GUC) and pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A) almost completely abolish U to \( \Psi \) conversion by yeast Pus7p. The same strong negative effect of base substitutions at positions -2 and +1 was also found in U2 snRNA (see supplemental material).

Hence, interaction with residues U-2 and A+1 in any RNA substrate is probably required to achieve one of the steps in the overall Pus7p catalytic process. These data are in perfect agreement with the strict conservation of U-2 and A+1 in all natural RNAs modified by Pus7p in vivo (Fig. 1B) and with previous data obtained for modification of the A. thaliana pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A) in a wheat germ extract (25).

Base substitutions at the four other positions, which are less strictly conserved (R-4, G/C-3, R-2, Y-2), had much less dramatic effects on the yield of U to \( \Psi \) conversion in all of the three substrates tested (Table 1 and Fig. S1, A–C). However, mutations at positions -3 and -4 had almost opposite effects in tRNA\(^{\text{Asp}}\)(GUC) and pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A). This may be linked to their respective RNA two-dimensional structures. Indeed, the base -3 in tRNA\(^{\text{Asp}}\)(GUC) is base-paired with the nucleotide in the D-stem, whereas nucleotide -3 in the pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A) is in the single-stranded region. On the other hand, position -4 in tRNA\(^{\text{Asp}}\)(GUC) is not involved in the interaction with other nucleotides, whereas the base-pairing of the nucleotide at position -4 in the pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A) is expected to stabilize the RNA two-dimensional structure (18) (Fig. 1A). In consequence, substitution of a nucleotide at position -3 in tRNA\(^{\text{Asp}}\)(GUC) had a greater effect on Pus7p activity than substitution at position -4, whereas a more important effect is observed when the nucleotide at position -4 was mutated in the pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A).

Interestingly, substitution of residue A+2 had a marked negative effect in both the tRNA\(^{\text{Asp}}\)(GUC) and pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A). In addition, both substrates, the A to U substitution had the lowest negative effect, whereas the A to C substitution had the greatest negative effect (Table 1 and Fig. 2). As expected, mutation at position -1, where any one of the four possible nucleotides may be found in Pus7p RNA substrates, had no effect on the yield of modification of both the tRNA\(^{\text{Asp}}\)(GUC) and pre-tRNA\(^{\text{Tyr}}\)(G\(\Psi\)A). This was also the case for the pyrimidine to purine substitution at position +3 (Table 1 and Fig. 2).

Restoration of the Consensus Sequence Converts Some tRNAs into Pus7p Substrates—Only three of the yeast cytoplasmic tRNAs that have a U residue at position 13 are not substrates of Pus7p (Fig. 3A). Interestingly, these three tRNAs (tRNA\(^{\text{Ala}}\)(IGC) and two isoacceptors of tRNA\(^{\text{Ala}}\)( mec3UCU) (29)) carry a C instead of a U residue at position 11 (position -2 in the consensus sequence of Pus7p substrates). Based on the above data, we expected that a C11U mutation in these tRNAs will convert them into Pus7p substrates. To test this hypothesis, we used the tRNA\(^{\text{Ala}}\)(IGC) as a model. The activity of the recombinant Pus7p was tested both on a WT tRNA\(^{\text{Ala}}\)(IGC) transcript and on a transcript with the C11U substitution. As illustrated in Fig. 3B, whereas an insignificant level of \( \Psi \) formation was detected in the WT RNA transcript, the C11U variant was almost completely modified (U to \( \Psi \) conversion of 95 ± 3%). These data further demonstrated the requirement of a U residue at position -2 for Pus7p activity.

Recombinant Pus7p Can Modify Mitochondrial tRNAs with U-2, U4, and A+2 Residues—Interestingly, despite the fact that \( \Psi \)13 residue was detected in yeast mitochondrial tRNAs (29), the sequence found for several of them around residue U13 fits to the consensus sequence of Pus7p RNA substrates (Fig. 3A). The absence of U13 pseudouridylation in mitochondria could therefore be due either to an inability of Pus7p to act on mitochondrial tRNAs, because of differences in two-dimensional structures as compared with cytoplasmic tRNAs, or simply due to the absence of Pus7p in mitochondria. To clarify this point, we tested the in vitro activity of Pus7p on one of the mitochondrial tRNAs having a U at position 13 surrounded by the sequence that is conserved in Pus7p substrates, namely tRNA\(^{\text{Tyr}}\)(UCA). A significant level of in vitro modification of this tRNA by Pus7p was obtained (77 ± 2%), showing that the absence of \( \Psi \)13 modification in mitochondrial tRNAs is probably due to the absence of Pus7p in mitochondria. This hypothesis is in agreement with the very low probability for a mitochondrial localization of Pus7p (30), which was also found when using various computer programs (PSORT I and II, SubLoc, ESLPred, and YPLS) developed for the prediction of protein subcellular localization (31–34).

\( \Psi \)13 Formation in tRNA\(^{\text{Asp}}\)(GUC) Does Not Depend on Correct tRNA Three-dimensional Structure but Is Increased by the Presence of RNA Helices—In the tRNA\(^{\text{Asp}}\)(GUC), the residues -4 (A9), +1 (A14), and +2 (A15) are involved in the stabilization of the RNA three-dimensional structure; A9 is involved in the A9-A13-U12 triple-base pair, A14 forms the conserved U8-A14 interaction, and A15 forms the semiconserved purine-pyrimidine Levitt base pair A15-U48 (17, 35). The residue A14 (+1) is particularly important for efficiency of Pus7p modification. However, since the same requirement was also found for pre-

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**FIGURE 3. The presence of a U-2 and A+1 residues in RNA substrate allows \( \Psi \)13 formation.** A, alignment of cytoplasmic and mitochondrial tRNAs that contain unmodified U residue at position 13. The names of the RNAs are given in the first column, and the anticodon sequence is shown (see the legend to Fig. 1). B and C, test for the tRNA\(^{\text{Ala}}\)-synthase activity of the recombinant Pus7p on the WT cytoplasmic tRNA\(^{\text{Ala}}\)(IGC) and its C11U variant and on the mitochondrial tRNA\(^{\text{Ala}}\)(UCA). Uniformly labeled RNA transcripts of the WT and mutated yeast cytoplasmic tRNA\(^{\text{Ala}}\)(IGC) (8) and the mitochondrial tRNA\(^{\text{Ala}}\)(UCA) C were incubated with 10 ns recombinant Pus7p for 90 min in the conditions described under “Experimental Procedures.” Control incubations were performed in the absence of recombinant protein. After incubation, the RNA substrates were digested with RNase T2, and the 3’-nucleotide monophosphates were fractionated by thin layer chromatography. The autoradiograms of the TLC plates are shown. Expected molar ratios of 3’-nucleotide monophosphates obtained after digestion are indicated on the two control plates. The mean value of the molar yield of \( \Psi \) residue formed/mol of tRNA obtained in three distinct experiments is indicated at the bottom of each two-dimensional plate with the confidence interval.
tRNA$^{\text{Asp}}$(GUC) and U2 snRNA, the strong negative effect upon the replacement of residue +1 is probably not related to the three-dimensional tRNA structure. In line with this, the mutation at position −4 (A$^\prime$) has almost no influence on the efficiency of modification, whereas its substitution affects tRNA three-dimensional folding. To verify that the tRNA three-dimensional structure is indeed not important for Pus7p activity and to test for a possible effect of the tRNA two-dimensional structure on this activity, we produced a large series of tRNA$^{\text{Asp}}$(GUC) variants.

The three-dimensional tRNA structure was disrupted to a different extent in all of these variants; the most important perturbation was introduced in the variant ΔΤΨ-SL (Fig. 4A) where the Ψ-stem-loop, which interacts with the D-loop, was deleted. In the variants ΔAC-SL and ΔAA-SL (Fig. 4A) the anticodon stem-loop and acceptor stem, respectively, were missing. In MC3 and MC4 variants, the anticodon stem was either slightly stabilized (MC3) or slightly destabilized (MC4). For each of these RNAs, three independent series of in vitro modification experiments were performed in the excess of RNA as compared with Pus7p (Fig. 4A).

As already demonstrated above, point mutations that destabilize the tRNA D-stem have only a limited effect on the Pus7p activity (Table 1 and Fig. 2). To go one step further in the destabilization of this stem, the sequence of its 3′-strand was completely mutated in variant OD-SL (Fig. 4A). Finally, two minisubstrates corresponding to the D-stem-loop of tRNA$^{\text{Asp}}$(GUC) and to the anticodon stem-loop containing the intron of the pre-tRNA$^{\text{Tyr}}$(GΨA), respectively, were produced (Fig. 4B).

Altogether, the results of these experiments with all eight RNA variants produced (Fig. 4) confirmed that the tRNA three-dimensional structure is not really required for Pus7p activity. The deletion of the acceptor stem (ΔAA-SL) had almost no effect on the activity, whereas elimination of one of the stem-loop structures other than the D-stem-loop (ΔAC-SL, ΔΤΨ-SL) or the destabilization of the anticodon stem loop (MC4) only slightly decreased the yield of modification.

A stronger negative effect was observed when the conformation of the D-stem-loop where modification occurs was altered (the level of Ψ residue formed in OD-SL was divided by 2), and a quite low, but detectable, level of modification was obtained for the two minisubstrates (28 and 32%) (Fig. 4B). We noticed a slight increase of Ψ formation upon stabilization of the anticodon stem (variant MC3). These data suggested that at least...
one stem-loop structure, containing the residue to be modified, is required for Pus7p activity. In addition, the presence of other stable stem-loops, which may facilitate the overall folding of the tRNA, probably also favors Pus7p activity.

ψ35 Can Be Formed in the Mature tRNA Tyr(GΨA) in Vitro—Alignment of the sequence surrounding ψ35 in the mature S. cerevisiae tRNA Tyr(GΨA) with the sequences containing ψ13 in Pus7p tRNA substrates reveals that intronless tRNA Tyr(GΨA) fits almost perfectly to the consensus sequence of Pus7p RNA substrates (Fig. 5A). The only difference is the substitution of the pyrimidine at position +3 by an A residue. As shown above, substitution of this pyrimidine at position +3 by an A residue in both tRNA Asp(GUC) and pre-tRNA Tyr(GΨA) had no marked effect on the yield of U to ψ conversion by Pus7p in vitro. Therefore, one might expect that the recombinant Pus7p is able to modify the mature tRNA Tyr(GΨA) in vitro.

FIGURE 5. Comparison of the Pus7p activity on the precursor and mature tRNA Tyr(GΨA). A, the sequence around position 35 in the mature cytoplasmic tRNA Tyr(GΨA) fits to the consensus sequence established for all Pus7p substrates. B, modification of the tRNA Tyr(GΨA) precursor and mature tRNA Tyr(GΨA) using different concentration of the recombinant Pus7p for the incubation (1 nM, 10 nM, and 1 μM). The modified transcripts were analyzed as described in the legend to Fig. 2. The molar amounts of ψ residue formed/mol of tRNA are indicated at the bottom of the two-dimensional plates. A control assay was done with 1 μM of Pus7p and the tRNA Tyr U35C variant. C, modification of tRNA Tyr(GΨA) variants with an extended anticodon loop or an A38U point mutation. One (+A) or two (+AA) adenosine residues were inserted between nucleotides 31 and 32 (indicated by an arrow). Nucleotide A38 (-3) mutated into a U residue is indicated by an arrow. The results of in vitro modification in the presence of 10 nM Pus7p are given at the bottom of the autoradiograms.
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vitro. To verify this hypothesis, modification experiments were performed in parallel on the precursor and the mature tRNA\textsuperscript{Asp}(GUC), using the excess of RNA substrate. The yield of modification for both RNA transcripts was tested after incubation with either a 1 nM, 10 nM, or 1 \mu M concentration of the recombinant Pus7p. Interestingly, whereas 81\% of U\textsubscript{35} was converted into 35\textsuperscript{Ψ} in the pre-tRNA\textsuperscript{Asp}(GUC) upon incubation with 10 nM Pus7p, no significant 35\textsuperscript{Ψ} formation was detected in the mature RNA under these conditions (Fig. 5A). However, when the protein concentration was increased to 1 \mu M, even mature tRNA\textsuperscript{Asp}(GUC) transcript was modified to a significant extent (63\%). The modification occurred at position 35, since it was not observed in the same transcript bearing the U35C substitution (Fig. 5B).

How can one explain the difference in modification of tRNA\textsuperscript{Asp}(GUC) variants and mature tRNA\textsuperscript{Asp}(GUC)? Indeed, A\textsuperscript{+3} variants of tRNA\textsuperscript{Asp}(GUC) or pre-tRNA\textsuperscript{Asp}(GUC) are modified by 10 nM Pus7p to 76–79\% (Table 1), whereas mature tRNA\textsuperscript{Asp}(GUC), which has the same sequence surrounding U\textsubscript{35}, remains unmodified. One possible explanation could be the highly constrained three-dimensional structure of the anticodon loop of the mature tRNA\textsuperscript{Asp}(GUC), since yeast Pus7p is presently the RNA:Ψ-synthase with the largest spectrum of identified RNA substrates (tRNA, pre-tRNA\textsuperscript{Asp}(GUC), U2 snRNA, and 5 S rRNA). Our data obtained in vitro point out the importance of a given sequence in a flexible RNA segment for efficient Pus7p recognition and modification. However, both in vitro and in vivo, many potential U to Ψ conversion sites are not modified by Pus7p. Even if the sequence of these RNA segments is suitable for recognition, their flexibility may not be sufficient for correct access of the target uridine into the Pus7p active site. In addition, such potential target uridines may be buried inside of stable RNA structures or covered by numerous associated proteins in vivo. The competition between the cognate and near cognate RNA substrates may also influence the outcome of the reaction in the cell. All of these parameters probably play an important role in the choice of RNA substrates by Pus7p.

The Presence of Two-dimensional Structural Motifs Is Required for Efficient Modification of U2 snRNA in Vitro—in the 5'-terminal region of yeast U2 snRNA, residue U\textsubscript{155} is flanked by two stem-loop structures, I and IIa. Stem-loop IIa is immediately followed by a third stem-loop structure, IIb, and a pseudoknot can be formed between the terminal stem-loop IIa and the single-stranded segment located downstream from stem-loop IIb (Fig. 6A). An additional potential target uridine surrounded by a consensus sequence is present in yeast U2 snRNA at position 56; however, this nucleotide is modified neither in vivo nor in vitro, using the recombinant Pus7p.

To test the importance of the U2 snRNA two-dimensional structure for Pus7p activity, we first eliminated the sequence downstream from position 85 in order to disrupt the pseudoknot structure (variant U2-(1–85)). Then the stem-loop structures I and IIb were individually or simultaneously deleted (variants U2-(1–85) \textit{ΔI}, U2-(1–85) \textit{ΔIIB}, and U2-(1–85) \textit{ΔIΔIIB}), and all three stem-loop structures were eliminated in the RNA variant U2-(28–47) (Fig. 6B). Disruption of the pseudoknot structure only slightly decreased the yield of 35\textsuperscript{Ψ} formation. In agreement with previous data (4), the elimination of stem-loop structure I also had only a limited effect on the yield of 35\textsuperscript{Ψ} formation, whereas deletion of stem-loop IIb alone or together with stem-loop I decreased the level of modification by a factor of about 2. In addition, Pus7p did not modify the RNA variant U2-(28–47) without any double-stranded region. Therefore, altogether the data obtained on both U2 snRNA and the tRNA\textsuperscript{Asp}(GUC) strongly suggested that the presence of stable two-dimensional structural motifs increases the level of in vitro activity of Pus7p.

DISCUSSION

In this study, we performed a deep characterization of the RNA sequence and structure requirements needed for modification of three distinct RNA substrates by the yeast RNA:Ψ-synthase Pus7p. The data obtained are especially important, since yeast Pus7p is presently the RNA:Ψ-synthase with the widest spectrum of identified RNA substrates (tRNA, pre-tRNA\textsuperscript{Asp}(GUC), U2 snRNA, and 5 S rRNA). Our data obtained in vitro point out the importance of a given sequence in a flexible RNA segment for efficient Pus7p recognition and modification. However, both in vitro and in vivo, many potential U to Ψ conversion sites are not modified by Pus7p. Even if the sequence of these RNA segments is suitable for recognition, their flexibility may not be sufficient for correct access of the target uridine into the Pus7p active site. In addition, such potential target uridines may be buried inside of stable RNA structures or covered by numerous associated proteins in vivo. The competition between the cognate and near cognate RNA substrates may also influence the outcome of the reaction in the cell. All of these parameters probably play an important role in the choice of RNA substrates by Pus7p.

Point Sequence Determinants Are Required for Pus7p Activity on Any of Its Substrates—Sequence alignments of all yeast Pus7p substrates reveals that the target U residue is located in a 7 nt-long sequence with three strictly conserved residues (the target U residue, U at position −2, and A at position +1), the other residues being semiconserved or partially conserved, except the nucleotide at position −1. Here, we demonstrate that the three strictly conserved residues are absolutely required for Pus7p activity on three of its substrates (tRNA\textsuperscript{Asp}(GUC), pre-tRNA\textsuperscript{Asp}(GUC), and U2 snRNA). Both purine-purine and pyrimidine-pyrimidine replacements at positions −2 and +1, respectively, abolish the activity. Therefore, we show that the U\textsubscript{155}A\textsubscript{156} sequence previously found to be required for Ψ-forming activity in plant pre-
tRNA\text{Tyr}(G\Psi A) is needed for all Pus7p substrates. These residues may be necessary for stabilization of “active” RNA substrate conformation as well as for stable anchoring of the RNA substrate onto the enzyme or for catalytic activity. The residue at position +2 in the consensus sequence is important for the yield of modification but is not essential as are residues U\text{-}2 and A\text{-}1. The identities of the other partially conserved residues of the 7-nt sequence only have a moderate effect on the modification yield, and these effects vary from one substrate to another, probably due to differences in their respective RNA two-dimensional structures. In the present \textit{in vitro} tests, we compared the efficiency of variant RNA modification using an end point measurement. These differences in modification efficiency may be even higher in terms of the initial modification rate.

\textbf{Remodeling of the \textit{tRNA} Structure Is Expected to Occur for the $\Psi$ Formation by Pus7p}—In the L-shaped canonical three-dimensional structure of \textit{tRNA}s, the essential U\text{-}2 and A\text{-}1 residues in RNA substrates are not accessible for the enzyme. Both of them are involved in base pair interactions; U\textsuperscript{11} (U\text{-}2) base-pairs with A\textsuperscript{24} in the D-stem, whereas A\textsuperscript{14} (A\textsuperscript{+}1) forms a tertiary interaction with U\textsuperscript{8}. Furthermore, they are buried into the molecule due to global \textit{tRNA} three-dimensional structure. Thus, the isomerization of U to $\Psi$ at position 13 requires considerable structural remodeling. The three-dimensional structure of the complex formed by a \textit{tRNA} and another RNA modification enzyme acting on a residue in the D-loop has been solved, namely the complex formed by the archaenal \textit{tRNA}-guanine transglycosylase, which converts G at position 15 into archaeosine in many archaenal \textit{tRNA}s and one of its \textit{tRNA} substrates (Fig. 7) (36). This \textit{tRNA} carries a U\textsuperscript{11}, U\textsuperscript{13}, and A\textsuperscript{14} residues like Pus7p substrates. In the three-dimensional structure, it fits into the enzyme active site in an open conformation called the $\lambda$-\textit{tRNA} conformation (36). This conformation is characterized by the opening of the D-stem loop and the interaction of its 3\textsuperscript{-}strand with the variable loop (36). By inspection of yeast \textit{tRNA} sequences, we noticed that 3–5 contiguous and noncontiguous base pairs can always be formed between the 3\textsuperscript{-}strand of the D-stem and the variable loop, so that all Pus7p \textit{tRNA} substrates may be able to adopt a $\lambda$-shape (data not shown). In this conformation, the 5\textsuperscript{-}strand of the D-stem containing U\textsuperscript{11} and U\textsuperscript{13} would be free of base pair interactions, and these two essential residues as well as residue A\textsuperscript{14} would become easily accessible as compared with the canonical L-shaped \textit{tRNA} structure (Fig. 7). Moreover, the conformation of the \textit{E. coli} TruD active site observed in the crystal does not allow fitting the \textit{tRNA} substrate in canonical L-form, whereas the alternative $\lambda$-form can be accommodated (37). Taking these arguments together, one can guess that by interaction with its \textit{tRNA} substrate, Pus7p converts the L-shaped \textit{tRNA} into a
λ-like shaped tRNA so that the three identity elements (U11, U13, and A14) become available, allowing U13 to Ψ13 conversion by the catalytic Asp256 residue.

The Intron in tRNA^{Tyr}(GΨA) Precursor May Avoid Three-dimensional Structural Constraints Limiting Pus7p Activity on the Mature tRNA^{Tyr}(GΨA) — The presence on an intron in pre-tRNA^{Tyr}(GΨA) as well as the presence of a Ψ35 residue in mature tRNA^{Tyr}(GΨA) are general features of Eukarya. Previous work by the group of J. Abelson (38) demonstrated that the absence of the intervening sequence in the yeast RNATyr ochre suppressor gene (SUP6) leads to the absence of Ψ35 modification in the mature tRNA^{Tyr}(GΨA) in vivo. Later, studies performed by RNA microinjection in Xenopus laevis oocytes showed that Ψ35 could be formed in both X. laevis and Drosophila pre-tRNA^{Tyr}(GΨA), despite the strong sequence and length differences of the introns of these two precursors (39–41). The conclusion of this study was that the tRNA:Ψ35-synthase needs the intron for tRNA^{Tyr}(GΨA) modification but that Ψ formation is not dependent on the size and sequence of the intervening sequence. A similar observation was made for the plant pre-tRNA^{Tyr}(GΨA); the presence of an intron is required to obtain a modification, and only the identity of residues U13, A36, and A37 have a strong influence on the activity (25). Further studies of Ψ35 formation in plant tRNA^{Tyr} pointed out the moderate importance of C32 (position −3) and the length of the intron; the minimal size still supporting modification was found to be 7 nt (42).

Here we show that the recombinant Pus7p can form Ψ35 residue in the mature tRNA^{Tyr}(GΨA) but only when used at rather high concentration (1 μM), which is probably much superior to that found in vivo. Indeed, experimental measurements have indicated that Pus7p is moderately expressed in S. cerevisiae; its estimated amount is about 4000 molecules/cell (see Yeast GFP Fusion Localization Database, University of California). We could markedly increase the activity of Pus7p on the mature tRNA^{Tyr}(GΨA) by insertion of one or two A residues in the anticodon loop, suggesting that the low activity of Pus7p on the mature tRNA^{Tyr}(GΨA) is probably due to the inability of
Pus7p to disrupt the three-dimensional structure of the tRNA$^{\text{Tyr}(G\Psi A)}$ anticodon loop. The presence of an intron in the anticodon loop of all eukaryal pre-tRNA$^{\text{Tyr}(G\Psi A)}$ probably relieves the three-dimensional structural constraints and therefore allows Pus7p activity on this tRNAs. Therefore, Pus7p probably has the capability to remodel the L-shaped structure of the tRNAs into the more open $\lambda$-form but is not able to change the quite rigid conformation of the anticodon loop of mature tRNA. In this case, the solution retained in the course of evolution for modification at position 35 was the insertion of an intron in the pre-tRNA$^{\text{Tyr}(G\Psi A)}$.

**Stable Double-stranded RNA Regions Are Required for Pus7p Activity**—Whereas tight three-dimensional structure may be a handicap for Pus7p activity, as seen for tRNA$^{\text{Tyr}(G\Psi A)}$, a substrate without any double-stranded region is not modified by Pus7p, as evidenced for the single-stranded U2 snRNA segment 28–47 containing the conserved 7-nt-long sequence. More generally, all of the mutations that decreased the number of stem-loop structures in RNA substrate diminished the modification level upon incubation with Pus7p. This was the case for variants of both tRNA$^{\text{Asp}(G\Psi A)}$ (\$\Delta$T\$\Psi$\$\Delta$A-$\Delta$C-\$\Delta$A-SL and \$\Delta$A-$\Delta$C-SL and \$\Delta$A-SL) and the 5$'$-terminal region of U2 snRNA (U2-(1–85) \$\Delta$I, U2-(1–85) $\Delta$I\$\Delta$I\$\Delta$I\$\Delta$I\$\Delta$I\$\Delta$I, and U2-(28–47)). Similarly, minisubstrates corresponding to the D-stem-loop of tRNA$^{\text{Asp}(G\Psi A)}$ or the anticodon stem-loop of the pre-tRNA$^{\text{Tyr}(G\Psi A)}$ were modified at a low level. In the case of U2 snRNA, deletion of one stem-loop structure, IIb, had a particularly strong effect on RNA modification. Mutation at position -4 in the pre-tRNA$^{\text{Tyr}(G\Psi A)}$, which disrupts the intron structure, had a strong negative effect as well as disruption of the D-stem-loop structure in tRNA$^{\text{Asp}(G\Psi A)}$. Hence, either Pus7p has numerous specific binding sites for recognition of each of its substrates in their integrity, or, more likely, it contains binding sites able to recognize one stem-loop structure, including or very close to the target residue and site recognizing with a looser specificity. The specific recognition of substrate may be ensured by interaction with the three point identity determinants and one stem-loop structure. The presence of poor specific recognition sites might reinforce the stability of the RNA-protein complex and therefore the yield of the reaction.

Numerous Pus7p orthologs have been identified in all kingdoms of life (2). Comparison of these proteins reveals an increase of size from bacteria to archaea and a greater one from archaea to eukarya. This size increase may be linked to the presence of additional domains needed to accommodate a larger number of RNA substrates in eukarya compared with bacteria (pre-tRNA$^{\text{Tyr}(G\Psi A)}$, 5 S rRNA, and U2 snRNA). Alternatively, these additional domains may help to maintain the specificity of eukaryotic Pus7p and to avoid unproductive modification of noncognate RNA substrates. Further studies are required to verify these hypotheses.

Inspection of the amino acid sequence of Pus7p-like enzymes does not reveal the presence of a domain belonging to the double-stranded RNA binding domain family that might explain a reinforced binding in the presence of several stem-loop structures. We did not detect strong sequence homologies between the Pus7p-like proteins outside of the catalytic domain. Further structural analysis of Pus7p is required to understand how this enzyme recruits a large variety of substrates and accommodates them in its active site.

**Mitochondrial Yeast tRNAs Carry the Conserved Sequence of Pus7p Substrates But Are Not Modified in Vivo**—Interestingly, 6 of 17 yeast mitochondrial tRNAs carry a U$^{\text{13}}$ residue with a surrounding sequence that fits perfectly to the consensus sequence of Pus7p substrates. Although we show that Pus7p can modify them in vitro, they do not carry a U$^{\text{13}}$ residue in vivo. This is probably explained by the absence of import of Pus7p in the mitochondria. Indeed, expression of GFP-Pus7p fusions revealed that its location is mainly nuclear (43). Thus, pre-tRNA$^{\text{Tyr}(G\Psi A)}$, 5 S rRNA, and probably other tRNAs are modified by Pus7p in the nuclear compartment, since only a small fraction of GFP-Pus7p protein was detected in the cytoplasm (43).

**Functional Importance of Pus7p-catalyzed Modification of Cellular RNAs**—The exact functional roles of $\Psi^{\text{50}}$ in 5 S rRNA and $\Psi^{\text{13}}$ in several tRNAs are not clearly established; however, these pseudouridine residues may stabilize the local RNA conformation and/or favor interactions with protein partners. In contrast, more experimental data were accumulated for the importance of $\Psi^{\text{35}}$ in eukaryotic tRNA$^{\text{Tyr}(G\Psi A)}$. First of all, the conformation of the anticodon loop of tRNA$^{\text{Tyr}(G\Psi A)}$ is stabilized by the presence of $\Psi^{\text{35}}$ (44, 45). In addition, the presence of $\Psi^{\text{35}}$ in the middle anticodon position of tRNA$^{\text{Tyr}}$ influences its suppressor activity toward stop codons UAG and UAA (41). These data show that $\Psi^{\text{35}}$ plays an important role in the stabilization of the codon-anticodon interaction between mRNA and tRNA$^{\text{Tyr}(G\Psi A)}$ and thus increases its translation efficiency. On the other hand, $\Psi^{\text{35}}$ in U2 snRNA should play an important role in the efficiency of the first step of the splicing reaction (46). Structural data clearly indicate that $\Psi^{\text{35}}$ in U2 snRNA induces particular conformation of the mRNA-U2 snRNA duplex and favors the extrahelical conformation of the branch point adenosine (47, 48). Hence, there are strong arguments explaining the need for a cellular Pus7p activity (at least production of functional U2 snRNA and tRNA$^{\text{Tyr}(G\Psi A)}$).

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