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Formation of the conserved pseudouridine at position 55 in archaeal tRNA

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ABSTRACT

Pseudouridine (Ψ) located at position 55 in tRNA is a nearly universally conserved RNA modification found in all three domains of life. This modification is catalyzed by TruB in bacteria and by Pus4 in eukaryotes, but so far the Ψ55 synthase has not been identified in archaea. In this work, we report the ability of two distinct pseudouridine synthases from the hyperthermophilic archaeon *Pyrococcus furiosus* to specifically modify U55 in tRNA *in vitro*. These enzymes are $_{pfu}$Cbf5, a protein known to play a role in RNA-guided modification of rRNA, and $_{pfu}$PsuX, a previously uncharacterized enzyme that is not a member of the TruB/Pus4/Cbf5 family of pseudouridine synthases. $_{pfu}$PsuX is hereafter renamed $_{pfu}$Pus10. Both enzymes specifically modify tRNA U55 *in vitro* but exhibit differences in substrate recognition. In addition, we find that in a heterologous *in vivo* system, $_{pfu}$Pus10 efficiently complements an *Escherichia coli* strain deficient in the bacterial Ψ55 synthase TruB. These results indicate that it is probable that $_{pfu}$Cbf5 or $_{pfu}$Pus10 (or both) is responsible for the introduction of pseudouridine at U55 in tRNAs in archaea. While we cannot unequivocally assign the function from our results, both possibilities represent unexpected functions of these proteins as discussed herein.

INTRODUCTION

Pseudouridine (Ψ) is the most abundant modified nucleoside in RNA. It has been identified in tRNAs, rRNAs, snRNAs and/or snoRNAs of all organisms that have been analyzed to date (1–4). Ψ is formed post-transcriptionally by enzymatic isomerization of uridine by a class of enzymes known as pseudouridine synthases. Most Ψ synthases have the ability to recognize and catalyze isomerization of one (or a few) unique target uridines (5).

For many years, there were thought to be only four families of Ψ synthases, corresponding to TruA, TruB, RluA and RsuA in bacteria (6). Members of these families were found in all three domains of life. Recently however, Ψ synthases that share little sequence homology to any of these families (such as *Escherichia coli* TruD) have been found, leading to the recognition of additional groups of Ψ synthases, as well as the idea that there may be many more Ψ synthases that are yet to be discovered (7).

Ψ55 in tRNA is a quasi-universally conserved modification found in all forms of life analyzed to date. The Ψ synthases responsible for this very highly conserved modification have been identified in eukaryotes and bacteria, and are members of the TruB/Pus4/Cbf5 family [Cluster of Orthologous Groups of proteins (COG) 0130 (8)]. In the yeast *Saccharomyces cerevisiae*, Pus4 was shown to be the enzyme responsible for this modification (9), and in *E.coli*, TruB is the enzyme responsible for formation of Ψ55 (10). Although Ψ55 is known to exist in archaeal tRNAs and the activity has been identified in *Pyrococcus furiosus* cell extract (11), no archaeal Ψ55 synthase has been identified to date. The goal of the present work was to identify the enzyme responsible for isomerization of U55 to Ψ55 in *P.furiosus*, our model archaeal system. We have tested two candidate Ψ synthases, $_{pfu}$Cbf5 and $_{pfu}$Pus10 (previously known as PsuX).

The only identified archaeal protein within the TruB/Pus4/Cbf5 family was recently shown to function in RNA-guided pseudouridylation of rRNA (12,13). While most Ψ synthases are dedicated to modification of a single target uridine, in

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eukaryotes and archaea members of the Cbf5 sub-family modify multiple specific sites by a mechanism that depends on guide RNAs and a set of essential accessory proteins (14). Thus in most eukaryotes, the paralogous proteins Pus4 and Cbf5 function in tRNA U55 and rRNA pseudouridylation, respectively. The functional complex is comprised of Cbf5 (the enzyme) plus Nop10, Gar1 and L7Ae (essential accessory proteins) and an H/ACA guide RNA (12,13). The guide RNA mediates substrate recognition by basepairing with nucleotides surrounding the target uridine. Multiple guide RNAs provide for pseudouridylation of numerous individual rRNA sites. Once recruited to the substrate rRNA by the guide RNA, the complex catalyzes formation of Ψ at the targeted uridine residue (12). There is no existing evidence that Cbf5 functions in the absence of a guide RNA and accessory proteins, or that Cbf5 is involved in pseudouridylation of tRNA.

The second candidate Ψ55 synthase that we tested has been called PsuX (15). The *psuX* gene (hereafter the *pus10* gene) was identified by sequence similarity to an internal region (catalytic domain; residues G66–A271) of the *Euglena gracilis* Cbf5 protein (15). Pus10 (PF1139) belongs to a different Ψ synthase family than TruB, Pus4, and Cbf5 [COG 1258 for Pus10, and COG 0130 for the three other Ψ synthases (8)] and its function and specificity are unknown.

In this work, the activities of recombinantly expressed and purified $_{pfu}$Cbf5 and $_{pfu}$Pus10 enzymes were characterized and compared in *in vitro* assays. The results indicate that both enzymes are capable of selective modification of tRNA U55 *in vitro* under similar reaction conditions. We found that $_{pfu}$Pus10 can pseudouridylate a truncated tRNA substrate (miniS) and a tRNA lacking the 3′CCA, but that $_{pfu}$Cbf5 functions only on the full-length tRNA substrate including the 3′CCA end. Moreover, $_{pfu}$Pus10 efficiently complements tRNA Ψ55 formation in an *E.coli truB* knockout strain. These results suggest two novel and interesting candidates for the pseudouridylation of tRNA U55 in archaea.

MATERIALS AND METHODS

Strains and plasmids

To construct the ML100 strain (BL21(DE3)*truB*), the *truB*2422::mini-Tn*10*Cm allele from strain GOB113 (16) was transferred by P1 transduction into strain BL21(DE3). Transductants were selected on LB medium supplemented with chloramphenicol (30 μg/ml). The GOB113 strain (Hfr P4X *sdr*+ *truB*2422::mini-Tn*10*Cm) carrying a mutation in the *truB* gene making this strain defective for TruB activity was kindly provided by Michael Wikström (University of Umeå, Sweden). The construction of the pET21d plasmid allowing T7-expression in *E.coli* of the *P.furiosus* Cbf5 protein ($_{pfu}$Cbf5; ORF = PF1785) and purification of the recombinant protein was described previously (12).

The plasmid pML156 allowing *in vitro* transcription of *P.furiosus* tRNA^{Asp} by T7 RNA polymerase is a pUC18 derivate with the fragment containing the *P.furiosus* tRNA^{Asp} gene downstream of a T7 promoter cloned in its BamHI/HindIII sites. This plasmid was kindly provided by Sylvie Auxilien (CNRS, Gif-sur-Yvette, France). The corresponding *P.furiosus* tRNA^{Asp}(U55C) mutant was obtained with the QuickChange site-directed mutagenesis kit (Stratagene) using pML156 as a template. The oligonucleotides used were 5′-CGACCCGGGTCCAAATCCCGG-3′ and 5′-CCGGGATTTGGACCCGGGTCG-3′. The sequence of the mutant was verified.

Cloning the *P.furiosus pus10* gene, expression and purification of the corresponding $_{pfu}$Pus10 protein

The gene encoding *P.furiosus* Pus10 (ORF = PF1139), was amplified by PCR from genomic DNA and cloned into the NdeI/XhoI sites of pET28b. The primers used in this PCR were 5′-GTCATATGATACTTGAAAAAGCCA GAG-3′ and 5′-GTCTCGAGTCAATTATCTCC CTCAACATCG-3′. The resulting plasmid (pET28b-*pus10*) allowed T7-expression in *E.coli* [strain Rosetta(DE3)] of the *P.furiosus* Pus10 protein ($_{pfu}$Pus10) bearing an N-terminal His-tag. The transformed cells were grown to an optical density at 660 nm of 0.5, and the expression of the protein was induced with 1 mM isopropylthio-β-D-galactoside (IPTG) for 3 h at 37°C. Cells were then harvested and resuspended in buffer A (20 mM Na phosphate pH 7.0, 1 M NaCl) and lyzed by a 30 min sonication at 4°C using a Vibracell 75 041 sonicator (40% amplitude). The lysate was cleared by centrifugation (20 000 *g* for 30 min) and was applied to a column of Chelating Sepharose Fast Flow (1 × 30 cm; Amersham Biosciences) charged with Ni^{2+}. The column was washed with buffer A and adsorbed material was eluted with a linear gradient (from 0 to 1 M) of imidazole in buffer A. Eluted fractions were analyzed by SDS–PAGE. Fractions containing the $_{pfu}$Pus10 protein were pooled, concentrated to 3 mg/ml in buffer containing 1 M NaCl and 200 mM imidazole and kept at −80°C until used.

Preparation of radiolabeled tRNA substrates

The general procedure for generating (^{32}P) radiolabeled tRNA transcripts as substrates for enzymatic reactions is based on the method described previously (17). Radioactive (^{32}P) *in vitro* transcripts were prepared as described elsewhere (18) using MvaI (for generating tRNA^{Asp} substrate) or NarI (for generating tRNA^{Asp}-3′CA substrate) digested plasmids as templates. [α-^{32}P]UTP, [α-^{32}P]CTP, [α-^{32}P]ATP and [α-^{32}P]GTP (400 Ci/mmol) were from ICN Biomedicals and T7 RNA polymerase was from Roche Diagnostics. Radioactive transcripts were purified by 10% polyacrylamide gel electrophoresis. The radioactive band corresponding to full-length transcript was eluted from the gel with water and finally precipitated by ethanol in the presence of 0.3 M Na acetate pH 5. The *P.furiosus* tRNA^{Asp} minisubstrate (TΨ-loop & stem—see Figure 2A) was prepared by *in vitro* transcription of a single stranded DNA template (minus stand), following the procedure described previously (19).

tRNA pseudouridine synthase assay

Purified recombinant $_{pfu}$Cbf5 (5 μg or 2 μg) or $_{pfu}$Pus10 (5 μg or 0.5 μg) was incubated in a 400 μl reaction mixture with the *in vitro* transcribed (^{32}P)-labeled tRNA for 1 h at 0°C in buffer B (20 mM HEPES pH 7.0, 500 mM KCl and 1.5 mM $MgCl_2$). The reaction was terminated by the addition of an equal volume of phenol and the RNA recovered by ethanol precipitation. The modified tRNA was then treated

and analyzed for its Ψ content as described previously (20). In brief, each RNA sample was hydrolyzed by nuclease P1 or RNase T2 and the resulting nucleotides were analyzed by 1D-(20 cm) or 2D-(10 cm × 10 cm) thin layer chromatography (TLC) on cellulose plates (Merck). First dimension was developed with solvent A (isobutyric acid/concentrated NH_4OH/water; 66/1/33; v/v/v); the second dimension was developed with solvent B (concentrated HCl/isopropanol/water; 18/68/14; v/v/v). When only one dimension was performed as in Figure 3, to reduce background, the incubated tRNA was digested with RNase T1, and the fragments were separated on a 20% polyacrylamide gel and the fragment corresponding to the TΨ-loop was extracted from the gel, eluted, ethanol precipitated and then subjected to nuclease P1 digestion and TLC, as outlined above, except using an acidic solvent for TLC (concentrated HCl/isopropanol/water; 15/70/15; v/v/v).

rRNA pseudouridine synthase assay

Recombinant $_{pfu}$Cbf5 (2 μg) was incubated with 0.75 pmol unlabeled guide (Pf9) sRNA and 0.05 pmol [α-^{32}P]UTP-labeled-target rRNA as described previously (12). For experiments with accessory proteins present, the additional proteins were added at stoichiometric amounts equivalent to 2 μg $_{pfu}$Cbf5. Incubation was at 70°C for 1 h. Resulting RNA was phenol extracted, digested with nuclease P1 for 1 h at 37°C and run on a 1D (20 cm) TLC plate in acidic solvent (concentrated HCl/isopropanol/water; 15/70/15; v/v/v). The results were analyzed by autoradiography.

CMC/RT assay for mapping pseudouridine residues

Total tRNA was isolated from *E.coli* cells that were either BL21(DE3), ML100, or ML100 transformed with the pET plasmids containing respectively the *cbf5* or *pus10* genes. cells (100 ml) were grown to OD 0.4, then protein expression was induced with 0.05 mM IPTG for 3 h at 37°C. Cells were then rapidly transferred from 37 to 50°C and incubated for an additional 2 h. tRNA was isolated according to (21). CMC modification was performed on 2 μg of total tRNA as described previously (22). The resulting modified tRNA samples were then subjected to primer extension using 5′ end labeled primers. Primer for $_{eco}$tRNA^{Cys} was 5′-TGGAGGCGCGTCCGG-3′. Primer for $_{eco}$tRNA^{Phe} was 5′-TGGTGCCCGGACTCGG-3′. Primer (0.5 pmol) was annealed to tRNA in 50 mM Tris–HCl pH 8.6, 60 mM NaCl and 10 mM DTT for 3 min at 70°C, 5 min at 37°C and at least 2 min at 0°C. Primer extension was performed using 2 μl of the above annealing reaction in a 5 μl reaction mixture containing 50 mM Tris–HCl pH 8.6, 60 mM NaCl, 10 mM DTT and 2.4 mM $MgCl_2$, 330 μM dNTPs and 20 U reverse transcriptase (Promega). Reaction was carried out for 30 min at 37°C. For sequencing reactions, 10 μg of unmodified wild type total tRNA was used in the same annealing reaction conditions as before, except using 1.5 pmol of labeled primer. Extension was performed in the same buffer as above, except with the addition of 1 mM individual ddNTPs in four different reactions. The resulting fragments were separated on a 15% sequencing acrylamide gel (7 M urea). Results were analyzed by autoradiography.

RESULTS

Both $_{pfu}$Cbf5 and $_{pfu}$Pus10 can form Ψ55 in tRNA *in vitro*

As described above, bioinformatic searches yielded two candidate enzymes for U55 modification in archaea. $_{pfu}$Cbf5 is a member of the TruB/Pus4/Cbf5 family of enzymes that are responsible for Ψ55 modification in bacteria and eukaryotes. $_{pfu}$Pus10 (previously PsuX) is a member of another family of pseudouridine synthases with unknown specificity. To test whether these enzymes are tRNA Ψ55 synthases, we cloned the *P.furiosus cbf5* and *pus10* genes, expressed and purified the $_{pfu}$Cbf5 and $_{pfu}$Pus10 proteins and assayed their activity. Both enzymes catalyzed the formation of Ψ *in vitro* (Figure 1). More extensive tests were then performed to further analyze the activity of $_{pfu}$Cbf5 and $_{pfu}$Pus10. Both enzymes were found to work under the same reaction conditions, with the optimal activity observed at 1 h at 70°C (Figure 1C–F). $_{pfu}$Pus10 efficiently produced 1 mol of Ψ for each mol of tRNA in the reaction, suggesting complete modification of a single site in the tRNA. The maximum extent of modification observed with $_{pfu}$Cbf5 in this assay was ∼0.6 mol Ψ per mole of substrate RNA (Figure 1A, C and E). This limitation could reflect the sensitivity of Cbf5 to alterations in the substrate RNA or the requirement of Cbf5 for accessory proteins (see below).

We determined the sites of tRNA modification for each enzyme by performing a combination of nearest neighbor analysis and mutational analysis. Wild type *in vitro* transcribed $_{pfu}$tRNA^{Asp} was (^{32}P)-radiolabeled with one of the four nucleotides. The RNA was incubated with the protein under the optimal reaction conditions (Figure 1). The modified tRNA was isolated and digested with either RNase T2 or nuclease P1 as indicated (Figure 2C and see Figure 2B). The resulting mononucleotides were analyzed by two-dimensional thin layer chromatography (2D-TLC) followed by autoradiography. For both $_{pfu}$Cbf5 and $_{pfu}$Pus10, Ψ is only detected when tRNA is either UTP-labeled and digested with nuclease P1, or CTP-labeled and digested with RNase T2 (Figure 2C, panels 1–4 and 9–12 and data not shown). These results indicate that the modified uridine is 5′-adjacent to a cytidine. This limits the modified uridine to positions 22, 35, 39, 55 and/or 60 [numbered according to yeast tRNA^{Phe} (4)]. To test whether the modified uridine is formed at position 55, a U55C mutant substrate was constructed in which U55 was replaced with C55, which cannot be modified. For $_{pfu}$Cbf5 (Figure 2C, panels 5–6), this mutation completely abolished activity. For $_{pfu}$Pus10 (Figure 2C, panels 13–14), this mutation also dramatically reduced the amount of Ψ produced. The small amount of Ψ seen with $_{pfu}$Pus10 may be owing to inefficient modification of other sites on the tRNA under the *in vitro* reaction conditions. These results clearly indicate that both $_{pfu}$Cbf5 and $_{pfu}$Pus10 can specifically modify U55 to Ψ55 *in vitro*.

Previously, we identified U55 modification activity in a crude *P.furiosus* cell extract (11). This extract was active on both a wild-type tRNA substrate and a truncated mini-substrate (miniS) that contains only the TΨ-arm and the acceptor stem (outlined in Figure 2A) (11). To test the ability of the two candidate proteins to act on this substrate, [α-^{32}P]UTP-radiolabeled transcripts of the miniS were incubated with the $_{pfu}$Cbf5 or $_{pfu}$Pus10 proteins, and analyzed as described

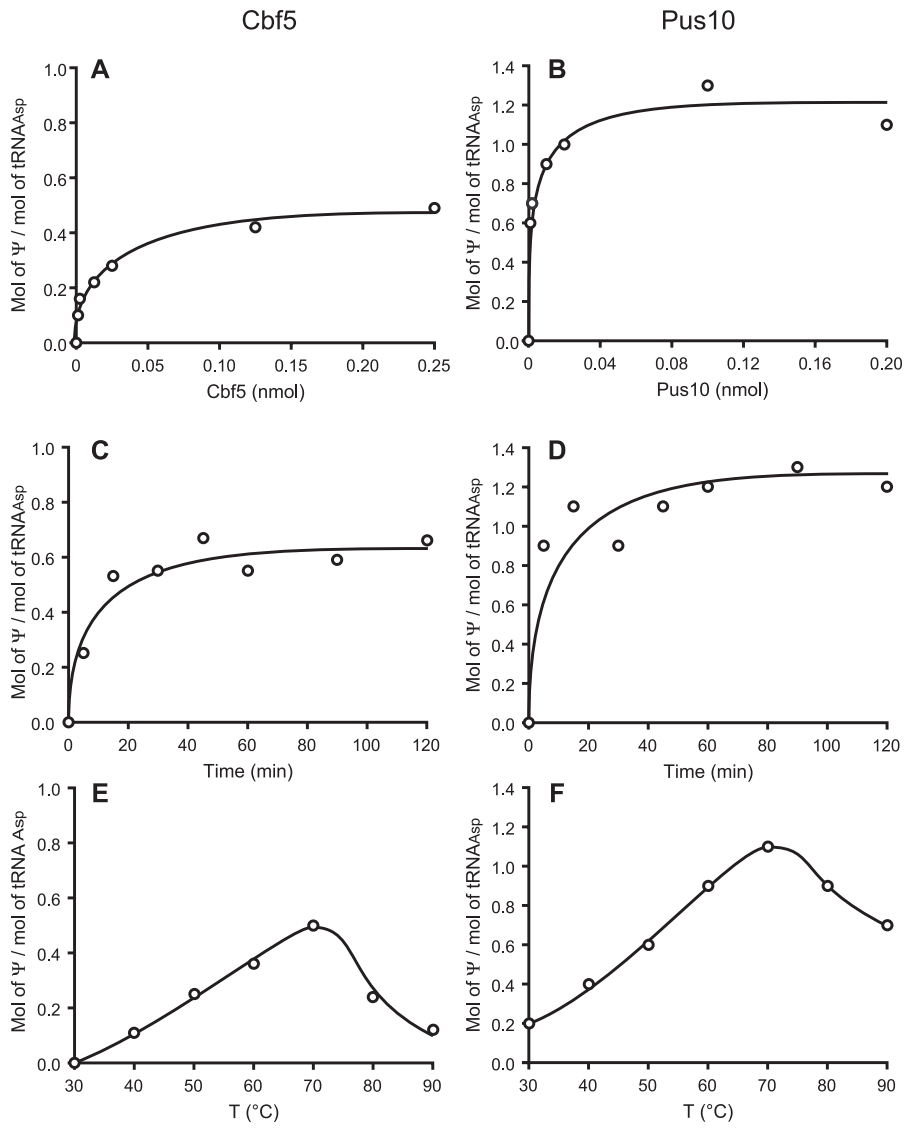

Figure 1. Ψ formation by $_{pfu}$Cbf5 and $_{pfu}$Pus10 in $_{pfu}$tRNA^{Asp}. (**A** and **B**) Increasing molar amounts of $_{pfu}$Cbf5 and $_{pfu}$Pus10 were incubated at 70°C for 60 min. (**C** and **D**) 0.12 nmol (5 μg) of $_{pfu}$Cbf5 and 0.10 nmol (5 μg) of $_{pfu}$Pus10 were incubated with T7-transcribed $_{pfu}$tRNA^{Asp} for increasing time intervals at 70°C. (**E** and **F**) 0.12 nmol (5 μg) of $_{pfu}$Cbf5 and 0.10 nmol (5 μg) of $_{pfu}$Pus10 were incubated at increasing temperatures for 60 min. After incubation, the tRNA was recovered, hydrolyzed by nuclease P1 and the resulting nucleotides were separated by 2D-TLC chromatography (see Materials and Methods). Mol of Ψ produced per mol of tRNA was measured after counting the radioactivity in the spots corresponding to UMP and ΨMP on the chromatogram and taking into account the nucleotide composition of the tRNA substrate. Precision is estimated to be ∼10%. The results shown are representative of two similar experiments.

above. As observed in Figure 2C panel 7, $_{pfu}$Cbf5 was unable to modify any uridine residue in the mini-substrate. In contrast, $_{pfu}$Pus10 was active on the mini-substrate (Figure 2C, panel 15).

The binding of $_{pfu}$Cbf5 to H/ACA guide RNAs (for rRNA modification) was previously found to depend on the presence of an intact ACA sequence near the 3′ terminus of the guide RNA (box ACA) and mutations in this element prevented Cbf5 activity toward rRNA substrate (12,13). tRNAs contain a CCA sequence at the 3′ end. To determine whether the 3′CCA plays an important role in recognition of tRNA by $_{pfu}$Cbf5, we generated a tRNA that lacked the terminal CA (see dashed outline in Figure 2A). As shown in Figure 2C (panels 8 and 16), $_{pfu}$Cbf5 did not modify the tRNA −3′CA mutant. At the same time, the −3′CA mutation did not affect the ability of $_{pfu}$Pus10 to modify the substrate tRNA.

Ψ55 formation by $_{pfu}$Cbf5 in the absence and presence of accessory proteins

While the known tRNA pseudouridylations are introduced by dedicated, single-subunit enzymes, we know that in order to function with guide RNAs to modify rRNA in archaea and eukaryotes, Cbf5 requires accessory proteins: Gar1, L7Ae and Nop10 (12,13). In order to determine whether the accessory proteins could also be important in tRNA U55 modification, pseudouridylation assays were performed in the absence and presence of these proteins.

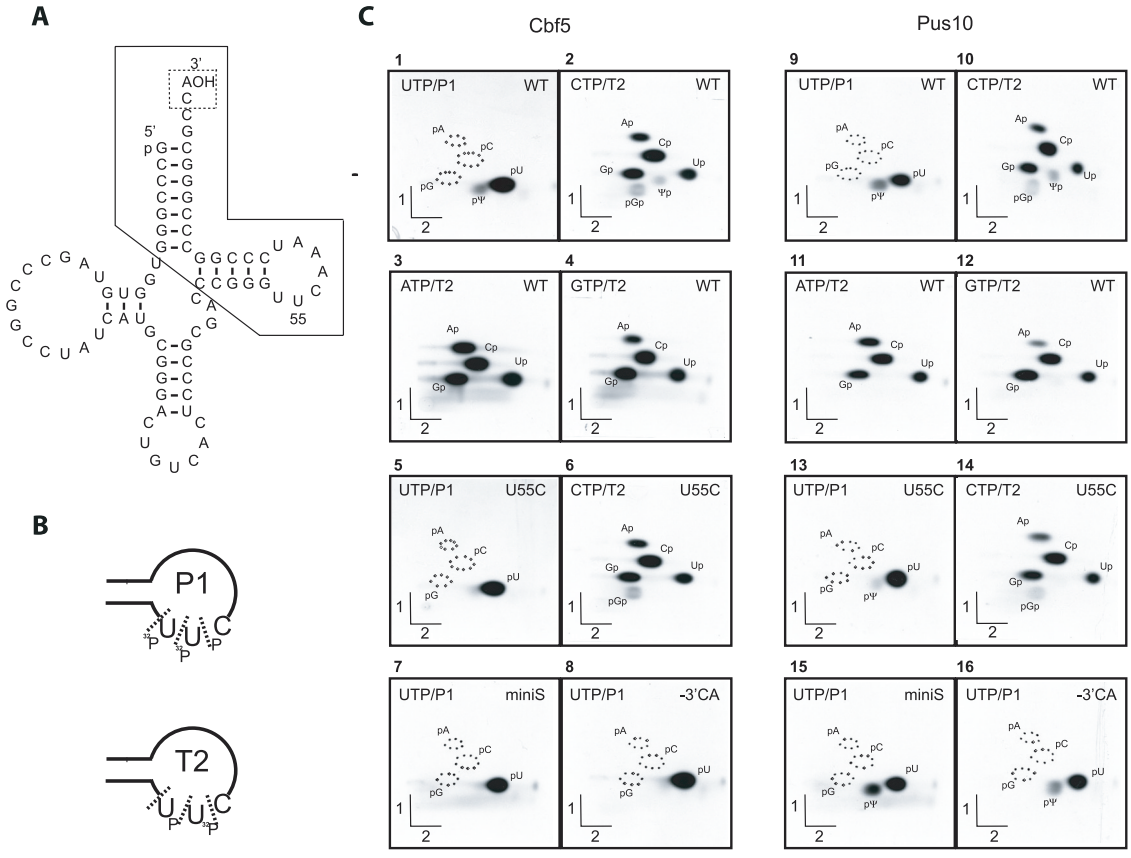

Figure 2. Analysis of $_{pfu}$Cbf5 and $_{pfu}$Pus10 modification of various tRNA substrates. (**A**) Cloverleaf structure of tRNA^{Asp} of *P.furiosus* used as the wild-type tRNA substrate in this work. The universal numbering system for nucleotides in tRNA corresponds to that of (4). U55 is indicated. C75 and A76 (missing in −3′CA substrates) are indicated by a dashed box. The portion of the tRNA sequence included in the miniS substrate is indicated by the plain box. (**B**) The expected patterns of nuclease P1 and RNase T2 cleavage in the U55 region of an [α-^{32}P]UTP- and [α-^{32}P]CTP-labeled tRNA, respectively. Nuclease P1 generates 5′-phosphate-nucleosides while RNase T2 generates 3′phosphate-nucleosides. (**C**) 2D-TLC analysis of $_{pfu}$Cbf5 and $_{pfu}$Pus10 modification of various tRNA substrates. WT indicates wild-type $_{pfu}$tRNA^{Asp} (panels 1–4, 9–12); U55C indicates U55 replacement mutant (panels 5,6,13,14); miniS indicates mini-substrate (panels 7,15) and −3′CA indicates 3′ terminal CA deletion (panels 8,16). See text for more details. UTP/CTP/ATP and GTP refer to the (^{32}P)-labeled nucleotide incorporated at transcription. Incubation was for 1 h at 70°C in the presence of 0.12 nmol (5 μg) of $_{pfu}$Cbf5 (panels 1–8) and 0.01 nmol (0.5 μg) of $_{pfu}$Pus10 (panels 9–16). After incubation, the RNA was digested by nuclease P1 or RNase T2 (as indicated in each panel) and the resulting nucleotides were analyzed by 2D-TLC on cellulose plates and autoradiography. Circles in dotted lines show the migration of the canonical nucleotides used as UV markers.

As has been shown previously (12) and is demonstrated in Figure 3 (right panel, rRNA), in rRNA $_{pfu}$Cbf5 converts a radiolabeled uridine (targeted by a specific guide RNA) to Ψ only in the presence of the three accessory proteins (C+ lane).

Uniformly [α-^{32}P]UTP-radiolabeled archaeal $_{pfu}$tRNA^{Asp} was also incubated with $_{pfu}$Cbf5 in the absence (C) and presence (C+) of stoichiometric amounts of the accessory proteins (Figure 3, left panel). After incubation, the tRNA was isolated and digested with RNase T1, and the TΨ-arm fragment was obtained by gel purification. The TΨ-arm fragment consists of nucleotides 54–65 and contains three uridines. The isolated fragment was digested with nuclease P1 and the mononucleotides were separated by single dimension TLC. As shown in Figure 3 (left panel), $_{pfu}$Cbf5 produced ∼0.6 mol of Ψ per mol of tRNA in the absence of the accessory proteins, and ∼0.9 mol of Ψ per mol of tRNA in the presence of the other proteins. The same extent of enhancement (∼50% increase) was observed in two experiments.

In order to determine which of the three uridines within the TΨ arm of the tRNA was modified in this assay, a mutant $_{pfu}$tRNA^{Asp} in which U55 was replaced by C55 was tested (Figure 3, middle panel, tRNAU55C). No pseudouridylation was observed in the tRNA lacking U55, indicating that the pseudouridylation activity observed is directed specifically at uridine 55.

Our findings indicate that $_{pfu}$Cbf5 can modify tRNA U55, but not rRNA, in the absence of Gar1, Nop10 and L7Ae. However, the activity of $_{pfu}$Cbf5 toward tRNA U55 is enhanced in the presence of the accessory proteins, suggesting that a multi-protein Cbf5 complex may function in Ψ55 synthesis in *P.furiosus*.

$_{pfu}$Pus10 can efficiently form Ψ55 in tRNA in *E.coli*

Our results indicate that both $_{pfu}$Cbf5 and $_{pfu}$Pus10 can catalyze tRNA Ψ55 formation *in vitro*. Unfortunately, because *P.furiosus* is not currently amenable to genetic analysis, we are unable to unequivocally determine which protein is responsible for U55 modification in this organism. Therefore we tested for the ability of the enzymes to modify tRNA U55 in a heterologous system—an *E.coli* strain harboring a null

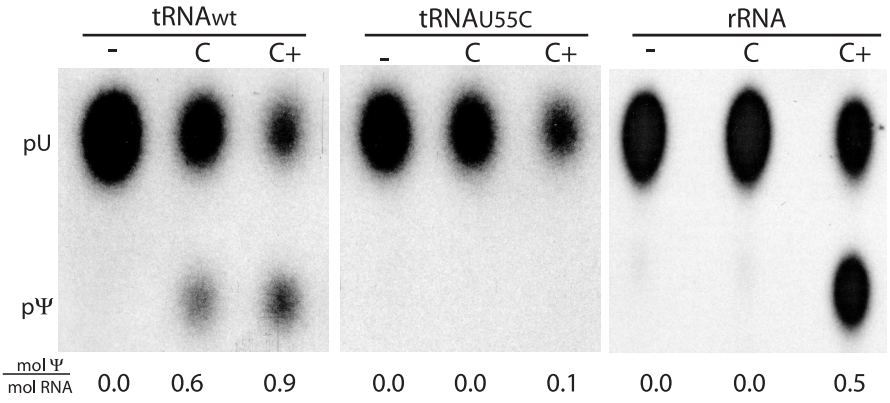

Figure 3. $_{pfu}$Cbf5 modification of tRNA in the absence and presence of accessory proteins. [α-^{32}P]UTP-labeled wild-type (wt) or U55 mutant (U55C) tRNA (left and middle panels) was incubated with no protein (-), $_{pfu}$Cbf5 alone (C), or $_{pfu}$Cbf5 plus *P.furiosus* accessory proteins Gar1, Nop10, and L7Ae (C+). After incubation, the tRNA was digested with RNase T1 and the fragment corresponding to the TΨ-loop (nt 54–65) was excised and purified from a 20% denaturing gel. This fragment was digested with nuclease P1, and Ψ and U were separated and analyzed by TLC and phosphoimaging. In the right panel, site-specifically radiolabeled target rRNA was incubated with the same combinations of proteins in the presence of Pf9 guide rRNA. The resulting RNA was digested with nuclease P1 and analyzed as for tRNA. The number of moles of Ψ incorporated per mole of RNA substrate (taking into account the number of uridines in the region analyzed) is indicated below each lane.

allele of the *truB* gene (16). It has been demonstrated that TruB is necessary (and sufficient) for the formation of Ψ55 *in vivo*, affording us the opportunity to test if expression of either of the two archaeal enzymes would complement U55 modification of *E.coli* tRNAs (23). The *truB* strains were transformed with plasmids containing the *P.furiosus pus10* or *cbf5* gene and the effect of the expression of each of these proteins on tRNA modification was assayed by primer extension after CMCT treatment (22). The cells were incubated for 1 h at 50°C [a compromise between the high temperature optimum for enzymatic activity (see Figure 1E–F) and optimal *E.coli* growth temperature]. tRNA was isolated from these cells and Ψ residues within tRNA^{Cys} and tRNA^{Phe} were identified using the CMC/RT assay system (22). Similar results were obtained with both tRNAs. As expected, Ψ55 was observed in the wild type (wt) strain but not in the *truB* (KO) strain (Figure 4). *truB* cells transformed with a plasmid containing the *cbf5* gene showed no significant modification of U55 (data not shown), but because we were not able to detect expression of the Cbf5 protein in the *E.coli*, no conclusion can be drawn from the lack of activity. Nearly wild type levels of U55 modification were observed in the *truB* strains transformed with a plasmid containing the *pus10* gene (+Pus10). These results indicate that $_{pfu}$Pus10 can efficiently catalyze formation of tRNA Ψ55 in this heterologous system.

DISCUSSION

Identification of archaeal enzymes that modify U55 in tRNA

Until the present work, no protein was attributed to the role of pseudouridylation of tRNA U55 in archaea. U55 modification activity was demonstrated in a cell-free extract of *P.furiosus*, but no protein was implicated as the source of the activity (11). We have identified two archaeal proteins capable of site-specific modification of U55: $_{pfu}$Cbf5, the only member of the TruB/Pus4/Cbf5 family (COG 0130) found in archaea, and $_{pfu}$Pus10 (previously PsuX, belonging to COG 1258), which was previously uncharacterized. The evidence reported here indicates that both of these archaeal enzymes are capable of specifically performing this modification. It is possible that either or both of these proteins introduce the conserved Ψ55 in tRNA in *P.furiosus*.

Pus10 (alias PsuX) is a previously uncharacterized enzyme. The data presented here clearly show that this enzyme can efficiently modify U55 both *in vitro* within a variety of substrates (wild type, −3′CA mutant and miniS tRNA^{Asp}), and *in vivo* within *E.coli* tRNAs. While Pus10 is not a member of the (TruB/Pus4/Cbf5) family of Ψ synthases that carry out tRNA U55 pseudouridylation in bacteria and eukaryotes, Pus10 orthologs are found in all sequenced archaeal genomes, consistent with the notion that this may be the conserved archaeal enzyme responsible for tRNA Ψ55 (15). Pus10 homologs are not present in bacteria (which employ TruB for Ψ55 synthesis), but do also appear to exist in certain higher eukaryotes (which employ Pus4 for Ψ55 synthesis) (15). Pus10 is renamed here as the 10th Ψ synthase acting on tRNA [for review on Pus1–9, see (24,25)]. The implication of Pus10 in tRNA Ψ55 formation (based on our findings) represents the first evidence for function of a protein outside the TruB/Pus4/Cbf5 family in introduction of this highly conserved modification.

The results of our studies indicate that $_{pfu}$Cbf5 also catalyzes specific tRNA Ψ55 formation *in vitro*. Cbf5 is the only enzyme in archaea that bears significant sequence similarity to the known Ψ55 synthases in bacteria and eukaryotes. Therefore, it is the most logical choice for the relevant enzyme. However, a role for Cbf5 in RNA-guided rRNA modification has been firmly established in archaea (12,13) as well as eukaryotes (14). Our finding that Cbf5 may also function in tRNA U55 modification suggests an unexpected dual function for Cbf5 in archaea. There is some precedent for this concept in the dual specificity of Pus1p for tRNA and U2 snRNA in yeast (26).

Pseudouridylation of rRNA by Cbf5 depends upon the accessory proteins Gar1, Nop10 and L7Ae (12,13). In this work we found that the activity of Cbf5 toward tRNA is

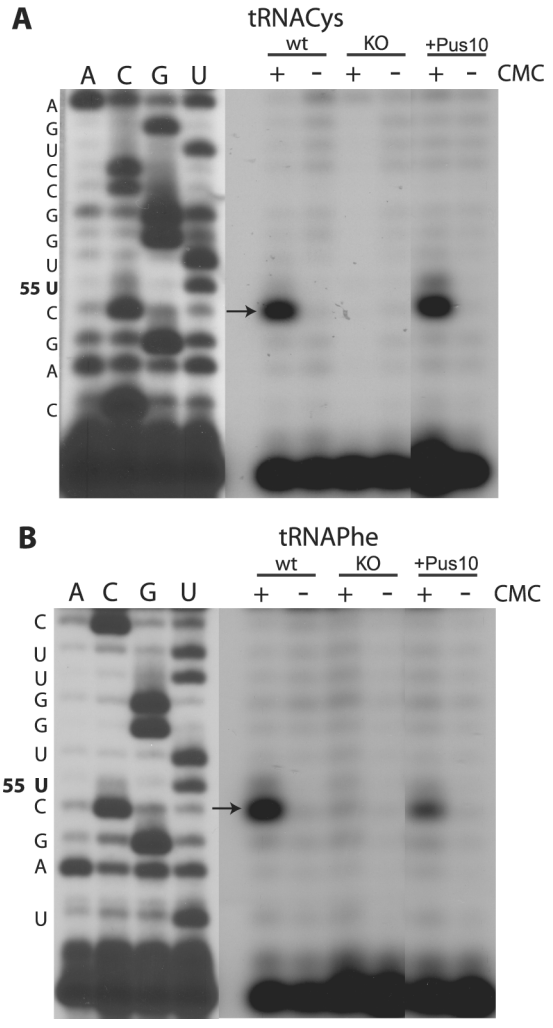

Figure 4. Detection of Ψ in *E.coli* $tRNA^{Cys}$ and $tRNA^{Phe}$. (**A**) CMC/RT analysis of $tRNA^{Cys}$ of *E.coli*. tRNA was isolated from wild-type (wt), *truB* (KO) or *truB* transformed with a plasmid containing the $_{pfu}$*pus10* gene (+Pus10) *E.coli* and CMC modified. Primer extension was performed as described in Materials and Methods with a primer complementary to $_{eco}tRNA^{Cys}$, nucleotides 61–76. The arrow indicates a strong stop at Ψ55. (**B**) CMC/RT analysis of $_{eco}tRNA^{Phe}$ as described above using a primer complementary to nucleotides 61–76 of $_{eco}tRNA^{Phe}$.

enhanced in the presence of the accessory proteins. Thus, in both cases the relevant enzyme may be a multi-protein complex.

The dual function of archaeal Cbf5 in tRNA and rRNA modification may account for structural similarities that have been noted between components of these pseudouridylation reactions from different systems. The X-ray crystal structures of bacterial TruB and archaeal Cbf5 are remarkably similar (27–31). Moreover, the structure of the regions of the substrate RNAs recognized and modified by TruB and Cbf5, the TΨ-arm of tRNA and guide RNA/rRNA duplex respectively, are strikingly comparable (29,32–34). Indeed this similarity was recently used to model the interaction between $_{pfu}$Cbf5 and the guide RNA/rRNA complex (29). Our findings suggest that $_{pfu}$Cbf5 may also recognize a common feature of tRNAs and H/ACA guide RNAs outside the area of modification—the CCA and ACA at the 3′ ends of tRNAs and H/ACA RNAs, respectively [Figure 3 and (12)]. These conserved structural features between tRNA and rRNA modification systems may be a consequence of the dual function of Cbf5 in both processes in archaea. Alternatively, the observed activity of Cbf5 toward tRNA may reflect general conservation between Ψ synthases.

Ψ55 synthases in the three domains of life

Modification of U55 in tRNA to Ψ55 occurs in all three domains of life. In bacteria, TruB is responsible for this modification (10,35). In eukaryotes (with few exceptions including *Drosophila melanogaster* and *Caenorhabditis elegans*) the tRNA Ψ55 synthase is Pus4 (15). The results presented here implicate Cbf5 or Pus10 (or both) as the enzyme responsible for this modification in archaea. Cbf5 also functions in H/ACA RNA-guided modification of rRNA in archaea (12,13). rRNA modifications are also introduced by Cbf5 (by an RNA-guided mechanism) in eukaryotes (14). Pseudouridylation of rRNA is carried out by a set of site-specific enzymes in bacteria (36). Pus10 is more distantly related to these other Ψ synthases.

The overall domain organization of Pus10 differs significantly from the other Ψ55 synthases (Figure 5). Sequence analysis of Pus10 reveals the presence of several distinct domains (15). CXXC Zn binding motifs are found in the N-terminal region of Pus10 proteins (15). A similar motif is present in Pus1, a distinct tRNA Ψ synthase in *S.cerevisiae*, which contains one atom of Zn essential for the native conformation and tRNA recognition (37). A THUMP domain (present in some THioUridine synthases, Methylases and Pseudouridine synthases) is located adjacent to the CXXC Zn binding motifs of Pus10 (38). This characteristic motif is proposed to be involved in RNA binding (38). A recent study indicates that the THUMP motif alone cannot bind RNA, but facilitates the interaction of the catalytic domain of the archaeal Trm-G10 methyltransferase enzyme with tRNA substrates (39). The Pus10-related proteins of *Drosophila* and *Caenorhabditis* lack this THUMP domain (38). The catalytic domain of Pus10 appears to be found in the C-terminal region of the protein (15).

TruB and Cbf5 (but not Pus4 of *S.cerevisiae*) contain a characteristic PUA domain (so called because it is present in PseudoUridine synthases and Archaeosine transglycosylase) within the C-termini of the proteins (40,41) (Figure 5). In archaeal Cbf5, the PUA domain plays a crucial role in binding H/ACA guide RNAs [(28), D. Baker, R. M. Terns and M. P. Terns, unpublished data]. This RNA binding depends on the presence of an ACA sequence at the 3′ end of the guide RNA (28,29). We have found that archaeal Cbf5 also requires an intact 3′ terminal CCA sequence for activity toward tRNA (Figure 2).

Evolutionary considerations

The observations made in this study may provide some insight on the evolutionary origin of the present-day RNA Ψ synthases, especially the tRNA Ψ55 synthases. The finding that in archaea Cbf5 may function in tRNA U55 pseudouridylation as well as RNA-guided pseudouridylation of rRNA supports the idea that Cbf5 is a direct descendant of a primordial TruB/Pus4-like tRNA Ψ synthase. This primordial Ψ synthase

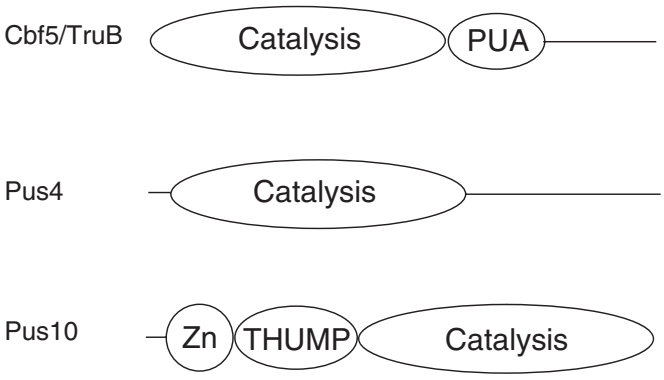

Figure 5. Domain organization of Cbf5 and Pus10. Schematic representation of predicted domains of Cbf5, TruB, Pus4 and Pus10 proteins. Conserved catalytic, PUA, Zn and THUMP domains are indicated. See text for discussion.

may have had the ability to also act on rRNA (and some dependence on guide RNAs and accessory proteins) in a common ancestor of eukaryotes and archaea. After the divergence of eukaryotes and archaea, a gene duplication in eukaryotes may have allowed separation of the functions in Cbf5 and Pus4. An analogous situation exists in the cases of the RNA-guided machinery catalyzing the formation of 2′*O*-methylribose in both rRNA and tRNA (42,43).

As we have indicated, our results suggest the possibility that Cbf5 and Pus10 provide redundant function in formation of the highly conserved tRNA U55 modification in archaea. Therefore it is also possible that the Pus10-related proteins present in higher eukaryotes also function redundantly in tRNA Ψ55 synthesis (in this case with Pus4).

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