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 pfuCbf5, a protein known to play a role in RNA-guided modification of rRNA, and pfuPsuX, a previously uncharacterized enzyme that is not a member of the TruB/Pus4/Cbf5 family of pseudouridine synthases. pfuPsuX is hereafter renamed pfuPus10. 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, pfuPus10 efficiently complement an Escherichia coli strain deficient in the bacterial Ψ55 synthase TruB. These results indicate that it is probable that pfuCbf5 or pfuPus10 (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 modification of Ψ55 (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, pfuCbf5 and pfuPus10 (previously known as PusX).

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 pseudouridylate, 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 \( \Psi \) 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 pseudouridylate of tRNA.

The second candidate \( \Psi \)55 synthase that we tested has been called PseX (15). The PseX gene (hereafter the \( \psi \)us10 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 \( \Psi \) synthase family than TruB, Pus4, and Cbf5 [COG 1258 for Pus10, and COG 0130 for the three other \( \Psi \) synthases (8)] and its function and specificity are unknown.

In this work, the activities of recombinantly expressed and purified \( \psi \)uCbf5 and \( \psi \)uPus10 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 \( \psi \)uPus10 can pseudouridylate a truncated tRNA substrate (miniS) and a tRNA lacking the 3′CCA, but that \( \psi \)uCbf5 functions only on the full-length tRNA substrate including the 3′CCA end. Moreover, \( \psi \)uPus10 efficiently complements tRNA \( \Psi \)55 formation in an \( \psi \)coli truB knockout strain. These results suggest two novel and interesting candidates for the pseudouridylate of tRNA U55 in archaea.

**MATERIALS AND METHODS**

**Strains and plasmids**

To construct the ML100 strain (BL21(DE3)truB), the truB2422::mini-Tn/0Cm allele from strain GOB113 (16) was transferred by PI transduction into strain BL21(DE3). Transductants were selected on LB medium supplemented with chloramphenicol (30 mg/μl). The GOB113 strain (Hfr P4X sdr+ truB2422::mini-Tn/0Cm) 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 \( \psi \)coli of the \( \psi \)furiosus Cbf5 protein (\( \psi \)uCbf5; ORF = PF1785) and purification of the recombinant protein was described previously (12).

The plasmid pML156 allowing in vitro transcription of \( \psi \)furiosus tRNA\(_{\text{A}\text{SP}}\) by T7 RNA polymerase is a pUC18 derivate with the fragment containing the \( \psi \)furiosus tRNA\(_{\text{A}\text{SP}}\) 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 \( \psi \)furiosus tRNA\(_{\text{A}\text{SP}}\)(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′-CCGGGAT-TTGACCCGGGTTCG-3′. The sequence of the mutant was verified.

**Cloning the \( \psi \)furiosus pus10 gene, expression and purification of the corresponding \( \psi \)uPus10 protein**

The gene encoding \( \psi \)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′-GTCTAGATGACCCGGCCAAGGCA GAG-3′ and 5′-GTTCAGATCCATTATCTCC CTCaACATCG-3′. The resulting plasmid (pET28b-pus10) allowed T7-expression in \( \psi \)coli (strain Rosetta(DE3)) of the \( \psi \)furiosus Pus10 protein (\( \psi \)uPus10) 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-\( \beta \)-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 lysed by a 30 min sonication at 4°C using a VibraCell 75 (414 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 \( \psi \)uPus10 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 \( ^{32} \text{P} \) radiolabeled tRNA transcripts as substrates for enzymatic reactions is based on the method described previously (17). Radioactive \( ^{32} \text{P} \) in vitro transcripts were prepared as described elsewhere (18) using Mval (for generating tRNA\(_{\text{A}\text{SP}}\) substrate) or Narl (for generating tRNA\(_{\text{A}\text{SP}}\)-3′CA substrate) digested plasmids as templates. \([\alpha-^{32} \text{P}]\text{UTP}, [\alpha-^{32} \text{P}]\text{CTP}, [\alpha-^{32} \text{P}]\text{ATP and } [\alpha-^{32} \text{P}]\text{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 \( \psi \)furiosus tRNA\(_{\text{A}\text{SP}}\) minisubstrate (TP′-loop & stem—see Figure 2A) was prepared by \( \text{in vitro} \) transcription of a single stranded DNA template (minus stand), following the procedure described previously (19).

**tRNA pseudouridine synthase assay**

Purified recombinant \( \psi \)uCbf5 (5 μg or 2 μg) or \( \psi \)uPus10 (5 μg or 0.5 μg) was incubated in a 400 μl reaction mixture with the \( \text{in vitro} \) transcribed \( ^{32} \text{P} \)-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...
RESULTS

Both pfuCbf5 and pfuPus10 can form Ψ55
in tRNA in vitro

As described above, bioinformatic searches yielded two candidate enzymes for U55 modification in archaea. pfuCbf5 is a member of the TruB/Pus4/Cbf5 family of enzymes that are responsible for Ψ55 modification in bacteria and eukaryotes. pfuPus10 (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 pfuCbf5 and pfuPus10 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 pfuCbf5 and pfuPus10. 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). pfuPus10 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 pfuCbf5 in this assay was ~0.6 mol Ψ per mole of substrate tRNA (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 pfaRNA\^\text{\textsubscript{~\frown}} was (\textsuperscript{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 pfuCbf5 and pfuPus10, 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\textsuperscript{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 pfuCbf5 (Figure 2C, panels 5–6), this mutation completely abolished activity. For pfuPus10 (Figure 2C, panels 13–14), this mutation also dramatically reduced the amount of Ψ produced. The small amount of Ψ seen with pfuPus10 may be owing to inefficient modification of other sites on the tRNA under the in vitro reaction conditions. These results clearly indicate that both pfuCbf5 and pfuPus10 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 Ψ5'-arm and the acceptor stem (outlined in Figure 2A) (11). To test the ability of the two candidate proteins to act on this substrate, [\textsuperscript{\textsuperscript{32}P}]UTP-radiolabeled transcripts of the miniS were incubated with the pfuCbf5 or pfuPus10 proteins, and analyzed as described.
above. As observed in Figure 2C panel 7, pfuCbf5 was unable to modify any uridine residue in the mini-substrate. In contrast, pfuPus10 was active on the mini-substrate (Figure 2C, panel 15).

The binding of pfuCbf5 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 pfuCbf5, we generated a tRNA that lacked the terminal CA (see dashed outline in Figure 2A). As shown in Figure 2C (panels 8 and 16), pfuCbf5 did not modify the tRNA/CCA mutant. At the same time, the –3′CA mutation did not affect the ability of pfuPus10 to modify the substrate tRNA.

Ψ55 formation by pfuCbf5 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.
As has been shown previously (12) and is demonstrated in Figure 3 (right panel, rRNA), in rRNA pfuCbf5 converts a radiolabeled uridine (targeted by a specific guide RNA) to $\Psi$ only in the presence of the three accessory proteins (C+ lane).

Uniformly $[^{32}\text{P}]$UTP-radiolabeled archaeal pfuRNA$\text{Agsp}$ was also incubated with pfuCbf5 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, 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-32P]UTP- and [α-32P]CTP-labeled tRNA, respectively. Nuclease P1 generates 5'-phosphate-nucleosides while RNase T2 generates 3'-phosphate-nucleosides. (C) 2D-TLC analysis of pfuCbf5 and pfuPus10 modification of various tRNA substrates. WT indicates wild-type pfuRNA$\text{Agsp}$ (panels 1–4, 9–12); U55C indicates U55 replacement mutant (panels 5,6,13,14); miniS indicates miniS-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 (32P)-labeled nucleotide incorporated at transcription. Incubation was for 1 h at 70°C in the presence of 0.12 nmol (5 μg) of pfuCbf5 (panels 1–8) and 0.01 nmol (0.5 μg) of pfuPus10 (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.

Our findings indicate that pfuCbf5 can modify tRNA U55, but not rRNA, in the absence of Gar1, Nop10 and L7Ae. However, the activity of pfuCbf5 toward tRNA U55 is enhanced in the presence of the accessory proteins, suggesting that a multi-protein Cbf5 complex may function in $\Psi$55 synthesis in P.furiosus.

**pfuPus10 can efficiently form $\Psi$55 in tRNA in E.coli**

Our results indicate that both pfuCbf5 and pfuPus10 can catalyze tRNA $\Psi$55 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
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 tRNAΨ55 and tRNAΨ5 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 (α-Pus10). These results indicate that 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: pus10, the only member of the TruB/Pus4/Cbf5 family (COG 0130) found in archaea, and 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 and in vivo. The implication of Pus10 in tRNA modification is that it 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 (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 pus10 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 in 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

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**Figure 3.** Cbf5 modification of tRNA in the absence and presence of accessory proteins. [t-32P]UTP-labeled wild-type (wt) or U55 mutant (U55C) tRNA (left and middle panels) was incubated with no protein (-), pus10 alone (C), or pus10 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 Ψ-loop (nt 54–65) was excised and purified from a 20% denaturing gel. This fragment was digested with nuclease P1, and number of moles of 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.

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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 substrates RNAs recognized and modified by 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...
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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REFERENCES
1. Rozenstki,J., Crain,P.F. and McCloskey,J.A. (1999) The RNA modification database. Nucleic Acids Res., 27, 196–197.
2. Massenet,S., Mougin,A. and Branlant,C. (1998) Posttranscriptional modifications in the U small nuclear RNAs. In Grosjean,H. and Benne,R. (eds), Modification and Editing of RNA. ASM Press, Washington DC, pp. 201–227.
3. McCloskey,J.A. and Rozenstki,J. (2005) The small subunit rRNA modification database. Nucleic Acids Res., 33, 135–138.
4. Sprinzl,M. and Vassilenko,K.S. (2005) Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res., 33, 139–140.

5. Ferré-D’Amare,A.R. (2003) RNA-modifying enzymes. Curr. Opin. Struct. Biol., 13, 49–55.
6. Koonin,E.V. (1996) Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. Nucleic Acids Res., 24, 2411–2415.
7. Kaya,Y. and Ofengand,J. (2003) A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. RNA, 9, 711–721.
8. Tatusov,R.L., Fedorova,N.D., Jackson,J.D., Jacobs,A.R., Kryutin,B., Koonin,E.V., Krylov,D.M., Mazumder,R., Mehedov,S.L. etc. (2003) The COG database: an updated version includes eukaryotes. BMC Bioinformatics, 4, 41.
9. Becker,H.F., Motorin,Y., Planta,R.I. and Grosjean,H. (1997) The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of psi55 in both mitochondrial and cytoplasmic tRNAs. Nucleic Acids Res., 25, 4499–4500.
10. Nurse,K., Wrzesinski,J., Bakin,A., Lane,B.G. and Ofengand,J. (1995) Purification, cloning and properties of the tRNA psi 55 synthase from Escherichia coli. RNA, 1, 102–112.
11. 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.
12. Baker,D.L., Youssaf,O.A., Chastalfsky,M.L., Dy,D.A., Terns,R.M. and Terns,M.P. (2005) RNA-guided RNA modification: functional organization of the RNA-modifying enzymes from the archaean H/ACA RNP. Genes Dev., 19, 1238–1248.
13. Charpentier,B., Muller,S. and Branlant,C. (2005) Reconstitution of archaean H/ACA small ribonucleoprotein complexes active in pseudouridylation. Nucleic Acids Res., 33, 3133–3144.
14. Yu,Y.-T., Terns,R.M. and Terns,M.P. (2005) Mechanisms and functions of RNA-guided RNA modification. In Grosjean,H. (ed.), Fine-Tuning of RNA Functions by Modifications and Editing. Springer-Verlag, Berlin Heidelberg, Germany, pp. 223–262.
15. Watanabe,Y. and Gray,M.W. (2000) Evolutionary appearance of genes encoding proteins associated with box H/ACA snoRNAs: Chf5p in Euglena gracilis an early diverging eukaryote, and candidate Gar1p and Nop10 homologs in archaeabacteria. Nucleic Acids Res., 28, 2342–2352.
16. Byland,G.O., Wipemo,J.C., Lundberg,L.A.C. and Wikstrom,P. (1998) RimM and RhaA are essential for efficient processing of 16S rRNA in Escherichia coli. J. Bacteriol., 180, 73–82.
17. Reyes,V.M. and Abelson,J. (1987) A synthetic substrate for tRNA pseudouridylation from Salmonella typhimurium. Anal. Biochem., 166, 90–106.
18. Roovers,M., Wouters,J., Bujnicki,J.M., Tricot,C., Stalon,V., Grosjean,H. and Droogmans,L. (2004) A primordial RNA modification enzyme: the case of tRNA (m1A) methyltransferase. Nucleic Acids Res., 32, 465–476.
19. Becker,H.F., Motorin,Y., Sissler,M., Florentz,C. and Grosjean,H. (1997) Major identity determinants for enzymatic formation of ribothymidine and pseudouridine in the T psi-loop of yeast tRNAs. J. Mol. Biol., 274, 505–518.
20. Grosjean,H., Keith,G. and Droogmans,L. (2004) Detection and quantification of modified nucleotides in RNA using thin-layer chromatography. Methods Mol. Biol., 265, 357–391.
21. Buck,M., Connick,M. and Ames,B.N. (1983) Complete analysis of tRNA-modified nucleosides by high-performance liquid chromatography: the 29 modified nucleosides in Salmonella typhimurium and Escherichia coli tRNA. Anal. Biochem., 129, 1–13.
22. Ofengand,J., Del Campo,M. and Kaya,Y. (2001) Mapping pseudouridines in RNA molecules. Methods, 25, 365–373.
23. Kinghorn,S.M., O’Byrne,C.P., Booth,L.R. and Stansfield,I. (2002) Physiological analysis of the role of trud in Escherichia coli: a role for RNA modification in extreme temperature resistance. Microbiology, 148, 320511–320535.
24. Behm-Ansment,L., Grosjean,H., Massenet,S., Motorin,Y. and Branlant,C. (2004) Pseudouridylation at position 32 of mitochondrial and cytoplasmic tRNAs requires two distinct enzymes in Saccharomyces cerevisiae. J. Biol. Chem., 279, 52998–53006.
25. Johansson,M.J.O. and Byström,A.S. (2005) Transfer RNA modifications and modifying enzymes in Saccharomyces cerevisiae. In Grosjean,H. (ed.), Fine-Tuning of RNA Functions by Modifications and Editing. Springer-Verlag, Berlin Heidelberg, Germany, pp. 87–120.
26. Massenet,S., Motorin,Y., Lafontaine,D.L.J., Hurt,E.C., Grosjean,H. and Branlant,C. (1999) Pseudouridine mapping in the Saccharomyces
cerevisiae spliceosomal U small nuclear RNAs (snRNAs) reveals that pseudouridine synthase Pus1p exhibits a dual substrate specificity for U2 snRNA and tRNA. Mol. Cell. Biol., 19, 2142–2154.

27. Hamma,T., Reichow,S.L., Varani,G. and Ferre-D’Amare,A.R. (2005) The Cbf5-Nop10 complex is a molecular bracket that organizes box H/ACA RNPs. Nat. Struct. Mol. Biol., 12, 1101–1107.

28. Manival,X., Charon,C., Fourmann,J.B., Godard,F., Charpentier,B. and Branlant,C. (2006) Crystal structure determination and site-directed mutagenesis of the Pyrococcus abyssi aChf5-aNop10 complex reveal crucial roles of the C-terminal domains of both proteins in H/ACA sRNA activity. Nucleic Acids Res., 34, 826–839.

29. Rashid,R., Liang,B., Baker,D.L., Youssef,O.A., He,Y., Phipps,K., Terns,R.M., Terns,M.P. and Hong,L. (2006) Crystal structure of a Cbf5-Nop10-Gar1 complex and implications in RNA-guided pseudouridylation and Dyskeratosis Congenita. Mol. Cell, 21, 249–260.

30. Chaudhuri,B.N., Chan,S., Perry,L.J. and Yeates,T.O. (2004) Crystal structure of the apo forms of psi55 tRNA pseudouridine synthase from Mycobacterium tuberculosis: a hinge at the base of the catalytic cleft. J. Biol. Chem., 279, 24585–24591.

31. Hoang,C., Hamilton,C.S., Mueller,E.G. and Ferre-D’Amare,A.R. (2005) Precursor complex structure of pseudouridine synthase TruB suggests coupling of active site perturbations to an RNA-sequestering peripheral protein domain. Protein Sci., 14, 2201–2206.

32. Hoang,C. and Ferre-D’Amare,A.R. (2001) Co-crystal structure of a tRNA psi55 pseudouridine synthase: Nucleotide flipping by an RNA-modifying enzyme. Cell, 107, 929–939.

33. 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. USA, 100, 12648–12653.

34. 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.

35. Conrad,J., Niu,L., Rudd,K., Lane,B.G. and Ofengand,J. (1999) 16S ribosomal RNA pseudouridine synthase RusA of Escherichia coli: deletion, mutation of the conserved Asp102 residue, and sequence comparison among all other pseudouridine synthases. RNA, 5, 751–763.

36. Ofengand,J., Mailhotra,A., Remme,J., Gutgsell,N.S., Del Campo,M., Jean-Charles,S., Peil,L. and Kaya,Y. (2001) Pseudouridines and pseudouridine synthases of the ribosome. Cold Spring Harb. Symp. Quant. Biol., 66, 147–159.

37. Arluzion,V., Hountondji,C., Robert,B. and Grosjean,H. (1998) Transfer RNA-pseudouridin synthase Pus1 of Saccharomyces cerevisiae contains one atom of zinc essential for its native conformation and tRNA recognition. Biochemistry, 37, 7268–7276.

38. Aravind,L. and Koonin,E.V. (2001) THUMP-a predicted RNA-binding domain shared by 4-thiouridine, pseudouridine synthases and RNA methylases. Trends Biochem. Sci., 26, 215–217.

39. Gabant,G., Auxilien,S., Tuszynska,I., Locard,M., Gajda,M.J., Chaussinand,G., Fernandez,B., Deleu,A., Grosjean,H., Golinelli-Pimpaneau,B. and Bujnicki,J.M. et al. (2006) THUMP from archaeal tRNA: m7G10 methyltransferase a genuine autonomously folding domain. Nucleic Acids Res., 34, pp. 2483–2494.

40. Aravind,L. and Koonin,E.V. (1999) Novel predicted RNA-binding domains associated with the translation machinery. J. Mol. Evol., 48, 291–302.

41. Iyer,L.M., Burroughs,A.M. and Aravind,L. (2006) The ASCH superfamily: novel domains with a fold related to the PUA domain and a potential role in RNA metabolism. Bioinformatics, 22, 257–263.

42. Tran,E., Brown,J. and Maxwell,E.S. (2004) Evolutionary origins of the RNA-guided nucleotide-modification complexes: from the primitive translation apparatus? Trends Biochem. Sci., 29, 343–350.

43. Clouet d’Orval,B., Gaspin,C. and Mougin,A. (2005) Two different mechanisms for tRNA ribose methylation in Archaea: a short survey. Biochimie, 87, 889–895.