Pseudouridine formation in archaeal RNAs: The case of *Haloferax volcanii*

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ABSTRACT

Pseudouridine (Ψ), the isomer of uridine, is commonly found at various positions of noncoding RNAs of all organisms. Ψ residues are formed by a number of single- or multisite specific Ψ synthases, which generally act as stand-alone proteins. In addition, in Eukarya and Archaea, specific ribonucleoprotein complexes, each containing a distinct box H/ACA guide RNA and four core proteins, can produce Ψ at many sites of different cellular RNAs. Cbf5 is the core Ψ synthase in these complexes. Using *Haloferax volcanii* as an archaeal model organism, we show that, contrary to eukaryotes, the Cbf5 homolog (HVO_2493) is not essential in this archaeon. The Cbf5-deleted strain of *H. volcanii* completely lacks Ψ at positions 1940, 1942, 2605, and 2591 (*Escherichia coli* positions 1915, 1917, 2572, and 2586) of its 23S rRNA, and contains reduced steady-state levels of some box H/ACA RNAs. Archaeal Cbf5 is known to have tRNA Ψ 55 synthase activity in vitro but we could not confirm this activity in vivo in *H. volcanii*. Conversely, the Pus10 (previously PsuX) homolog (HVO_1979), which can produce tRNA Ψ 55, as well as Ψ 54 in vitro, is shown here to be essential in *H. volcanii*, whereas the corresponding tRNA Ψ 55 synthases, Pus4 and TruB, are not essential in yeast and *E. coli*, respectively. Finally, we demonstrate that HVO_1852, the TruA/Pus3 homolog, is responsible for the pseudouridylation of position 39 in *H. volcanii* tRNAs and that the corresponding gene is not essential.

Keywords: pseudouridine synthase; RNA modification; Cbf5; Pus10; TruA; Pus3

INTRODUCTION

Pseudouridine (Ψ), the post-transcriptional C5-ribosyl isomer of uridine, is one of the most common modifications identified in a wide range of RNA species and in all domains of life (Charette and Gray 2000; Grosjean 2009; Cantara et al. 2011). Ψ modifications are very abundant in tRNAs. In addition to the near ubiquitous Ψ 55 found in tRNAs of Archaea, Bacteria, Eukarya, and most of the organelles, Ψ is found at several other positions of tRNA (Juhling et al. 2009). Ψ modifications are plentiful in rRNAs, particularly in eukaryotes with $\sim$ 100 identified in mammals and $\sim$ 50 found in *Saccharomyces cerevisiae* (Cantara et al. 2011). Ψ residues appear to be located specifically at functionally

important positions (Ofengand et al. 2001b; Decatur and Fournier 2002; Karijolich and Yu 2010).

Although the biological role of many Ψ residues remains unknown, the modification is thought to be required for RNA structure and stability. Indeed, the ability of Ψ residues to form an additional hydrogen bond compared to uridine, coupled with the property of augmenting local RNA stacking, causes the localized phosphodiester structure to become more rigid, contributing to overall RNA structure stabilization (Davis 1995; Yarian et al. 1999). Structural studies have suggested that, while the presence of pseudouridine modifications does not affect the overall three-dimensional structure of tRNA (Harrington et al. 1993; Arnez and Steitz 1994), the local structure near the site of modification is affected (Auffinger and Westhof 1997). The presence of Ψ in the anticodon helps foster proper codon–anticodon interactions, which may increase translational accuracy, and the presence of Ψ at positions 38/39 of tRNA has a direct influence on codon reading and frameshifting (Zerfass and Beier 1992; Lecointe et al. 2002; Namy et al. 2005). Ψ modifications in rRNAs can affect both ribosome biogenesis and

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translation accuracy (Decatur and Fournier 2002; Baudin-Baillieu et al. 2009).

Six distinct pseudouridine synthase families have been identified to date on the basis of protein sequence similarity: TruA, TruB, TruD, RluA, RsuA, and Pus10 (Mueller and Ferre-D'Amare 2009). Predicting the substrate specificity of any given pseudouridine synthase is, however, difficult. Different proteins of the same family can produce Ψ at different positions (Hamma and Ferre-D'Amare 2006; Mueller and Ferre-D'Amare 2009). The annotation of Ψ modification genes is all the more difficult given that examples of non-orthologous displacements are frequent. In *Escherichia coli* and *S. cerevisiae* pseudouridylation of position 55 of tRNA is catalyzed by TruB and Pus4, respectively (Nurse et al. 1995; Becker et al. 1997), both members of Cluster of Orthologous Group (COG) 0130 (Tatusov et al. 2003). In Archaea however the unrelated Pus10 family of proteins belonging to COG 1258 seems to modify both positions 54 and 55 of tRNA (Roovers et al. 2006; Gurha and Gupta 2008).

Specific Ψ residues in eukaryal and archaeal rRNAs and in eukaryal snRNAs can also be introduced by box H/ACA RNPs. The H/ACA RNPs comprise four core proteins and a modification-specific guide RNA containing between one and three hairpins (Dennis and Omer 2005; Yu et al. 2005; Meier 2006; Karjilovich and Yu 2008; Li 2008; Hamma and Ferre-D'Amare 2010; Kiss et al. 2010). Cbf5, a member of the TruB family (COG 0130), is the pseudouridine synthase in the RNP, which together with Nop10, L7Ae (Nhp2 in Eukarya), and Gar1 forms a site-specific complex. Box H/ACA RNA-guided in vitro pseudouridylation systems using recombinant *Pyrococcus furiosus*, *Pyrococcus abyssi*, and *Methanocaldococcus jannaschii* proteins have been established (Baker et al. 2005; Charpentier et al. 2005; Gurha et al. 2007; Muller et al. 2008). However, Cbf5, in addition to its RNA-guided activity, also produces Ψ at position 55 in tRNAs and in a 23S rRNA fragment in vitro in a guide RNA-independent manner. This guide RNA-independent activity of Cbf5 either requires or is enhanced by association with Gar1 and Nop10 (Roovers et al. 2006; Gurha et al. 2007; Muller et al. 2008).

In Eukarya the Cbf5 encoding gene is essential (Jiang et al. 1993; Giordano et al. 1999), which may be due to multiple roles of H/ACA RNPs in ribosome biogenesis, pre-mRNA splicing, and telomere maintenance (Meier 2005; Karjilovich and Yu 2008). Indeed, Cbf5 (Nap57 in mice) was originally identified in *S. cerevisiae* as a low affinity centromere binding protein, which is essential for cell viability (Jiang et al. 1993; Meier and Blobel 1994; Heiss et al. 1998). Orthologs of *cbf5* are essential in yeast and *Drosophila melanogaster*, and its disruption is embryonic lethal in mice (Jiang et al. 1993; Phillips et al. 1998; Giordano et al. 1999; He et al. 2002).

Most of the information on the role of Cbf5 and protein-alone Ψ synthases in Archaea is based on bioinformatic, biochemical, or structure-based data. The essentiality and

function of these proteins in vivo is therefore still undetermined in this domain of life. We set out to determine the in vivo role of Cbf5 in Ψ synthesis as well as of other Ψ synthases using the model archaeon *H. volcanii*. Indeed, sequences of the nearly complete set of tRNAs with associated modifications have been determined in this archaeon (Gupta 1984, 1986) and the positions of Ψ s in 23S rRNA can be inferred by comparison with other haloarchaea (Ofengand and Bakin 1997; Del Campo et al. 2005; Kirpekar et al. 2005 and <http://people.biochem.umass.edu/fournierlab/3dmodmap/heneqslu.php>). *H. volcanii* 16S rRNA does not contain Ψ (Kowalak et al. 2000). Ψ synthases for most of these Ψ have also been predicted in the *H. volcanii* genome using a bioinformatic approach (Grosjean et al. 2008). In this work, we achieved the deletion of *cbf5* (*HVO_2493*) (*H. volcanii* ORFs are as annotated in <http://archaea.ucsc.edu> and Hartman et al. 2010) and demonstrated that Cbf5 is responsible for the introduction of Ψ at multiple positions in the 23S rRNA, but for none of the Ψ s at common positions of tRNAs. We also made efforts to create genomic-deletions of certain other Ψ synthases, to determine their role in producing these non-Cbf5-dependent Ψ s in tRNAs. We show that *pus10* (*HVO_1979*), the gene for tRNA Ψ 54 and Ψ 55 synthase (Gurha and Gupta 2008), is essential in *H. volcanii*. We also show that the *H. volcanii* TruA/Pus3 homolog (*HVO_1852*) is indeed responsible for the introduction of Ψ 39 in tRNA. Finally we show that Cbf5 is needed for stabilization of box H/ACA RNAs that are predicted to guide Ψ formation in 23S rRNA.

RESULTS AND DISCUSSION

HVO_2493, encoding the Cbf5 homolog, is not essential in *H. volcanii*

The *H. volcanii* *cbf5* homolog *HVO_2493* was deleted using a published method (Allers et al. 2004), modified slightly to expedite the preliminary cloning steps (Blaby et al. 2010). Deletion of *HVO_2493* was confirmed by PCR using two primer pairs, one designed to anneal outside the deleted fragment and the other within (Fig. 1B,C), and independently verified by Southern hybridization (Fig. 1D). These data indicate that *cbf5* is dispensable in *H. volcanii*. However, as shown in Figure 1E, deletion of *cbf5* caused a slight decrease in growth rate (from 0.03 h⁻¹ in the wild-type [WT] strain to 0.02 h⁻¹ in the mutant), although the final growth yield is not affected. To rescue the effects of the *cbf5* deletion, a derivative of the $\Delta cbf5$ strain was created (Fig. 1A,D), which contained a plasmid-borne copy of *cbf5*. The growth rate was restored to WT levels when *cbf5* was expressed in *trans* in the mutant strain (Fig. 1E). A well-established role of Ψ residues is to maintain tertiary structure. If the absence of Cbf5 influenced the stability of the rRNA molecule, the *H. volcanii* $\Delta cbf5$ strain could become more sensitive to altered salt concentrations or to temperature extremes.

FIGURE 1. *cbf5*-deleted strain of *H. volcanii* is viable. Deletion of the *cbf5* gene was confirmed by three independent methods. (A) Schematics to show *HVO_2493* (thick lines) in the genomes of wild-type (WT) and *cbf5*-deleted ($\Delta cbf5$) strains and on plasmid *pHCbf5*. *EcoRI* and *NdeI* (in plasmid) sites are marked. Positions of primers 1–5 (Cbf5_Ext_Fwd, Cbf5_Ext_Rev, Cbf5_Int_Fwd, Cbf5_Int_Rev, and HVCBF5-F, respectively) are shown by arrows. The arrow direction indicates 5' to 3' direction of the primers. Segment between broken lines is deleted in the $\Delta cbf5$ strain. The His-tag at the C terminus of the Cbf5 protein is indicated by an asterisk. Approximate lengths of the segments are indicated but are not drawn to a scale. (B) PCR products using primers designed to anneal outside the gene (primers 1 and 2 in A) confirm a genomic rearrangement of correctly predicted sizes in WT and mutant strains. (C) PCR products using primers designed to anneal within the target gene (primers 3 and 4 in A) show the absence of the *HVO_2493* segment in the mutant and its presence in the WT strain. (D) Southern hybridization is consistent with loss of the gene in mutant strain. Blots of *EcoRI*-digested DNAs of WT and $\Delta cbf5$ strains, and $\Delta cbf5$ strain transformed with *pHCbf5* were probed with [$5'$ - 32 P]-labeled HVCBF5-F primer. The predicted sizes of the hybridized fragments are indicated beside the panel. (E) Growth curves of WT, $\Delta cbf5$, and $\Delta cbf5$ +*pHCbf5* strains of *H. volcanii* are shown. OD₆₀₀ was measured using a Bioscreen C apparatus. Each point is the mean of three independent cultures; error bars represent standard error. H26 and VDC2364 were used as WT and $\Delta cbf5$ strains.

However, deletion of *cbf5* did not lead to any obvious phenotype when analyzing growth on solid medium at 20, 30, 37, 44, and 50°C or with total salt concentrations ranging from 12% (1.812 M NaCl, 65.28 mM MgCl₂, 63 mM MgSO₄, and 41.64 mM KCl) to 25% (3.775 M NaCl, 136 mM MgCl₂, 131.25 mM MgSO₄, and 86.75 mM KCl) (compared to the standard YPC media with a final concentration of 18% salt; data not shown). It is presently unclear whether slower growth of the *cbf5*-deleted strain is due to the absence of Cbf5 itself or due to the loss of Cbf5-catalyzed modifications.

The nonessentiality of *cbf5* in *H. volcanii* is in stark contrast to eukaryotes, where the gene is essential and point mutations lead to pleiotropic effects. Point mutations in yeast *cbf5* can abolish in vivo pseudouridylation of rRNA (Zebardjian et al. 1999) and mutations in NAP57/dyskerin in mice decrease pseudouridylation of rRNA and assembly of box H/ACA RNP (Ruggero et al. 2003; Grozdanov et al. 2009). RNAi silencing of *Trypanosoma brucei* Cbf5 depleted box H/ACA RNAs, and caused defects in rRNA processing and *trans*-splicing (Barth et al. 2005). Partial loss-of-function

mutations in the *D. melanogaster* ortholog *minifly* result in small size and developmental delay (Giordano et al. 1999). Mutations in the human ortholog dyskerin result in the disorder dyskeratosis congenita (Heiss et al. 1998; Knight et al. 1999; Mitchell et al. 1999). The number of Ψ residues produced by Cbf5 is much larger in eukaryotes than in Archaea. Therefore, the essentiality of *cbf5* in complex eukaryotic systems may be due to the vital role of certain Cbf5-mediated Ψ residues in biological processes, e.g., translation, pre-mRNA splicing, telomere maintenance, etc. Alternatively Cbf5 may have some other functions in addition to pseudouridylation in eukaryotes that may be essential. At least in yeast the essential nature of Cbf5 is not due to its pseudouridylation activity, because cells containing a mutant Cbf5 with the catalytic aspartate mutated to alanine lack Ψ in their rRNA, but show a temperature-sensitive growth phenotype (Zebarjadian et al. 1999). Another possibility is a role of Cbf5 in maintaining essential H/ACA RNAs in eukaryotes. U17/E1/snrR30 is a nonpseudouridylating H/ACA snoRNA that is essential for rRNA processing (and consequently cell growth) (Meier 2006). A Cbf5 depletion can destabilize this RNA. If the only activity of Cbf5 in “simpler” archaeal systems is to produce Ψ , then it might not be absolutely necessary under normal growth conditions.

Cbf5 is needed for pseudouridylation of the large subunit rRNA residues of *H. volcanii*

Ψ residues are found at four positions in the 23S rRNA of *Halobacterium salinarium* (*Halobacterium halobium*) (Ofengand and Bakin 1997) corresponding to positions 1940, 1942, 2591, and 2605 in *H. volcanii*. The 23S rRNA of *Haloarcula marismortui* contains only three Ψ (Del Campo et al. 2005; Kirpekar et al. 2005). Position 2607 of *H. marismortui* (corresponding to *H. volcanii* 2591) contains an unmodified U. In *E. coli* the corresponding residues in the 23S rRNA are m³ Ψ 1915, Ψ 1917, A2572, and U2586 (<http://people.biochem.umass.edu/fournierlab/3dmodmap/heneqlsu.php>). Based on the correlation with *E. coli* and *H. marismortui* 23S rRNAs, *H. volcanii* residues 1940 and 1942 are in the loop of helix 69 of domain IV, residue 2605 is between helix 90 and 93 of domain V, and residue 2591 is in a bulge in the stem of helix 90 (<http://people.biochem.umass.edu/fournierlab/3dmodmap/heneqlsu.php>). We sought to determine which of the four *H. volcanii* positions contained Ψ , and which of these were Cbf5-mediated. Both CMCT and U-reaction based approaches were used to determine whether a Ψ or an unmodified U is present at each of these four positions in the WT strain. Furthermore, when Ψ is present at a specific position, we determined whether it was replaced by an unmodified U in the *cbf5*-deleted strain and, if so, whether it reverts to Ψ in the complemented strain and is thus Cbf5-mediated.

Figure 2A,B shows that positions 1940 and 1942 of 23S rRNA contain Ψ in the WT strain. However, the same

positions contain unmodified U residues in the $\Delta cbf5$ strain, which reverts back to Ψ when *cbf5* is added back in *trans*. These results suggest that production of Ψ at both positions 1940 and 1942 is Cbf5-mediated. Similar data presented in Figure 2C,D suggest that Cbf5 is also responsible for the formation of Ψ at position 2605. Presence of Ψ at these three positions is in agreement with the results of *H. salinarium* and *H. marismortui* (Ofengand and Bakin 1997; Del Campo et al. 2005; Kirpekar et al. 2005). These three Ψ modifications were predicted to be catalyzed by Cbf5 in an RNA-guided manner (Grosjean et al. 2008). The results presented here confirm that Cbf5, at least, is critical in the production of these three Ψ residues.

Analysis for the presence of Ψ at position 2591 in 23S rRNA is less clear. Based on the data presented in Figure 2E,F, it appears that the formation of Ψ 2591 is Cbf5-mediated as it is present only if the *cbf5* gene is present in *cis* or *trans*. We believe that conversion of U to Ψ at this position is not 100% for the following reasons. Neither U nor Ψ should produce a strong stop in untreated samples (see lanes 1 in Fig. 2A,C). However, presence of stops in lanes 1 of WT and $\Delta cbf5$ +pHCbf5 (Fig. 2E) suggests that position 2591 contains a residue other than U or Ψ , such as a secondary modification of Ψ (see below). On the other hand lanes 2 (U-reaction) of the same strains should not show stops unless there is an unmodified U (or an unknown modification of U that behaves like an unmodified U in aniline/hydrazine treatment) at position 2591. Similar incomplete conversion to Ψ has also been observed in certain *H. volcanii* tRNAs (Gupta 1984) as well as in other RNAs (discussed in Grosjean 2005). Recently it has been shown that pseudouridylation of certain residues of *S. cerevisiae* U2 snRNA can be conditionally induced in response to nutrient deprivation and heat shock (Wu et al. 2011). At present we cannot be certain whether 23S rRNA with and without Ψ 2591 in our RNA preparations reflect a mixed population of rRNAs in each cell or a mixed population of cells, each containing only one type of rRNA. In the former case, it would raise questions as to whether each cell has two or more types of differentially modified ribosomes, each one representing different translation machinery or translating different groups of mRNAs.

Finally a secondary modification of Ψ seems to occur at position 2591. Untreated RNAs of WT and $\Delta cbf5$ +pHCbf5 strains, but not of $\Delta cbf5$ strain, show a band (primer-extension stop) at position 2591 (Fig. 2E, lanes 1). Primer-extension stops with untreated RNAs do not appear to be due to the presence of a strong secondary structure in this region of the RNAs, as the untreated RNA of the $\Delta cbf5$ strain does not show this stop, and Ψ normally does not inhibit the extension. So far only four modifications of Ψ have been identified, 1-methylpseudouridine (m¹ Ψ) in Archaea, 1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine (m¹acp³ Ψ) in Eukarya, 3-methylpseudouridine (m³ Ψ) in Bacteria, and 2'-O-methylpseudouridine (Ψ m)

in both Archaea and Eukarya (<http://rna-mdb.cas.albany.edu/RNAmdb/> and <http://modomics.genesilico.pl/modifications/>). The first three are base modifications, whereas the last one is a ribose modification. These base modifications require U to Ψ conversion, before production of secondarily modified Ψ . It is difficult to conclude at this point whether the secondary modification of Ψ 2591 in *H. volcanii* 23S rRNA is one of the three known base modifications of Ψ or is a new modification.

As mentioned above, *H. salinarium* also contains Ψ (Ψ 2590) at the equivalent position to *H. volcanii* Ψ 2591 (Ofengand and Bakin 1997). It is possible that *H. salinarium* may also have this secondary modification of Ψ , since CMCT reactions alone may not be able to distinguish between Ψ and certain modified Ψ . Again as mentioned above, *H. marismortui* is not reported to contain Ψ at position 2607 (equivalent to *H. volcanii* 2591) (Del Campo et al. 2005; Kirpekar et al. 2005). However, Kirpekar's laboratory (Kirpekar et al. 2005) does see a medium strong stop at position 2607 of *H. marismortui* in their CMCT reactions but could not confirm the presence of Ψ at this position by MALDI-MS study. It is possible that, like *H. volcanii*, *H. marismortui* may also contain a subpopulation of 23S rRNAs that contain either a Ψ or a secondarily modified Ψ at their position 2607, while the rest contain unmodified U at that position.

Position 2619 in *H. marismortui* 23S rRNA has been shown to contain 3-methyluridine (m^3U) (Kirpekar et al. 2005), which inhibits primer extension. Residues at its equivalent positions in *E. coli* and human 23S rRNAs are U2584 and m^3U 4500, respectively (<http://people.biochem.umass.edu/fournierlab/3dmodmap/heneqslu.php>). Lack of this m^3U modification in *H. salinarium* has been shown to confer sparsomycin resistance (Lazaro et al. 1996; Kirpekar et al. 2005). Its equivalent position in *H. volcanii* is 2603, which shows strong primer-extension stops across all lanes in all strains (Fig. 2C,D). Taken together, this suggests that *H. volcanii* also has m^3U 2603 in its 23S rRNA.

FIGURE 2. (Legend on next page)

H. volcanii tRNA Ψ 55 residues are produced even in the absence of Cbf5

Positions of Ψ residues in nearly all tRNAs of *H. volcanii* have been determined and compiled (Gupta 1984, 1986; Grosjean et al. 2008). However, determination of the presence or absence of these Ψ in native tRNAs by primer-extension-based methods is technically challenging. Strong secondary structure of tRNAs in general and presence of certain modified residues in single-stranded regions pause or halt reverse transcriptase reactions (Motorin et al. 2007). Furthermore, the presence of partial modifications at a given site (Gupta 1984) can make primer extension reactions difficult to interpret. Therefore, a combination of strategies was used to determine the effect of *cbf5*-deletion in production of Ψ s at known positions of tRNAs.

Two panels in Figure 3A reflect nucleotide composition of total tRNA extracted from WT and $\Delta cbf5$ cells. The amount of Ψ appears to be the same in both strains. Most of the Ψ in the tRNA population comes from Ψ at positions 55 (in all tRNAs), and 13 and 39 (in several tRNAs) (Grosjean et al. 2008). Therefore, it appears that Ψ modifications at these three positions are not Cbf5-mediated.

Cbf5 either alone or in association with other H/ACA RNP core proteins has been shown to produce Ψ 55 in a guide RNA-independent manner in vitro in full-size tRNAs or tRNA fragments containing T Ψ C stem-loop (Roovers et al. 2006; Gurha et al. 2007; Muller et al. 2007; Zhou et al. 2011). To test whether this reaction occurs in vivo, we analyzed *H. volcanii* tRNA^{Trp}. This tRNA contains only one Ψ (Ψ 55) and one modified Ψ ($m^1\Psi$ 54) (Gupta 1984). The $\Psi/m^1\Psi$ ratio of tRNA^{Trp} remains the same even after deletion of *cbf5*, suggesting that Cbf5 is not the primary enzyme producing tRNA Ψ 55 (Fig. 3B) in vivo. As attempts to delete Pus10 were not successful (see below) the question whether Ψ 55 might be modified in vivo by Cbf5, in absence of active Pus10, remains open.

Cbf5 belongs to the TruB/Pus4 family of Ψ synthases that produce tRNA Ψ 55 in Bacteria/Eukarya. Apparently archaeal Cbf5 protein, although specialized for RNA-guided activity, seems to have retained its capacity to produce tRNA

Ψ 55, though it does not seem to be its normal function. It is possible that there was a common ancestral mechanism for recognition of tRNA for Ψ 55 modification by TruB/Pus4 and archaeal Cbf5. A similar example exists for archaeal and eukaryal tRNA splicing endonucleases (Fabbri et al. 1998; Fruscoloni et al. 2001), where the enzymes are homologous but have evolved to use different features of the pre-tRNAs to determine their cleavage sites (which are generally symmetric in Archaea and asymmetric in Eukarya). However, the eukaryal enzyme has retained the ability to use archaeal recognition signals. Alternatively, archaeal Cbf5 may produce Ψ in rRNAs in vivo in both RNA-guided and guide RNA-independent manners, the latter reaction recognizing a structure somewhat similar to the T Ψ C stem-loop of tRNA. Structures similar to this stem-loop are indeed present in rRNAs (Nagaswamy and Fox 2002; Krasilnikov and Mondragon 2003), and U2603 in a fragment of *P. abyssi* 23S rRNA has been shown to be converted by Cbf5 along with other H/ACA core proteins in a guide RNA-independent manner (Muller et al. 2008). The sequence surrounding this U2603 can indeed be folded into a stem-loop similar to the T Ψ C stem-loop of tRNA.

Pus10, a tRNA Ψ 55 and Ψ 54 synthase, is essential in *H. volcanii*

Another archaeal protein, Pus10, has been shown to produce Ψ 54 and Ψ 55 in tRNA in vitro and Ψ 55 in heterologous in vivo conditions (Roovers et al. 2006; Gurha and Gupta 2008). Despite several attempts, we failed to delete the *H. volcanii* *pus10* homolog, *HVO_1979*, using our standard protocol. This led us to postulate that the gene might be essential. To address this, the gene was placed under control of the inducible *P_{trp}* promoter. A strain carrying the chromosomal *HVO_1979* deletion could then be constructed in the presence of the gene in *trans* as described in the Methods section (Fig. 4). This strain required tryptophan for growth, hence providing strong evidence for *pus10* essentiality in *H. volcanii* (Fig. 4). The in vitro data that showed Pus10 can introduce Ψ residues at positions 54 and 55 in tRNA combined with evidence presented above that Ψ 55 is present in tRNAs of *cbf5*-deleted strains suggest that Pus10 is primarily responsible for the production of Ψ 55 and Ψ 54 in cellular tRNAs.

The essentiality of *pus10* was unexpected as genes responsible for the insertion of Ψ 55 in the other domains of life, at least *truB* and *pus4*, are dispensable in *E. coli* and yeast, respectively (Becker et al. 1997; Gutsell et al. 2000). The strains lacking *truB* and *pus4* are viable and have unmodified U55 in the tRNAs. This suggests that the essential nature of Pus10 in *H. volcanii* may be

FIGURE 2. Pseudouridylation of *H. volcanii* 23S rRNA is Cbf5-mediated. (A) U-specific analyses to determine the modification status of U1940 and U1942 of 23S rRNA were done using primer HVLSUR1 and total RNAs of WT and $\Delta cbf5$ strains, and $\Delta cbf5$ strain transformed with pHCb5. (Lanes 1,2) Primer extensions without and with U-specific reactions, respectively. Positions of certain U in 23S rRNA are indicated on the *side*; those in bold indicate Cbf5-mediated modification while that in italic indicates an unmodified U (used as indicator for the positions). (B) CMCT-primer extension analyses to determine the modification status of U1940 and U1942 of 23S rRNA were done using primer HVLSUR1 and total RNAs of WT and $\Delta cbf5$ strains, and $\Delta cbf5$ strain transformed with pHCb5. Total RNAs were treated with (+) or without (–) CMCT for the indicated time (in minutes), followed by alkali (OH[–]) treatment (+) or no treatment (–). Positions of Cbf5-mediated modifications are indicated on the *side*. (C,D) Analyses similar to those in A and B, respectively, using primer HVLSUR2, to determine the modification status of U2605 of 23S rRNA. (E,F) Analyses similar to those in A and B, respectively, using primer HVLSUR3, to determine the modification status of U2591 of 23S rRNA.

FIGURE 3. *cbf5* deletion does not significantly reduce the amount of Ψ in *H. volcanii* total tRNAs and at tRNA position 55. Nuclease P1 digests of uniformly labeled tRNAs were resolved by thin layer chromatography. pA, pC, pG, pU, p Ψ , and pm¹ Ψ indicate 5'-phosphorylated A, C, G, U, Ψ , and m¹ Ψ , respectively. (A,B) Digests of total tRNA and tRNA^{Trp}, respectively, from WT (upper panels) and $\Delta cbf5$ strains (lower panels). Ratio of Ψ and m¹ Ψ is indicated in each panel.

either due to its role in tRNA Ψ 54 synthesis or in Ψ production at a presently unidentified position in other cellular RNAs. Alternatively Pus10 may be essential because of a function not related to pseudouridylation such as a possible chaperone function during tRNA maturation or being part of a multiprotein complex having some non-pseudouridylation function. Furthermore, Pus10 might not be essential in other Archaea. The in vitro Ψ 54 activity of Pus10 from *M. jannaschii* is more robust than of Pus10 from *P. furiosus*, which may be a reflection of their in vivo functions (Gurha and Gupta 2008). Indeed, Thermococcales seem to have mostly 5-methyluridine (m⁵U or rT) as 5-methyl-2-thiouridine (m⁵s²U or s²T) in tRNA and few Ψ 54 (or m¹ Ψ 54) (Edmonds et al. 1991; Kowalak et al. 1994; Constantinesco et al. 1999; Urbonavicius et al. 2008). If this were the case, then it would be interesting to test if *pus10* is essential in *Pyrococcus* sp.

Cbf5 is not needed for most of the tRNA Ψ residues of *H. volcanii*, but TruA/Pus3 homolog is needed for Ψ 39

We determined the Ψ status at certain other positions of tRNAs in Cbf5-deleted strains of *H. volcanii*, in addition to the Ψ 55 mentioned above, by primer extension following U-specific and CMCT reactions with RNAs of WT and $\Delta cbf5$ strains using elongator tRNA^{Met}-specific primers (Fig. 5).

Both strains show the presence of Ψ 13 and Ψ 22 in tRNA^{Met} (Fig. 5A,B), suggesting that neither the formation of Ψ 13 nor Ψ 22 are Cbf5-mediated. Probably Ψ 13 is produced by TruD/Pus7 because a homolog is present in *H. volcanii* (Grosjean et al. 2008), and *P. abyssi* Pus7 can produce Ψ 13 in vitro (Muller et al. 2009).

Similar analysis using CMCT reactions suggests that Ψ 39 in elongator tRNA^{Met} is also not Cbf5-mediated (Fig. 5C,D) thus is not guide RNA dependent, whereas 2'-O-methylation of U at the same position 39 in tRNA^{Trp} of *H. volcanii* is guide RNA dependent (Clouet d'Orval et al. 2001; Singh et al. 2004). Ψ 39 in tRNAs of Bacteria and Eukarya is produced by TruA and Pus3, respectively. Therefore, we deleted the gene encoding their *H. volcanii* homolog, *HVO_1852* (data not shown). Primer extension following U-specific and CMCT reactions using WT and ΔHVO_1852 strains (Fig. 5C,D) shows that indeed as for Bacteria and Eukarya the TruA/Pus3 homolog in *H. volcanii* catalyzes the formation of Ψ 39 in tRNA, further con-

firming that it is not Cbf5-mediated. Furthermore, these results show that *pus3* in *H. volcanii* is not essential.

In addition to Ψ at positions 13, 39, and 55 (and m¹ Ψ 54), Ψ is also present at four other positions in *H. volcanii* tRNAs and only in one tRNA for each of these additional positions. These are Ψ 22 in elongator tRNA^{Met}, Ψ 28 in tRNA^{Ile}, Ψ 38 in tRNA^{Pro}, and Ψ 52 in tRNA^{Lys}, the last three being only in the major isoacceptor for these amino acids (Gupta 1984; Grosjean et al. 2008). While we have demonstrated above that Ψ 22 is not Cbf5-mediated, we were unable to obtain clear evidence as to whether Cbf5 has any role in the production of Ψ 28. We did not attempt to determine Ψ 38 and Ψ 52 production in this analysis, because both of these show partial modification of tRNAs (Gupta 1984). The ratio of U vs. Ψ can vary from a tRNA preparation to another, which can affect the analysis of the data. However, based on the activities of *E. coli* TruA and *S. cerevisiae* Pus3 (Kammen et al. 1988; Nurse et al. 1995; Lecoite et al. 2002), we can assume that Ψ 38 is also produced by their homolog in *H. volcanii*. At present it is difficult to predict any Ψ synthase for the other three positions (22, 28, and 52) with any certainty.

cbf5 deletion reduces the amounts of guide box H/ACA RNAs in *H. volcanii*

Two box H/ACA guide RNAs have been proposed for production of Ψ in *H. volcanii* 23 rRNA: one for U2605

FIGURE 4. *pus10* is essential in *H. volcanii*. *pus10* (HVO_1979) could only be deleted from the chromosome when expressed in *trans*. (A) H26 (WT) and two independent isolates VDC2606 and VDC2607 (Δ HVO_1979 with plasmid-borne copy of HVO_1979 under P_{tna} control) were serially diluted from 10^0 to 10^{-4} and spotted onto Hv-Min media plates, plus and minus 500 μ M tryptophan. Significant growth of the mutant is only observed in the presence of tryptophan. (B) Deletion of the chromosomal copy of HVO_1979 was further verified by PCR using primers Pus10_Ext_Fwd and Pus10_Ext_Rev. Predicted sizes of the amplicon are indicated above each band.

and another one for both U1940 and U1942 (Grosjean et al. 2008). Both of these RNAs are present in the cell as shown by Northern hybridizations of WT RNA using appropriate probes (Fig. 6B). Interestingly, signals for the probes were greatly reduced in the Δ *cbf5* strain, but the signals return to WT levels when the mutant strain is supplemented with a plasmid-borne copy of *cbf5*. This suggests that, in the absence of Cbf5, H/ACA RNAs are destabilized and get degraded. This would be expected if these RNAs normally formed H/ACA RNP complexes with Cbf5 and other proteins and were thereafter protected against cellular RNA nucleases. Similar situations are observed in yeast, where genetic depletion of Cbf5 leads to depletion of all box H/ACA snoRNAs (Lafontaine et al. 1998) and in *T. brucei*, where RNAi-mediated depletion of Cbf5 destabilized box H/ACA snoRNAs (Barth et al. 2005). Our results in Figures

2A–D and 6B together suggest that Ψ 1940, 1942, and 2605 in 23S rRNA are produced by H/ACA RNPs. This is confirmed for the position 2605 by our in vitro pseudouridylation assays using appropriate guide RNAs, and a 23S rRNA fragment containing U2605 and recombinant *M. jannaschii* proteins (M Majumder and R Gupta, unpubl.).

A double stem-loop box H/ACA RNA in *H. volcanii*

Both probes used for Northern analyses seemed to hybridize to one RNA of $\sim$ 200 bases (Fig. 6B). The two sRNA sequences are located near each other in the *H. volcanii* genome (<http://archaea.ucsc.edu>) and are separated by a 59-base spacer region between the two stem-loops. Therefore, we used the same two probes to determine the 5' ends of the two RNAs. As seen in Figure 6C,D, both RNAs have the same 5' end, suggesting that both RNAs are part of the same molecule. Reduced signals in the Δ *cbf5* strain (Fig. 6C,D) again confirm the reduced level of this box H/ACA RNA in the mutant strain and the recovery to original levels when the strain is supplemented with a plasmid-borne copy of *cbf5*. Archaeal H/ACA RNAs mostly have either one or three stem-loops, though two stem-loops have also been observed, albeit rarely (Dennis and Omer 2005; Muller et al. 2008). This *H. volcanii* RNA is a rare case of archaeal H/ACA RNA containing two stem-loops. Normally spacer regions between the stem-loops of archaeal H/ACA RNAs are very short (Grosjean et al. 2008; Muller et al. 2008). However, this double stem-loop RNA has a spacer of 59 bases (or 56 bases excluding ACA of the stem-loop on the 5' side). The spacer length between homologs of these two stem-loops among haloarchaea varies from 21 to 142 bases, but other Archaea have smaller spacers from 0 to 5 bases (Grosjean et al. 2008). This spacer of *H. volcanii* potentially can form a pseudoknot structure (inset in Fig. 6A). Interestingly, this spacer of all haloarchaeal species can also be folded into a pseudoknot structure (data not shown). Generally pseudoknots have some kind of regulatory role but it is not yet known if this structure between the two H/ACA RNAs forms in vivo and whether it has any role there.

CONCLUSIONS

In conclusion, we present here the analysis and confirmation of several proteins previously predicted to be involved in pseudouridylation of ribonucleic acids in *H. volcanii*. We demonstrate that Cbf5 is responsible for making Ψ at multiple positions of the large subunit of rRNA. Contrary to suggestions made by previous in vitro based assays Cbf5 does not appear to be involved in tRNA modification. Furthermore, while essential in yeast, *cbf5* is dispensable in *H. volcanii*, although its deletion does result in a slight growth defect. However, another protein suspected to be involved in pseudouridylation of at least U55 of tRNAs,

FIGURE 5. *H. volcanii* tRNA Ψ 13, Ψ 22, and Ψ 39 are not Cbf5-mediated and Ψ 39 is TruA/Pus3-mediated. (A) U-specific analyses to determine the modification status of U13 and U22 of elongator tRNA^{Met} were done using primer HVMETCR2 and total tRNAs of WT and $\Delta cbf5$ strains. (Lanes 1,2) Primer extensions without and with U-specific reactions, respectively. Positions of certain Us and Ψ 13 and Ψ 22 in tRNA are indicated on the side. (B) CMCT-primer extension analyses to determine the modification status of U13 and U22 of tRNA^{Met} were done using primer HVMETCR2 and total tRNAs of WT and $\Delta cbf5$ strains. Total tRNAs were treated with (+) or without (–) CMCT for the indicated time (in minutes), followed by alkali (OH⁻) treatment (+) or no treatment (–). Positions of Ψ 13 and Ψ 22 are indicated on the side. (C,D) Analysis similar to those in A and B, respectively, using primer VM5/6R, to determine the modification status of U39 of tRNA^{Met} in WT (H26), $\Delta pus3$ (VDC2465), and $\Delta cbf5$ (VDC2364) strains.

Pus10, is necessary for viability. Finally we demonstrate that HVO_1852, the TruA homolog, is responsible for Ψ 39 in tRNA.

It is important to note that the results reported here might be specific to halophilic Archaea. Compared to *E. coli* or yeast, *H. volcanii* clearly contains a lower number of RNA modifications (both in tRNA and rRNA) and comparative genomics analysis suggests the number of RNA modifications in certain halophilic Archaea could be reduced even more. Indeed, as far as tRNAs are concerned, *H. volcanii* has already lost the *tyw1* signature gene for the imG-14 modification (Waas et al. 2005) that is present in

H. salinarium (de Crécy-Lagard et al. 2010). *Haloquadratum walsbyi*, which lives in an environment with a much more stable salinity level than *H. volcanii* (Burns et al. 2007), has lost not only the *tyw1* gene like *H. volcanii* but also the *tgtA* gene involved in the formation of archaeosine (Watanabe et al. 1997) and the gene encoding COG 1444 that has been shown to be involved in ac⁴C formation (El Yacoubi et al. 2009). A possibility is that high salt concentration can replace specific modifications in these organisms (discussed in Grosjean et al. 2008). It is therefore crucial to repeat the experiments reported here with non-halophilic Archaea before any generalization is made.

FIGURE 6. Levels of a double stem-loop box H/ACA guide RNA are reduced in the absence of Cbf5. (A) Primary sequence and predicted secondary structures of two H/ACA guide RNAs proposed (Grosjean et al. 2008) to be responsible for guiding pseudouridylation of U2605 and U1940/U1942 of *H. volcanii* 23S rRNA. The 59-base region that separates the two RNAs in the genome can potentially form a pseudoknot (*inset*). Pseudoknot is drawn according to pknotsRG-mfe (<http://bibiserv.techfak.uni-bielefeld.de/pknotsrg/>). Conserved box H and box ACA are underlined. G•A pairs of K-turns are boxed. Positions for probe 1 (HV1940/42HAR) and probe 2 (HV2605HAR) are indicated by lines along the sequences. (B) Northern blot analysis of total RNA isolated from WT and $\Delta cbf5$ strains, and $\Delta cbf5$ transformed with pHCBf5. Blot was hybridized using probes 1 and 2 (shown in A). Both probes hybridized to an ~200-base RNA. 5S rRNA-specific probe was used as a loading control. Quantity of RNA in each hybridized band is indicated. (C) Primer extension and sequencing reactions using probe 2 to determine 5' ends of the RNAs. Equal amounts of total RNAs from WT and $\Delta cbf5$ strains and $\Delta cbf5$ transformed with pHCBf5 were used in the reactions. Sequence in the region of primer stop is indicated on the side. Asterisk G indicates the 5' end of the RNA. (D) Reactions similar to C, using probe 1.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, media, and transformation procedures

All strains, plasmids, and oligonucleotides used in this study are detailed in Supplemental Tables S1, S2, and S3, respectively. *H. volcanii* H26 was received from Julie Maupin-Furlow as well as from Thorsten Allers. Plasmids pJAM202 (Kaczowka and Maupin-Furlow 2003) and pMDS132, a derivative of pMLH32 (Holmes and Dyall-Smith 2000), were gifts from Julie Maupin-Furlow and Michael Dyall-Smith, respectively. H26 and VDC2364 (prepared in this work) were used in this study as WT and $\Delta cbf5$ strains of *H.*

volcanii. *E. coli* was routinely grown in LB (Fisher) or LB agar (Fisher) at 37°C, supplemented when required with ampicillin (100 µg/mL), isopropyl β-D-1-thiogalactopyranoside (0.2 mM), and bromo-chloro-indolyl-galactopyranoside (40 µg/mL). *H. volcanii* cells were routinely grown at 42°C–44°C (unless specified) in Hv-YPG, Hv-CA, and Hv-Min as described in the HaloHandbook (http://www.haloarchaea.com/resources/halo handbook/Halo handbook_2008_v7.pdf) or in a standard medium as described previously (Gupta 1984). Transformations of *E. coli* were performed by standard protocols (Sambrook and Russell 2001) or as described by the manufacturer (Invitrogen). Transformation of *H. volcanii* was performed as described in the HaloHandbook using the “standard PEG-mediated transformation of haloarchaea” protocol. Briefly, spheroplasts were made by adding 100 µL 0.5 M EDTA (pH 8.0) to 1 mL concentrated cells, incubating at room temperature for 10 min; 2 µg plasmid DNA was then added to 100 µL spheroplasts and incubated for 5 min; 100 µL 60% (v/v) PEG600 was added and mixed gently, and the mixture incubated at room temperature for 30 min. Cells were recovered by adding recovery solution, as described in the HaloHandbook, and incubated for 4 h at 42°C–44°C before plating onto the appropriate media. Phenotypes (growth rate, and growth at different salt concentrations and temperature) were tested as described previously (Blaby et al. 2010).

Deletion strain and plasmid construction

Plasmids to delete *H. volcanii* genes were constructed as described previously (Blaby et al. 2010). Briefly, regions of ~600 bp upstream of and downstream from the gene to be deleted, along with a small portion of the gene at both ends to avoid affecting neighboring genes, were PCR-amplified from *H.*

volcanii genomic DNA and recombined between the *Xho*I and *Eco*RI sites of pTA131 using InFusion (Clontech); 0.2 µL of the reaction mixture was transformed to TOP10 chemically competent cells (Invitrogen) and plated onto LB plus ampicillin (100 µg/mL), isopropyl β-D-1-thiogalactopyranoside (0.2 mM), and bromo-chloro-indolyl-galactopyranoside (40 µg/mL). Clones were screened by PCR using M13 primers (which anneal on either side of the multiple cloning site of pTA131) using 5' Master Mix (Eppendorf) as per the directions, and confirmed by sequencing using the UF Sequencing facility.

The plasmid generated to disrupt HVO_2493 (pIKB219) was constructed as described above using oligonucleotide pairs HVO2493_N_IfKO_Fwd, HVO2493_N_IfKO_Rev, HVO2493_C_IfKO_Fwd,

and HVO2493_C_IfKO_Rev. The plasmid constructed to delete HVO_1979 (pIKB180) was made using oligonucleotides HVO_1979_N_IfKO_Fwd, HVO_1979_N_IfKO_Rev, HVO_1979_C_IfKO_Fwd, and HVO_1979_C_IfKO_Rev. The plasmid used to delete HVO_1852 (pIKB199) was made using HVO_1852_N_IfKO_Fwd, HVO_1852_N_IfKO_Rev, HVO_1852_C_IfKO_Fwd, and HVO_1852_C_IfKO_Rev.

Once obtained, deletion plasmids were passed through a *dam*[−] *E. coli* (INV110; Invitrogen), transformed into *H. volcanii* H26 (or derivatives) as described above. Deletion of the targeted locus was selected in a two-step process as described previously (Allers et al. 2004). Briefly, insertion of the deletion plasmid by a single crossover event was selected for by ability to grow on Hv-CA (i.e., in the absence of uracil). Subsequent excision of the integrated plasmid and target gene by a second recombination event was selected for by plating onto Hv-CA supplemented with uracil (10 µg/mL) and 5-fluoroorotic acid (5-FOA; 50 µg/mL). Gene deletion candidates were screened using a PCR-based method as follows. One pair of primers (denoted by the suffix _Ext_Fwd and _Ext_Rev) was designed to anneal within the flanking regions of the gene to be deleted, and the amplicon compared to WT and predicted size. To confirm loss of the gene, a second pair of primers (suffixed _Int_Fwd and _Int_Rev) was designed to anneal within the gene to be deleted. Gene deletions were verified by amplification of the fragment in the WT, but inability in mutant strains.

Other plasmid and strain constructions

pHCbf5 was generated by replacing ~420 bp *NdeI*-*EcoRI* fragment of pMDS132 with an ~890 bp PCR fragment produced by amplifying HVO_2493 using HVCBF5ND-F2 and HVCBF5-R2 primers. An amplified gene contains six His codons just before the stop codon. The reaction mixture was used to transform DH5α cells. Plasmids were verified by PCR, restriction digestion, and sequencing, and then passed through *E. coli* GM2929 and transformed into *H. volcanii* strain VDC2364 (Δ *cbf5* strain). Transformants were selected for by plating onto Hv-YPC containing novobiocin (0.8 µg/mL).

A fragment from the *H. volcanii* genome containing the *P_{tna}* promoter was PCR-amplified using primers Ptna_5 and rev-Ptna, and inserted between the *BlnI* and *XbaI* sites of pTA131, to generate pPT002. *pus10* was subsequently PCR-amplified using primers Pus10_tna_Nde_Fwd and Pus10_tna_Rev from *H. volcanii* genomic DNA and inserted between the *NdeI* and *EcoRV* sites of pPT002 to generate pIKB487. Subsequent deletion of the chromosomal copy of *pus10* was performed as described by Allers and colleagues (Allers et al. 2004; Large et al. 2007) using pIKB180 to disrupt the gene in the strain transformed with pIKB487.

Mapping the sites of Ψ in RNAs

Presence of Ψ in RNA was analyzed by the 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT) modification technique (Ofengand et al. 2001a; Motorin et al. 2007). Briefly, 20 µg total RNA or 10 µg gel-purified tRNA was treated with CMCT for 2, 10, and 20 min each at 37°C, while an untreated sample was simply incubated at 37°C for 20 min as control. After precipitation of the RNA, alkali treatment was done for 3 h at 37°C for all samples except for the ones treated with CMCT for 2 min. Again after precipitation, the RNA was used for primer extension

using [5′-³²P]-labeled primers that hybridized close to and on the 3′ side of the position to be mapped for Ψ. M-MLV reverse transcriptase (Promega) was used for primer extension according to the manufacturer's instructions. The extension stops at one residue before the CMCT modified Ψ. A dark band in CMCT followed by alkali treatment lanes, with an increased intensity in the 20 min treatment lane, indicates the presence of Ψ at that position. The position of the band in the gel that corresponds to Ψ is determined by its distance from the end of the primer and sometimes the presence of darker bands for U and G in 2 min CMCT but no alkali treatment lane, and correlation with the known sequence of the RNA.

U-specific sequencing reactions (Peattie 1979; Gupta 1984) followed by primer extension were used to determine the positions of U (absence of Ψ) in the RNAs. Briefly, 20 µg total RNA or 10 µg gel-purified tRNA was treated with 50% hydrazine for 20 min on ice and precipitated. The RNA was then treated with acidified aniline at 60°C for 20 min. Precipitated RNA was then used for primer extension as above for the CMCT reaction. Here also the position of the primer extension band in the gel is one residue before the expected U. A dark band in the hydrazine-aniline treated lane but not in the untreated lane indicates the presence of an unmodified U at that position. A correlation between the position of the dark band in the gel relative to the end of the primer and positions of unmodified Us in the known sequence of the RNA determines the position of Ψ.

Quantitation of Ψ residues in tRNAs

Uniformly labeled total tRNA was prepared as described before (Gupta 1984; Joardar et al. 2008). When needed, uniformly labeled tRNA^{Trp} was isolated from this total tRNA according to previously published procedures (Joardar et al. 2008). Labeled tRNAs were digested with nuclease P1 and digests were resolved by two-dimensional thin-layer chromatography on cellulose plates (EM Science) using isobutyric acid/0.5 N NH₄OH (5:3, v/v) in the first dimension and isopropanol/HCl/H₂O (70:15:15, v/v/v) in the second dimension (Gupta 1984). Radioactivity in the plates was revealed and quantified by phosphorimaging. The amount of Ψ per tRNA was determined by the ratio of radioactivities in Ψ vs. m¹Ψ nucleotide spots. The reasons for using m¹Ψ spot for comparison are because nearly all *H. volcanii* tRNAs have one m¹Ψ residue per tRNA (Gupta 1984, 1986), Ψ for this m¹Ψ is produced by Pus10 (Gurha and Gupta 2008) not by Cbf5, and this nucleotide is well separated in our chromatography system.

Southern and Northern hybridizations, and mapping the 5′ ends of guide RNAs

The genomic deletion of *cbf5* was checked by Southern hybridization of *EcoRI*-digests of DNA using standard procedures (Sambrook and Russell 2001). The blots were hybridized with [5′-³²P]-labeled HVCBF5-F primer. For Northern analyses, total RNA was isolated from the cells using TRI reagent (Molecular Research Center, Inc). The RNA was separated by a 6% denaturing PAGE. The blots were prepared and hybridized to [5′-³²P]-labeled probes according to standard procedures (Sambrook and Russell 2001). The blot was stripped (incubated in 10 mM Tris-Cl pH 7.4, 0.2% SDS at 70°C for 2 h) after hybridization for use with a second probe. Radioactivity was detected and quantified (when needed) by phosphorimaging. The amount of H/ACA RNA in Northern blots was quantitated by using

a band for 5S rRNA in each lane as a loading control and normalizing each H/ACA RNA band in that lane against these controls. The amount of RNA in WT is considered 100% in each case. To determine 5' ends of the guide RNAs the primers used in Northern analyses were 5'-³²P labeled and used with total RNA for extension reactions.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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H. volcanii H26 was received from Julie Maupin-Furlow (University of Florida) as well as from Thorsten Allers (University of Nottingham, UK). Plasmids pJAM202 and pMDS132 were gifts from Julie Maupin-Furlow and Michael Dyall-Smith (Charles Sturt University, Australia), respectively. We thank Wayne Decatur for the help in correlating the positions of Ψ in *H. volcanii* with those of other haloarchaea and *E. coli*. We thank Patrick Thiaville for the construction of pTA002. H.G. holds a CNRS position of Emeritus Scientist at the University Paris-South in Orsay-France. This work was funded in part by National Science Foundation grant number MCB-05169448 to V.d.C.-L. and by NIH grant number GM055945 to R.G.

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