REPORT

Archaeal Pus10 proteins can produce both pseudouridine 54 and 55 in tRNA

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ABSTRACT

Pus10, a recently identified pseudouridine (Ψ) synthase, does not belong to any of the five commonly identified families of Ψ synthases. *Pyrococcus furiosus* Pus10 has been shown to produce Ψ 55 in tRNAs. However, in vitro studies have identified another mechanism for tRNA Ψ 55 production in Archaea, which uses Cbf5 and other core proteins of the H/ACA ribonucleoprotein complex, in a guide RNA-independent manner. Pus10 homologs have been observed in nearly all sequenced archaeal genomes and in some higher eukaryotes, but not in yeast and bacteria. This coincides with the presence of Ψ 54 in the tRNAs of Archaea and higher eukaryotes and its absence in yeast and bacteria. No tRNA Ψ 54 synthase has been reported so far. Here, using recombinant *Methanocaldococcus jannaschii* and *P. furiosus* Pus10, we show that these proteins can function as synthase for both tRNA Ψ 54 and Ψ 55. The two modifications seem to occur independently. Salt concentration dependent variations in these activities of both proteins are observed. The Ψ 54 synthase activity of *M. jannaschii* protein is robust, while the same activity of *P. furiosus* protein is weak. Probable reasons for these differences are discussed. Furthermore, unlike bacterial TruB and yeast Pus4, archaeal Pus10 does not require a U54•A58 reverse Hoogsteen base pair and pyrimidine at position 56 to convert tRNA U55 to Ψ 55. The homology of eukaryal Pus10 with archaeal Pus10 suggests that the former may also have a tRNA Ψ 54 synthase activity.

Keywords: pseudouridine synthase; TruB; Pus4; TrmA

INTRODUCTION

Pseudouridine (Ψ) is produced by post-transcriptional C5-ribosyl isomerization of uridine and is the most common modification observed in stable RNAs (Rozenski et al. 1999). All three domains of life contain single-protein Ψ synthases that recognize one or a few target uridines through conserved primary and/or secondary RNA structures (Hamma and Ferré-D'Amaré 2006). Based on protein sequence similarities these Ψ synthases have been classified into five families: TruA, TruB, TruD, RluA, and RsuA (Hamma and Ferré-D'Amaré 2006). Archaea and Eukarya also contain H/ACA ribonucleoproteins (RNPs) that can produce Ψ in an RNA-guided manner (Filipowicz and Pogacic 2002; Kiss 2002; Decatur and Fournier 2003; Henras et al. 2004; Dennis and Omer 2005; Meier 2005; Yu et al. 2005; Reichow et al. 2007). These RNPs contain

four core proteins: Cbf5 (dyskerin, NAP57), Nop10, Gar1, and Nhp2 (L7Ae in Archaea), and a guide RNA. The Cbf5 is the Ψ synthase in these RNPs. Archaeal and eukaryal Cbf5 are homologous and belong to the TruB family. Archaeal Cbf5 has also been shown to function without guide RNA (Roovers et al. 2006; Gurha et al. 2007; Muller et al. 2007, 2008). However, this protein-alone activity of archaeal Cbf5 is mostly observed in association with Gar1 and/or Nop10.

Bacterial TruB and its homolog Pus4 in yeast are Ψ synthases that convert the universally conserved U55 in the T Ψ C loop of tRNAs to Ψ 55 (except in eukaryal initiator tRNA) (Nurse et al. 1995; Becker et al. 1997a). Archaeal tRNAs also contain Ψ 55. Two different enzymes in Archaea, Cbf5 and Pus10 (PsuX), are able to catalyze the in vitro formation of Ψ 55 in tRNA in a protein-only manner (Roovers et al. 2006; Gurha et al. 2007; Muller et al. 2007). Since Ψ 55 synthesis by these two enzymes appears to be redundant in Archaea, the question remains whether these proteins also serve some other purpose. As mentioned above, archaeal Cbf5 is also the Ψ synthase of the H/ACA RNP. However, no additional role for archaeal Pus10 has been found yet. Pus10 does not belong to any of the five

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known families of Ψ synthases (Watanabe and Gray 2000). Pus10 homologs have been observed in nearly all sequenced archaeal genomes. Its homologs are also present in some higher eukaryotes, but not in yeast and bacteria (Watanabe and Gray 2000; Roovers et al. 2006; McCleverty et al. 2007). Interestingly, human Pus10 earlier had been identified as a hypothetical protein FLJ32312, gi:75516696 that has been implicated in TRAIL-induced apoptosis in humans (Aza-Blanc et al. 2003; McCleverty et al. 2007). This implies a dual functional role of Pus10 in higher eukaryotes.

Most bacterial and eukaryal tRNAs contain 5-methyluridine (m^5U , also termed rT for ribothymidine) at position 54 in their T Ψ C loop (Sprinzl and Vassilenko 2005). The products of the *trmA* gene in most bacteria and *TRM2* in yeast produce m^5U54 (T of T Ψ C loop) using AdoMet as the methyl donor (Greenberg and Dudock 1980; Ny and Bjork 1980; Nordlund et al. 2000). In certain bacteria, this m^5U54 is produced by the *trmFO* gene product, where N^5,N^{10} -methylenetetrahydrofolate is used as the 1-carbon donor and FADH₂ as the reducing agent (Delk et al. 1980; Urbanavicius et al. 2005). Most archaeal and some higher eukaryal tRNAs, on the other hand, contain $\Psi54$, instead of m^5U54 . This is commonly modified to 1-methyl- $\Psi54$ in Archaea (Gupta 1984, 1986; Sprinzl and Vassilenko 2005; Grosjean et al. 2008). However, no synthase for $\Psi54$ has yet been identified. So far no bacterial or yeast tRNA has been noted to contain $\Psi54$, which correlates with the absence of Pus10 in these organisms. Thus, it appears that Pus10, a unique Ψ synthase, is present only in the organisms that have $\Psi54$ in their tRNA. Therefore, we hypothesized that Pus10 may produce both $\Psi54$ and $\Psi55$ in archaeal tRNA, since some Ψ synthases are known to have multisite substrate specificity (Motorin et al. 1998; Behm-Ansmant et al. 2006; Hamma and Ferré-D'Amaré 2006). Using recombinant *Methanocaldococcus jannaschii* (Mj) and *Pyrococcus furiosus* (Pfu) Pus10 proteins, we show that these can convert U at both positions 54 and 55 of tRNA to Ψ . The $\Psi54$ synthase activity of the Mj protein is robust, but that of the Pfu protein is comparatively weak. The initial kinetics of reaction for the Mj protein is very fast and, at a 1:1 RNA:protein ratio, almost complete U to Ψ conversion occurs at both positions in about 5 min. This is the first identification of a tRNA $\Psi54$ synthase.

RESULTS AND DISCUSSION

Archaeal Pus10 proteins can produce both $\Psi54$ and $\Psi55$ in tRNA

$[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled *Haloferax volcanii* tRNA^{Trp} was incubated with equimolar amounts of Mj-Pus10 or Pfu-Pus10 proteins. TLC analyses of nuclease P1 digested products showed that both proteins can convert one or more U's to Ψ 's in this tRNA (Fig. 1B). Nuclease P1 will generate pU or modified pU from $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled

FIGURE 1. Archaeal Pus10 proteins can convert both U54 and U55 of tRNA to Ψ . (A) Sequence of in vitro transcribed *H. volcanii* tRNA^{Trp}. Mutations changing U55 and C56 are indicated by arrows. (B) $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled tRNA^{Trp} was incubated with (+) and without (-) Mj-Pus10 or Pfu-Pus10 proteins, as indicated. Nuclease P1 digests of purified products were resolved by TLC on cellulose plates. pU and p Ψ indicate 5'-phosphorylated U and Ψ , respectively. (C-E) $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ - or $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ -labeled (indicated in the panels) tRNA^{Trp} and its mutants U55A and C56A were incubated with Mj-Pus10 or Pfu-Pus10 proteins, as indicated. RNase T2 digests of purified products were resolved by using solvents I and II, as described in the Materials and Methods section. Ap, Cp, Gp, Up, and Ψ p indicate 3'-phosphorylated A, C, G, U, and Ψ , respectively. (F) tRNA^{Trp} after incubation with Mj-Pus10 was treated in the absence (-) or presence (+) of CMCT for indicated time in minutes. This was (+) or was not (-) followed by alkali (OH^-) treatment. Positions of U54 and U55 are indicated on the left side. Asterisks on the right indicate strong stops before CMCT-modified $\Psi54$ and $\Psi55$.

RNA. Therefore, it will reveal overall Ψ production. Nearest-neighbor analysis using RNase T2 digestion was done to determine the specific sites of Ψ production in the tRNA. RNase T2 digestion produces ribonucleoside 3'-monophosphates (Np) and in the process transfers the

labeled phosphate on the 5' side of a residue in the RNA to the 3' side of the preceding residue. TLC analyses of RNase T2 digested products of [α - 32 P]UTP-labeled tRNA^{Trp} showed that both these proteins can produce Ψ in tRNA (Fig. 1C). The labeled Ψ p in this case is derived from conversion of U54 to Ψ , because there is only one U (position 54) that precedes another U in this tRNA (see Fig. 1A). Conversion to Ψ is nearly complete using Mj-Pus10 but is only partial with Pfu-Pus10 (Fig. 1C). Site-specific production of Ψ 54 was further confirmed by RNase T2 digestion of [α - 32 P]ATP-labeled tRNA^{Trp}-U55A (a mutant with U55 changed to A55) treated with Mj-Pus10 (Fig. 1D). The presence of labeled Ψ p and near absence of labeled Up in this case confirms that U at position 54 is converted to Ψ . Normally there is no UA sequence in tRNA^{Trp}; hence, no labeled Up is observed in RNase T2 digests of [α - 32 P]ATP-labeled wild-type (WT) tRNA^{Trp} (data not shown).

Ψ 55 synthase activity of Pfu-Pus10 has been shown by others (Roovers et al. 2006). Here we show that Mj-Pus10 can also synthesize Ψ 55. RNase T2 digests of [α - 32 P]CTP-labeled tRNA^{Trp} were not useful for this analysis since the tRNA^{Trp} contains sequence UC at seven different places. Instead, we used [α - 32 P]ATP-labeled tRNA^{Trp}-C56A, a mutant where C56 is changed to A56, creating a unique UA sequence at positions 55–56. RNase T2 digests of this mutant treated with Mj-Pus10 also showed labeled Ψ p with near absence of labeled Up (Fig. 1E), suggesting conversion of U55 to Ψ 55. Production of Ψ at positions 54 and 55 in tRNA^{Trp} was further confirmed by primer extension analysis of RNA that was *N*-cyclohexyl-*N'*-(2-morpholinoethyl)-carbodiimide metho-*p*-toluosulfonate (CMCT) modified after treatment with Mj-Pus10 (Fig. 1F). Taken together, our data suggest that both Mj-Pus10 and Pfu-Pus10 proteins can produce both Ψ 54 and Ψ 55 in tRNAs.

Differences in the pseudouridylation activities of Mj-Pus10 and Pfu-Pus10 proteins

During initial optimization of the reaction conditions for both Mj-Pus10 and Pfu-Pus10 proteins, we observed that the production of total Ψ (Ψ 54 and Ψ 55) and specific Ψ 54 increased as the salt concentration in the reaction decreased (Fig. 2). Neither the total Ψ nor the Ψ 54 producing activity of Mj-Pus10 changed in the range 300–450 mM NaCl, but it decreased at 600 and 900 mM NaCl. Specific Ψ 54 production by Mj-Pus10 was about the same at 150 mM NaCl as at 300–450 mM (data not shown). However, the total Ψ synthase activity of Mj-Pus10 was about 2.8 Ψ /tRNA at 150 mM NaCl, but most of this increase was due to nonspecific conversion of U to Ψ at different sites in the tRNA (data not shown). Therefore, Ψ production only between 300 and 900 mM NaCl concentrations are shown in the figure. Overall, the salt-dependent patterns of Pfu-Pus10 activities were similar to those of Mj-Pus10 (Fig. 2). However, total Ψ production by Pfu-Pus10 at each salt

FIGURE 2. Pseudouridylation activity of Pus10 proteins versus salt concentration. Ψ production by Mj-Pus10 and Pfu-Pus10 was determined under different NaCl concentrations. Total Ψ 54 and Ψ 55 production was calculated from the TLC analyses of nuclease P1 digests as shown in Figure 1B. Ψ 54 production was calculated from the TLC analyses of RNase T2 digests as shown in Figure 1C.

concentration was much less than that for Mj-Pus10. Furthermore, Ψ 54 synthesis by Pfu-Pus10 was drastically reduced with increased salt concentration, which mainly accounted for the reduction of total Ψ synthesis under these conditions. Both total Ψ and specific Ψ 54 production by Pfu-Pus10 at 150 mM NaCl (see Fig. 3C) were higher than at 300 mM NaCl (Fig. 2). The Ψ 54-synthesizing activity of Pfu-Pus10 was probably not detected by Roovers et al. (2006) because their reactions contained 500 mM KCl, and at this salt concentration, we also could not detect any Ψ 54 synthesis.

Time course experiments using 1:1 tRNA^{Trp}:Mj-Pus10 protein showed efficient modification of both Ψ 54 and Ψ 55. In these conditions the reactions were nearly complete in ~5 min (Fig. 3A). The kinetics of Ψ 54 and Ψ 55 production in these experiments were similar. (Mutant tRNA^{Trp}-C56A was used here to confirm the specific Ψ production at position 55. The initial kinetics for this mutant in the figure appear somewhat different from those of tRNA^{Trp} because Ψ production at time points of 1 and 3 min were not determined for the C56A mutant.) Time course studies of Ψ 54 formation by Mj-Pus10 in three mutants of tRNA^{Trp} where U55 was changed to A, C, or G showed very similar kinetics for all three substrates (Fig. 3B). These curves (Fig. 3B) did not significantly differ from the curve for Ψ 54 synthesis in wild-type U55 containing tRNA^{Trp} shown in Figure 3A. Overall, these results suggest that syntheses of Ψ 54 and Ψ 55 by Mj-Pus10 are independent.

The specific Ψ 54 synthase activity of Pfu-Pus10 is very low, even at 300 mM NaCl, producing only ~0.2 Ψ /tRNA after 1 h (Fig. 2). Therefore, time course studies for this activity were done at 150 mM NaCl (Fig. 3C). The rate of reaction for Ψ 54 production with 1:1 tRNA^{Trp}:Pfu-Pus10 protein was slow. The total amount of Ψ 54 modification was ~0.5 Ψ /tRNA after 60 min even at this low salt

FIGURE 3. Time course of Ψ production by Pus10 proteins. (A) [α - 32 P]UTP- or [α - 32 P]CTP-labeled tRNA^{Trp} or [α - 32 P]ATP-labeled tRNA^{Trp}-C56A (labeling indicated in the parentheses) was incubated with Mj-Pus10. The product from each time point was analyzed by nuclease P1 (P1) or RNase T2 (T2) digestion and quantitated for Ψ. The location of the Ψ (position 54 or 55 or total) is indicated in parentheses. (B) Time course analyses similar to A, using [α - 32 P]UTP-labeled U55C, U55A, and U55G mutants of tRNA^{Trp} after digestion with nuclease P1. (C) Time course analyses similar to A, using [α - 32 P]UTP-labeled tRNA^{Trp} after incubation with Pfu-Pus10 (150 mM NaCl) followed by digestion with nuclease P1 or RNase T2.

concentration. As a control, time course studies for total Ψ synthase activity at 150 mM NaCl were also done (Fig. 3C). As with the Mj-Pus10, total Ψ synthase activity of Pfu-Pus10 was very high at 150 mM NaCl, but most of this

increase was due to nonspecific conversion of U to Ψ at different sites in the tRNA (data not shown).

The data presented in Figures 2 and 3 indicate that in vitro Ψ synthase activity of Mj-Pus10 is more robust and faster than that of Pfu-Pus10 at 68°C, more so for the Ψ54 synthesis. The difference in the Ψ54 synthase activities of the two proteins may be a reflection of the in vivo functions of these proteins. Unlike Mj-Pus10, Pfu-Pus10 may not be converting most of the U54 to Ψ54. Nearly all tRNAs of Bacteria and Eukarya contain m⁵U, which is always present at position 54 of tRNA. On the other hand, in Archaea, m⁵U (as 2-thio-m⁵U) is detected only in the tRNAs of *Thermococcus* sp. and *P. furiosus* (Edmonds et al. 1991; Kowalak et al. 1994), members of the order Thermococcales. Confirming this, AdoMet-dependent tRNA m⁵U54 activity has been demonstrated in the cell extracts of *P. furiosus* (Constantinesco et al. 1999). Recently, it has been shown that homologs of the bacterial *rumA* gene, which encodes the enzyme for m⁵U1939 in *Escherichia coli* 23 rRNA, are present in Archaea, but only in the members of Thermococcales and Nanoarchaea. Moreover, the product of this *rumA*-type gene has in vitro AdoMet-dependent tRNA m⁵U54 activity (Urbonavicius et al. 2008). Therefore, it is possible that most of the *P. furiosus* tRNAs contain m⁵U54, while a select few have Ψ54, as is the case for higher eukaryotes (Sprinzl and Vassilenko 2005). In such a case, the weak Ψ54 synthase activity of Pfu-Pus10 would be sufficient to produce Ψ54 in these few tRNAs. This raises the possibility that certain features of these tRNAs would affect recognition by Pfu-Pus10 to produce Ψ54. The differences in the activities of Mj-Pus10 and Pfu-Pus10 could be a reflection of the differences in specific sequences or overall structures of the two proteins. Sequence alignment of the two proteins shows that Pfu-Pus10 lacks certain residues in the N-terminal domain and “forefinger loop” (McCleverty et al. 2007) that are present in Mj-Pus10 (data not shown).

Pus10 protein recognizes tRNA differently than TruB/Pus4 to produce Ψ55

Mj-Pus10 can produce both Ψ54 and Ψ55 in *H. volcanii* elongator tRNA^{Met}. This was shown using wild-type and certain mutant transcripts (Fig. 4A; data not shown). The tRNA^{Met} contains G58 and G60 instead of conserved A and semiconserved pyrimidine, respectively, at these positions (Fig. 4A). This tRNA is a substrate for Pus10 protein, as shown here, and for archaeal Cbf5 protein, as shown previously (Gurha et al. 2007); however, it may not be a substrate for TruB/Pus4. A requirement for U54, U55, and A58 and a preference for C over U at position 56 has been noted for the Ψ55 synthetic activity of *E. coli* TruB (Gu et al. 1998). For its Ψ55 synthesis yeast Pus4, in addition, requires a specific G53–C61 pair and only accepts C56 (not U56) (Becker et al. 1997b). These bases are conserved in

FIGURE 4. Mj-Pus10 activity with different substrates. (A) Sequence of in vitro transcribed *H. volcanii* elongator tRNA^{Met} is shown on the left. TLC separation of RNase T2 and nuclease P1 (on cellulose plates) digests of [α -³²P]UTP-labeled tRNA^{Met} treated with Mj-Pus10 as done in Figure 1 are shown in the panels. (B) Sequence of the T-arm-Trp transcript, a 17-mer corresponding to the TΨC-arm (positions 49–65) of tRNA^{Trp} (Fig. 1A) is shown on the left. U's corresponding to U54 and U55 are underlined. TLC separation of RNase T2 (on cellulose plates) and nuclease P1 (on PEI-cellulose plates) digests of [α -³²P]UTP-labeled T-arm-Trp treated with Mj-Pus10 in different NaCl concentrations are shown in the panels. The table shows the amount of Ψ calculated from these panels. (R) untreated RNA. (ND) not detected.

bacterial and yeast tRNA sequences. TruB specifically recognizes the reverse Hoogsteen base pair formed between U54 and A58 of tRNA (Pan et al. 2003; Chaudhuri et al. 2004; Phannachet and Huang 2004; Hamma and Ferré-D'Amaré 2006). Pus4 does not convert U55 to Ψ55 in the eukaryal initiator tRNAs, because they have A54 instead of U54 (Sprinzl and Vassilenko 2005), which does not allow this reverse Hoogsteen base pairing. This U54•A58 pair would not form in tRNA^{Met}, because it contains G58 not A58. Therefore, the production of Ψ55 in elongator tRNA^{Met}, as shown here and reported previously (Gurha et al. 2007), suggests that the mechanism to recognize U55 for its conversion to Ψ55 (either by Pus10 or by Cbf5) in Archaea is different from the mechanisms in Bacteria and Eukarya. This is further confirmed by production of Ψ55 in the mutant tRNA^{Trp}-C56A by Pus10 (Fig. 1D) and by

archaeal Cbf5 (Gurha et al. 2007). This mutant tRNA cannot be a substrate for TruB/Pus4 because they require C (or U) at position 56, while this tRNA has A56.

Pus10 can produce Ψ in just the TΨC-arm (stem-loop) of tRNA

Previously we have shown that the archaeal Cbf5–Gar1 complex could produce the equivalent of Ψ55 in 3'-exon-Trp, a 39-base transcript containing approximately the 3' half of tRNA^{Trp} (Gurha et al. 2007). The equivalents of Ψ54 and Ψ55 in the same substrate could also be produced by Mj-Pus10 (data not shown). Furthermore, the amounts of these activities were about the same as for the full-size tRNA. T-arm-Trp, a 17-base RNA containing the TΨC-stem-loop of the tRNA^{Trp} (Fig. 4B) is not a substrate for Ψ synthesis by archaeal Cbf5 (Gurha et al. 2007). This RNA would be a good substrate for both *E. coli* TruB and Yeast Pus4 (Becker et al. 1997b; Gu et al. 1998). Mj-Pus10 converted both U's in the loop of this RNA (equivalent to U54 and U55) to Ψ's, albeit with a lower efficiency than for the full-size tRNA (Fig. 4B). The extent of conversion of both U's was higher at 150 mM than at 300 mM NaCl. Surprisingly, the conversion of Ψ54 was not detected at 450 mM NaCl, but total Ψ (essentially contributed by Ψ55) was observed at this concentration of salt. These data suggested that, although the TΨC-arm of tRNA is a good substrate for both Ψ54 and Ψ55 syntheses by Mj-Pus10, the production of Ψ55 was more efficient than that of Ψ54 in this substrate. This contrasts with full-size tRNA substrates, where both Ψ syntheses appeared to be equally efficient (Fig. 3A).

In vivo role of Pus 10

This study suggests that archaeal Pus10 proteins can produce both Ψ54 and Ψ55 in tRNAs. *P. furiosus* Pus10 can produce Ψ55 in tRNAs in a heterologous in vivo system by complementing an *E. coli* strain deficient in TruB activity (Roovers et al. 2006). However, in vitro studies have suggested that archaeal Cbf5 in complex with Gar1 and/or Nop10 protein can also produce Ψ55 in tRNA (Roovers et al. 2006; Gurha et al. 2007; Muller et al. 2007) as well as in 23S rRNA fragments (Muller et al. 2008) in a

guide-RNA-independent manner. The sequence containing the U to be modified in the 23S rRNA fragment can be folded into a stem-loop structure somewhat similar to the T Ψ C-arm of tRNA (Muller et al. 2008). We speculate that the in vivo guide-RNA-independent role of archaeal Cbf5 (in complex with accessory proteins) is to convert certain U's in rRNA to Ψ 's and archaeal Pus10 functions primarily as a Ψ 54 and Ψ 55 synthase for tRNA.

Ψ 54 synthase activity has been observed in *Xenopus laevis* oocytes upon microinjection of yeast tRNA^{Tyr} gene into their nuclei (Nishikura and De Robertis 1981). About 80% Ψ 54 and only 10% m⁵U54 were produced in these tRNAs. Furthermore, microinjection of a mini-helix containing only acceptor stem and T Ψ C-arm of yeast tRNA^{Asp} produced Ψ 54, while full-size and D-arm deleted versions of this tRNA produced m⁵U54 (Grosjean et al. 1996). However, no protein has been identified for this eukaryal Ψ 54 activity. The homology of eukaryal Pus10 with archaeal Pus10 suggests that the former may also have a Ψ 54 synthase activity for tRNA.

MATERIALS AND METHODS

Preparation of transcripts

Transcripts for *H. volcanii* tRNA^{Trp}, elongator tRNA^{Met}, 3'-exon-TRP, T-arm-TRP, and mutant tRNA^{Trp}-C56A were generated as described previously (Gurha et al. 2007). Mutants tRNA^{Trp}-U55A, tRNA^{Trp}-U55C, and tRNA^{Trp}-U55G were prepared by changing U55 of tRNA^{Trp} to A, C, and G, respectively, by the procedures described before (Gurha et al. 2007). Sequences of these transcripts are shown in the relevant figures. Both unlabeled and ³²P-labeled transcripts were prepared as needed.

Cloning and preparation of recombinant Pus10 proteins

Genes encoding the Pus10 proteins of *M. jannaschii* and *P. furiosus* were PCR amplified using corresponding genomic DNAs as templates and primer pairs containing NdeI and EcoRI sites on the 5' and 3' sides, respectively, of the protein-coding segment. After restriction digestion, the PCR fragments were cloned into the pET28a vector to produce genes for N-terminal His-tagged proteins. The proteins were expressed in *E. coli* Rosetta (DE3) pLysS. Recombinant proteins were prepared by nickel-affinity chromatography under native conditions as for the preparation of other proteins described before (Gurha et al. 2007). Proteins were quantitated and stored at -70°C .

Pseudouridylation assays

A typical 20 μL Mj-Pus10 pseudouridylation reaction consisted of 50 nM radiolabeled RNA and ~ 50 ng (~ 50 nM) protein in a buffer containing 20 mM Tris-Cl (pH 7.5), 450 mM NaCl, 0.75 mM DTT, 1.5 mM MgCl₂, 0.1 mM EDTA, and 10% glycerol at 68°C for 1 h, unless specified otherwise. NaCl concentration was reduced to 300 mM in typical reactions with Pfu-Pus10. For kinetics experiments, a total 180 μL reaction was set up. Samples

of 20 μL were withdrawn at different time points and processed as above. For salt titration experiments, varying amounts of NaCl (150 to 900 mM) were used in pseudouridylation assays. Both *M. jannaschii* and *P. furiosus* are marine organisms. Therefore, salt is required for the activity of their proteins. RNA was purified by phenol/chloroform extraction and ethanol precipitation and digested with RNase T2 or nuclease P1. RNase T2 digests were resolved by two-dimensional TLC either on Merck cellulose plates using solvent I (isobutyric acid/0.5 N NH₄OH, 5:3, v/v) for the first dimension and solvent II (isopropanol/HCl/H₂O, 70:15:15, v/v/v) for the second, or on Macherey-Nagel Cel 300 plates using solvent I for the first dimension and solvent III (0.1 M sodium phosphate at pH 6.8/ammonium sulfate/n-propanol, 100:60:2, v/w/v) for the second (Gupta 1984). Nuclease P1 digest was resolved by one-dimensional TLC either on Merck cellulose or on Macherey-Nagel PEI-cellulose plates using solvent II. Nuclease P1 digestion was done only for [α -³²P] UTP-labeled substrates. Radioactivity in the TLC spots was revealed and quantitated by phosphorimaging. All assays were repeated two to three times. The amount of Ψ produced in RNase T2 digests was determined as (radioactivity in Ψ p spot $\times$ number of U's in the RNA preceding the labeled nucleotide)/(sum of the radioactivity in Up and Ψ p spots). The amount of Ψ produced in nuclease P1 digests of [α -³²P] UTP-labeled RNAs was determined as (radioactivity in Ψ p spot $\times$ number of U's in the RNA)/(sum of the radioactivity in pU and p Ψ spots).

Mapping the sites of pseudouridylation

Pseudouridine formation in in vitro modified tRNAs was analyzed by the CMCT modification technique (Motorin et al. 2007). Varying amounts (2–20 pmol) of in vitro modified tRNA were treated with CMCT for different time points (2, 10, and 20 min) while an untreated sample was used as control. Alkali treatment was done for 3 h for all samples except for the ones treated with CMCT for 2 min. This was followed by primer extension using a 5'-labeled primer (5'-TGGTGGGGCCGGAG) specific for the 3' end of tRNA^{Trp}. The extension stops one base before the CMCT modified base under these conditions. Sequence ladders were generated using the same primer and pVT9P11 Δ i (Gurha et al. 2007) DNA as template.

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REFERENCES

- Aza-Blanc, P., Cooper, C.L., Wagner, K., Batalov, S., Deveraux, Q.L., and Cooke, M.P. 2003. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol. Cell* 12: 627–637.
- Becker, H.F., Motorin, Y., Planta, R.J., and Grosjean, H. 1997a. The yeast gene YNL292w encodes a pseudouridine synthase (Pus4)

- catalyzing the formation of Ψ 55 in both mitochondrial and cytoplasmic tRNAs. *Nucleic Acids Res.* **25**: 4493–4499.
- Becker, H.F., Motorin, Y., Sissler, M., Florentz, C., and Grosjean, H. 1997b. Major identity determinants for enzymatic formation of ribothymidine and pseudouridine in the T Ψ -loop of yeast tRNAs. *J. Mol. Biol.* **274**: 505–518.
- Behm-Ansmant, I., Massenet, S., Immel, F., Patton, J.R., Motorin, Y., and Branlant, C. 2006. A previously unidentified activity of yeast and mouse RNA:pseudouridine synthases 1 (Pus1p) on tRNAs. *RNA* **12**: 1583–1593.
- Chaudhuri, B.N., Chan, S., Perry, L.J., and Yeates, T.O. 2004. Crystal structure of the apo forms of Ψ 55 tRNA pseudouridine synthase from *Mycobacterium tuberculosis*: A hinge at the base of the catalytic cleft. *J. Biol. Chem.* **279**: 24585–24591.
- Constantinesco, F., Motorin, Y., and Grosjean, H. 1999. Transfer RNA modification enzymes from *Pyrococcus furiosus*: Detection of the enzymatic activities in vitro. *Nucleic Acids Res.* **27**: 1308–1315.
- Decatur, W.A. and Fournier, M.J. 2003. RNA-guided nucleotide modification of ribosomal and other RNAs. *J. Biol. Chem.* **278**: 695–698.
- Delk, A.S., Nagle Jr., D.P., and Rabinowitz, J.C. 1980. Methylenetetrahydrofolate-dependent biosynthesis of ribothymidine in transfer RNA of *Streptococcus faecalis*. Evidence for reduction of the 1-carbon unit by FADH₂. *J. Biol. Chem.* **255**: 4387–4390.
- Dennis, P.P. and Omer, A. 2005. Small noncoding RNAs in Archaea. *Curr. Opin. Microbiol.* **8**: 685–694.
- Edmonds, C.G., Crain, P.F., Gupta, R., Hashizume, T., Hocart, C.H., Kowalak, J.A., Pomerantz, S.C., Stetter, K.O., and McCloskey, J.A. 1991. Post-transcriptional modification of tRNA in thermophilic archaea (Archaeobacteria). *J. Bacteriol.* **173**: 3138–3148.
- Filipowicz, W. and Pogacic, V. 2002. Biogenesis of small nucleolar ribonucleoproteins. *Curr. Opin. Cell Biol.* **14**: 319–327.
- Greenberg, R. and Dudock, B. 1980. Isolation and characterization of m5U-methyltransferase from *Escherichia coli*. *J. Biol. Chem.* **255**: 8296–8302.
- Grosjean, H., Edqvist, J., Straby, K.B., and Giege, R. 1996. Enzymatic formation of modified nucleosides in tRNA: Dependence on tRNA architecture. *J. Mol. Biol.* **255**: 67–85.
- Grosjean, H., Gupta, R., and Maxwell, E.S. 2008. Modified nucleosides in archaeal RNAs. In *Archaea: New models for prokaryotic biology* (ed. P. Blum), pp. 171–196. Caister Academic Press, Norfolk, UK.
- Gu, X., Yu, M., Ivanetich, K.M., and Santi, D.V. 1998. Molecular recognition of tRNA by tRNA pseudouridine 55 synthase. *Biochemistry* **37**: 339–343.
- Gupta, R. 1984. *Halobacterium volcanii* tRNAs. Identification of 41 tRNAs covering all amino acids, and the sequences of 33 class I tRNAs. *J. Biol. Chem.* **259**: 9461–9471.
- Gupta, R. 1986. Transfer RNAs of *Halobacterium volcanii*: Sequences of five leucine and three serine tRNAs. *Syst. Appl. Microbiol.* **7**: 102–105.
- Gurha, P., Joardar, A., Chaurasia, P., and Gupta, R. 2007. Differential roles of archaeal box H/ACA proteins in guide RNA-dependent and independent pseudouridine formation. *RNA Biol.* **4**: 101–109.
- Hamma, T. and Ferré-D'Amaré, A.R. 2006. Pseudouridine synthases. *Chem. Biol.* **13**: 1125–1135.
- Henras, A.K., Dez, C., and Henry, Y. 2004. RNA structure and function in C/D and H/ACA s(no)RNPs. *Curr. Opin. Struct. Biol.* **14**: 335–343.
- Kiss, T. 2002. Small nucleolar RNAs: An abundant group of non-coding RNAs with diverse cellular functions. *Cell* **109**: 145–148.
- Kowalak, J.A., Dalluge, J.J., McCloskey, J.A., and Stetter, K.O. 1994. The role of posttranscriptional modification in stabilization of transfer RNA from hyperthermophiles. *Biochemistry* **33**: 7869–7876.
- McCleverty, C.J., Hornsby, M., Spraggon, G., and Kreusch, A. 2007. Crystal structure of human Pus10, a novel pseudouridine synthase. *J. Mol. Biol.* **373**: 1243–1254.
- Meier, U.T. 2005. The many facets of H/ACA ribonucleoproteins. *Chromosoma* **114**: 1–14.
- Motorin, Y., Keith, G., Simon, C., Foiret, D., Simos, G., Hurt, E., and Grosjean, H. 1998. The yeast tRNA:pseudouridine synthase Pus1p displays a multisite substrate specificity. *RNA* **4**: 856–869.
- Motorin, Y., Muller, S., Behm-Ansmant, I., and Branlant, C. 2007. Identification of modified residues in RNAs by reverse transcription-based methods. *Methods Enzymol.* **425**: 21–53.
- Muller, S., Fourmann, J.B., Loegler, C., Charpentier, B., and Branlant, C. 2007. Identification of determinants in the protein partners aCBF5 and aNOP10 necessary for the tRNA: Ψ 55-synthase and RNA-guided RNA: Ψ -synthase activities. *Nucleic Acids Res.* **35**: 5610–5624.
- Muller, S., Leclerc, F., Behm-Ansmant, I., Fourmann, J.B., Charpentier, B., and Branlant, C. 2008. Combined in silico and experimental identification of the *Pyrococcus abyssi* H/ACA sRNAs and their target sites in ribosomal RNAs. *Nucleic Acids Res.* **36**: 2459–2475.
- Nishikura, K. and De Robertis, E.M. 1981. RNA processing in microinjected *Xenopus* oocytes. Sequential addition of base modifications in the spliced transfer RNA. *J. Mol. Biol.* **145**: 405–420.
- Nordlund, M.E., Johansson, J.O., von Pawel-Rammingen, U., and Bystrom, A.S. 2000. Identification of the TRM2 gene encoding the tRNA(m⁷U₅₄)methyltransferase of *Saccharomyces cerevisiae*. *RNA* **6**: 844–860.
- Nurse, K., Wrzesinski, J., Bakin, A., Lane, B.G., and Ofengand, J. 1995. Purification, cloning, and properties of the tRNA Ψ 55 synthase from *Escherichia coli*. *RNA* **1**: 102–112.
- Ny, T. and Bjork, G.R. 1980. Cloning and restriction mapping of the trmA gene coding for transfer ribonucleic acid (5-methyluridine)-methyltransferase in *Escherichia coli* K-12. *J. Bacteriol.* **142**: 371–379.
- Pan, H., Agarwalla, S., Moustakas, D.T., Finer-Moore, J., and Stroud, R.M. 2003. Structure of tRNA pseudouridine synthase TruB and its RNA complex: RNA recognition through a combination of rigid docking and induced fit. *Proc. Natl. Acad. Sci.* **100**: 12648–12653.
- Phannachet, K. and Huang, R.H. 2004. Conformational change of pseudouridine 55 synthase upon its association with RNA substrate. *Nucleic Acids Res.* **32**: 1422–1429.
- Reichow, S.L., Hamma, T., Ferré-D'Amaré, A.R., and Varani, G. 2007. The structure and function of small nucleolar ribonucleoproteins. *Nucleic Acids Res.* **35**: 1452–1464.
- Roovers, M., Hale, C., Tricot, C., Terns, M.P., Terns, R.M., Grosjean, H., and Droogmans, L. 2006. Formation of the conserved pseudouridine at position 55 in archaeal tRNA. *Nucleic Acids Res.* **34**: 4293–4301.
- Rozenski, J., Crain, P.F., and McCloskey, J.A. 1999. The RNA Modification Database: 1999 update. *Nucleic Acids Res.* **27**: 196–197.
- Sprinzel, M. and Vassilenko, K.S. 2005. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.* **33**: D139–D140.
- Urbonavicius, J., Skouloubris, S., Myllykallio, H., and Grosjean, H. 2005. Identification of a novel gene encoding a flavin-dependent tRNA:m5U methyltransferase in bacteria—evolutionary implications. *Nucleic Acids Res.* **33**: 3955–3964.
- Urbonavicius, J., Auxilien, S., Walbott, H., Trachana, K., Golinelli-Pimpaneau, B., Brochier-Armanet, C., and Grosjean, H. 2008. Acquisition of a bacterial Ruma-type tRNA(uracil-54, C5)-methyltransferase by Archaea through an ancient horizontal gene transfer. *Mol. Microbiol.* **67**: 323–335.
- Watanabe, Y. and Gray, M.W. 2000. Evolutionary appearance of genes encoding proteins associated with box H/ACA snoRNAs: cbf5p in *Euglena gracilis*, an early diverging eukaryote, and candidate Gar1p and Nop10p homologs in archaeobacteria. *Nucleic Acids Res.* **28**: 2342–2352.
- Yu, Y.T., Terns, R.M., and Terns, M.P. 2005. Mechanisms and functions of RNA-guided RNA modification. In *Fine-tuning of RNA functions by modification and editing* (ed. H. Grosjean), pp. 223–262. Springer, New York.

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