Template-dependent 3′–5′ nucleotide addition is a shared feature of tRNA$^{\text{His}}$ guanylyltransferase enzymes from multiple domains of life

Maria G. Abad[a], Bhalchandra S. Rao[a,b], and Jane E. Jackman[a,b,c,1]

[a]Department of Biochemistry and Center for RNA Biology,[b]Molecular Cell and Developmental Biology Program, and [c]The Ohio State Biochemistry Program, The Ohio State University, Columbus, OH 43210

The presence of an additional 5′ guanosine residue (G_{-1}) is a unique feature of tRNA$^{\text{His}}$. G_{-1} is incorporated posttranscriptionally in eukarya via an unusual 3′–5′ nucleotide addition reaction catalyzed by the tRNA$^{\text{His}}$ guanylyltransferase (Thg1). Yeast Thg1 catalyzes an unexpected second activity: Watson–Crick-dependent 3′–5′ nucleotide addition that occurs in the opposite direction to nucleotide addition by all known DNA and RNA polymerases. This discovery led to the hypothesis that there are alternative roles for Thg1 family members that take advantage of this unusual enzymatic activity. Here we show that archaeal homologs of Thg1 catalyze G_{-1} addition, in vitro and in vivo in yeast, but only in a templated reaction, i.e. with tRNA$^{\text{His}}$ substrates that contain a C_{73} discriminator nucleotide. Because tRNA$^{\text{His}}$ from archaea contains C_{73}, these findings are consistent with a physiological function for templated nucleotide addition in archaeal tRNA$^{\text{His}}$ maturation. Moreover, unlike yeast Thg1, archaeal Thg1 enzymes also exhibit a preference for template-dependent U_{-1} addition to A_{73}-containing tRNA$^{\text{His}}$. Taken together, these results demonstrate that Watson–Crick template-dependent 3′–5′ nucleotide addition is a shared catalytic activity exhibited by Thg1 family members from multiple domains of life, and therefore, that this unusual reaction may constitute an ancestral activity present in the earliest members of the Thg1 enzyme family.

archaea | G-1 addition | reverse polymerase | S. cerevisiae

An additional G-residue (G_{-1}) at the 5′ end of tRNA$^{\text{His}}$ is a nearly universal feature, whose loss results in decreased aminoacylation of tRNA$^{\text{His}}$ both in vitro and in vivo, as observed in organisms from *E. coli* to yeast (1–6). The only organisms known to lack G_{-1} on tRNA$^{\text{His}}$ are 20 α-proteobacteria (7) in which there is a simultaneous alteration in the histidyl-tRNA synthetase (HisRS) that is suggested to compensate for loss of this universal tRNA feature (8, 9).

Although the presence of G_{-1} on tRNA$^{\text{His}}$ is highly conserved, the mechanism by which G_{-1} is incorporated into tRNA varies in different domains of life. In bacteria, G_{-1} is genomically encoded and present in the precursor-tRNA transcript (10). The presence of G_{-1} and a universally conserved C_{73} discriminator nucleotide results in formation of an unusual 8-base pair acceptor stem in bacterial tRNA$^{\text{His}}$ that directs the tRNA 5′-end maturation enzyme, RNase P, to cleave at an alternate site, leaving G_{-1} in the mature tRNA (Fig. 1*A*) (11). In eukarya, the presence of G_{-1} is the result of a posttranscriptional nucleotide addition reaction, catalyzed in yeast by the essential enzyme tRNA$^{\text{His}}$ guanylyltransferase (Thg1) (Fig. 1*B*) (12, 13). The widespread conservation of Thg1 throughout eukarya, including humans, suggests a similar mode of G_{-1} addition.

Thg1 adds G_{-1} by an unusual 3′–5′ nucleotide addition reaction, via an unknown molecular mechanism that can not be predicted, due to the lack of sequence similarity between Thg1 and any known enzyme family. In yeast, G_{-1} is added opposite an A_{73} discriminator nucleotide that is universally conserved throughout eukarya (Fig. 1*B*) (14). However, in vitro, wild-type yeast Thg1

exhibits a second biochemical activity with variant tRNA$^{\text{His}}$ substrates that contain alterations at A_{73}. Use of these substrates revealed that wild-type yeast Thg1 adds multiple G or C residues to the 5′ end of the tRNA in a reaction that uses the 3′ acceptor stem nucleotides as a template for 3′–5′ addition (Fig 1*B*) (15). Although nontemplated (G opposite A) nucleotide addition plays a well-documented physiological role in the maturation of tRNA$^{\text{His}}$, the biological significance of templated 3′–5′ nucleotide polymerization, in yeast or any other organism, has not been demonstrated. Yet the ability of yeast Thg1 to recognize and add Watson–Crick base-paired nucleotides was completely unexpected for an enzyme that seemingly only functions to add G_{-1} opposite A_{73} in tRNA$^{\text{His}}$. Moreover, the ability of yeast Thg1 to add deoxynucleotides as well as ribonucleotides to tRNA substrates, as well as the observation of templated 3′–5′ addition with other tRNA substrates in vitro suggest that alternative roles for templated addition are a possibility (15).

Both bacteria-like and eukarya-like systems for incorporation of G_{-1} into tRNA$^{\text{His}}$ appear to be present in archaea. Of 55 annotated archaeal tRNA$^{\text{His}}$ genes (16), 40 contain a genomically encoded G_{-1} residue and a C_{73} discriminator nucleotide, suggesting that G_{-1} could be incorporated into tRNA$^{\text{His}}$ using the RNase P-dependent pathway. However, the remaining 15 annotated archaeal tRNA$^{\text{His}}$ genes lack a G residue at the −1 position in their coding sequence. Notably, homologs to yeast Thg1 are present in all of these species with the exception of *N. equitans* (17), consistent with a requirement for enzymatic G_{-1} addition in these organisms (Fig. 2). Intriguingly, among the 40 archaea that already contain a genomically encoded G_{-1} residue, 7 of these also contain a Thg1 homolog (Fig. 2), thus these organisms may use either, or both, pathways for addition of G_{-1} to tRNA$^{\text{His}}$.

The discriminator nucleotide in archaeal tRNA$^{\text{His}}$ is universally a C_{73}, as it is in bacteria (14), raising the possibility that enzymatic G_{-1} addition in archaea could require a templated 3′–5′ nucleotide addition reaction similar to that previously associated with yeast Thg1 reverse (3′–5′) polymerization activity. Although templated 3′–5′ nucleotide addition activity appears to be required for an unusual form of 5′-tRNA editing in certain protozoan mitochondria (18, 19), the enzyme(s) that catalyze this reaction remain to be identified. Thus, archaeal Thg1 has the potential to be a unique example of a purified enzyme that catalyzes templated 3′–5′ nucleotide addition with a demonstrated biological relevance.

In this study, we investigated the biochemical function of Thg1 homologs from four archaea. The results show that both yeast
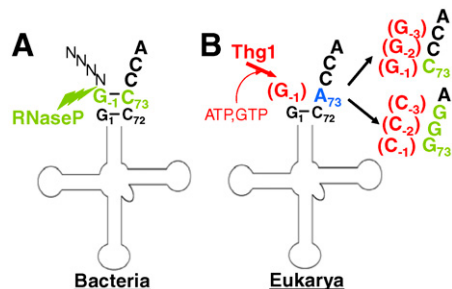
Fig. 1. Domain-dependent incorporation of G_{-1} into tRNA^{His}. (A) In bacteria, G_{-1} is genomically encoded and retained by RNase P during 5′-processing. (B) Thg1 adds G_{-1} to eukaryal tRNA^{His}, and catalyzes template-dependent 3′–5′ addition to tRNA^{His} variant substrates. Nucleotides added posttranscriptionally by Thg1 are indicated by parentheses. For clarity, only the altered 3′ end sequences of tRNA^{His} variant substrates are shown.

and archaeal Thg1 enzymes catalyze templated 3′–5′ nucleotide addition, therefore this property appears to be a shared feature of Thg1 enzymes from multiple domains of life. In contrast, the nontemplated reaction catalyzed by eukaryal Thg1 during maturation of tRNA^{His} may be a derived evolutionary trait unique to eukarya.

Results

Archaeal Thg1 Homologs Do Not Efficiently Add G_{-1} to A_{73}-Containing tRNA^{His}. To test the activities of archaeal Thg1 enzymes, we cloned, expressed, and purified homologs from *M. thermoautotrophicus* (MtThg1), *M. kandleri* (MkThg1), *M. acetivorans* (MaThg1), and *M. barkeri* (MbThg1) (see *SI Text*). These four archaea include two that are predicted to require enzymatic addition of G_{-1} to tRNA^{His} (Mt and Mk) and two in which G_{-1} is already encoded in the genome (Ma and Mb) (Fig. 2). The MaThg1 coding sequence
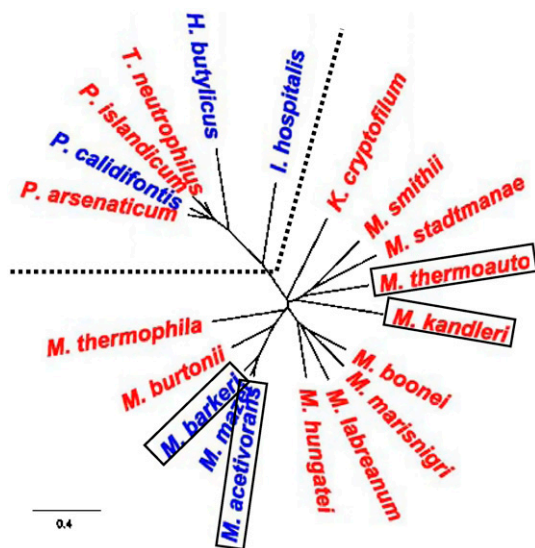

Fig. 2. Phylogeny of archaeal Thg1 homologs. An unrooted phylogenetic tree of the archaeal Thg1 homologs was constructed with Geneious Pro software (v.4.7.5) using the neighbor-joining method with a Jukes–Cantor genetic distance model and no outgroup. The tree was built upon a ClustalW alignment (36) of Thg1 sequences from 20 of 21 Thg1-containing archaea (*P. aerophilum* Thg1 is similar to the other three *Pyrobaculum* homologs and was omitted for clarity). Organism names are colored according to the presence of G_{-1} on the annotated tRNA^{His}; blue, G_{-1} is genomically encoded; red, G_{-1} is not present in the genomic sequence. The four archaea investigated in this work are indicated by boxes. The dashed line indicates the separation between crenarchaeal (*Upper Left*) and euryarchaeal (*Lower Right*) clades.

is interrupted by an in-frame amber stop codon, and appears to be a previously undocumented case of nonsense suppression by insertion of pyrrolysine at this position, as is seen with other proteins from methanobacteria, although the presence of pyrrolysine is not required for activity (see *SI Text*) (20).

We first tested the ability of each purified archaeal Thg1 to add G_{-1} to yeast tRNA^{His} that contains the universally conserved eukaryal A_{73} discriminator nucleotide. G_{-1} addition activity was tested using a previously described assay with 5′-^{32}P labeled monophosphorylated tRNA^{His} (p-tRNA^{His}) (21). Briefly, following incubation of purified Thg1 with the labeled tRNA, the reactions are treated with RNase A, followed by phosphatase, finally yielding inorganic phosphate (^{32}P$_i$) from unreacted substrate, and a labeled G_{-1}p*GpC trimeric oligonucleotide if the G_{-1} addition product is formed during the reaction (Fig. 3A). No products were observed in reactions with MtThg1 and this A_{73}-containing tRNA, whereas reaction products were observed with the other three archaeal enzymes (MkThg1, MaThg1, and MbThg1) (Fig. 3A; compare lane 2 with lanes 3–5). However, the primary reaction product of these enzymes' activity migrates slightly lower in the TLC solvent system used for this assay than the G_{-1}-containing product produced by yeast Thg1. This slower migrating product corresponds to the adenylylated-tRNA^{His} reaction intermediate (App*G_{+1}pC) produced by yeast Thg1 (13). The major reaction product observed with all of the archaeal enzymes (a representative analysis with MaThg1 is shown) is only dependent on addition of ATP (Fig. 3B), and migrates identically to the adenylylated tRNA^{His} species produced by yeast Thg1, as demonstrated by nuclease analysis of isolated reaction products (13).

Adenylylated-tRNA^{His} is the terminal product of archaeal Thg1 activity with A_{73}-tRNA^{His}. Measurements of total product formation as a function of decreasing Thg1 concentration reveal similar amounts of App-tRNA^{His} and G_{-1}-tRNA^{His} formed at the same low concentrations (0.4 µM) of MaThg1 or yeast Thg1, respectively (Fig. 3C), indicating that the overall activity of MaThg1 is not significantly lower than the yeast enzyme. Instead,
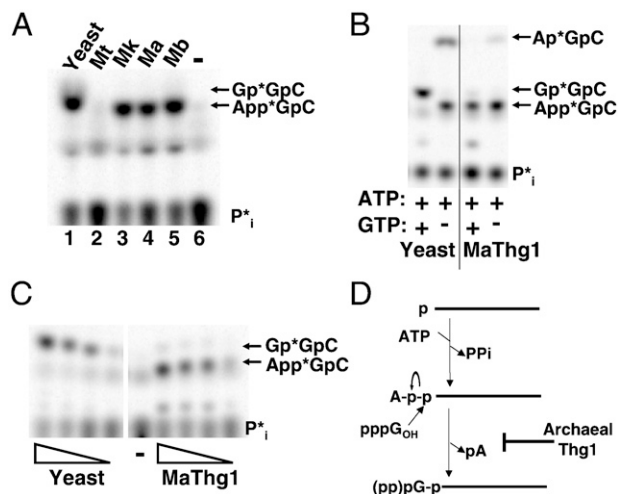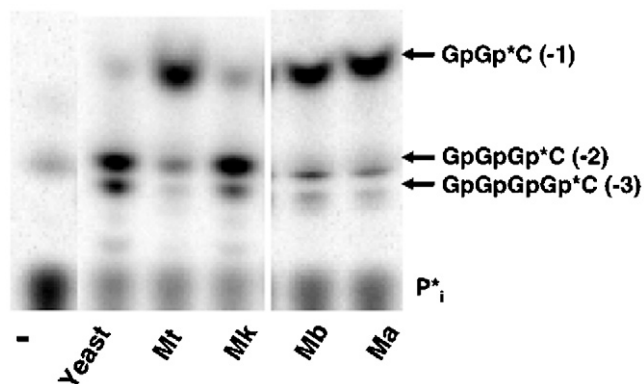

Fig. 3. Archaeal Thg1 homologs do not efficiently add G_{-1} to wild-type yeast tRNA^{His}. (A) Assays using 5′-^{32}P-labeled tRNA^{His} and 1 µL each Thg1, as indicated, were performed with 0.1 mM ATP and 1.0 mM GTP as described in *Methods*. Lane-, buffer only. Reaction products are labeled to the right of each panel, and indicated by arrows. (B) 5′-^{32}P-tRNA^{His} assay performed with 1 µL yeast or MaThg1. The presence or absence of ATP or GTP (1 mM) is denoted by (+) and (–). (C) Assays of 5′-^{32}P-tRNA^{His} with titrations (5-fold serial dilutions, starting with approximately ~50 M enzyme) of purified yeast or MaThg1; Lane-, buffer only control reaction. (D) Scheme of steps for G_{-1} addition to tRNA^{His} showing first, adenylylation of p-tRNA^{His}, followed by nucleotidyltransfer to yield G_{-1}-containing tRNA. The apparent block in the 2nd step of the reaction observed with archaeal Thg1 is as indicated.

the persistent accumulation of the App-tRNA^{His} intermediate even at the highest concentrations tested (46 μM) suggests that archaeal Thg1 enzymes can not catalyze the second, guanylyl-transfer step of the three-step Thg1 reaction with A$_{73}$-tRNA^{His} (Fig. 3D) (13).

Archaeal Thg1 homologs Efficiently Add G$_{-1}$ to C$_{73}$-Containing tRNA^{His} In Vitro.

We next tested the ability of archaeal Thg1 to add G$_{-1}$ to a yeast tRNA^{His} variant containing C$_{73}$ instead of A$_{73}$; this tRNA is a substrate for template-dependent $3'-5'$ polymerization by yeast Thg1 in vitro (Fig. 1B). In contrast to the inefficient addition of G$_{-1}$ to A$_{73}$-containing tRNA described above, all four archaeal Thg1 enzymes catalyzed efficient addition of G$_{-1}$ to this C$_{73}$-containing tRNA substrate (Fig. 4). Whereas yeast and MkThg1 catalyze the addition of multiple G-residues (G$_{-1}$, G$_{-2}$, and G$_{-3}$), the single G$_{-1}$ addition product is the predominant product observed with Ma, Mb, and MtThg1, and G$_{-2}$/G$_{-3}$ are added inefficiently, if at all (Fig. 4). No accumulation of adenylylated tRNA intermediate was observed with any of the archaeal Thg1 enzymes and C$_{73}$-tRNA^{His}.

To quantify the relative catalytic abilities of archaeal Thg1 enzymes, we measured the steady-state kinetic parameters for addition of G$_{-1}$ to C$_{73}$-containing tRNA^{His} catalyzed by MaThg1 and MtThg1, as representative enzymes from archaea that contain or lack, respectively, a genomically encoded G$_{-1}$ residue. For comparison, kinetic parameters for the same reaction with yeast Thg1 were also determined. Steady-state kinetic assays were performed with triphosphorylated (ppp − tRNA^{His}) substrates as previously described (21).

With yeast Thg1, k_{cat}/K_M for C$_{73}$-tRNA^{His} is essentially the same as for wild-type (A$_{73}$) tRNA^{His} (Table 1, *SI Text*). Despite the relatively slow k_{cat} exhibited by MaThg1 for C$_{73}$-tRNA^{His}, the significantly lower K_M results in only a 10-fold decreased k_{cat}/K_M for MaThg1 compared to the yeast enzyme (Table 1, *SI Text*). Initial determination of MtThg1 kinetic parameters similarly performed at 25 °C indicated very low activity, with a significantly decreased k_{cat} (*SI Text*). However, because *M. thermoautotrophicum* is a thermophile that grows optimally at 65 °C (22), MtThg1 activity was measured at 55 °C, where the enzyme exhibits maximal activity in the in vitro assay (*SI Text*). At this temperature, initial rates for MtThg1 activity at 2 μM and 4 μM tRNA were essentially unchanged, consistent with the K_M measured at 25 °C (*SI Text*). Thus, the k_{cat}/K_M calculated for MtThg1 under optimal temperature conditions is within 20-fold of that exhibited for yeast Thg1 (Table 1).
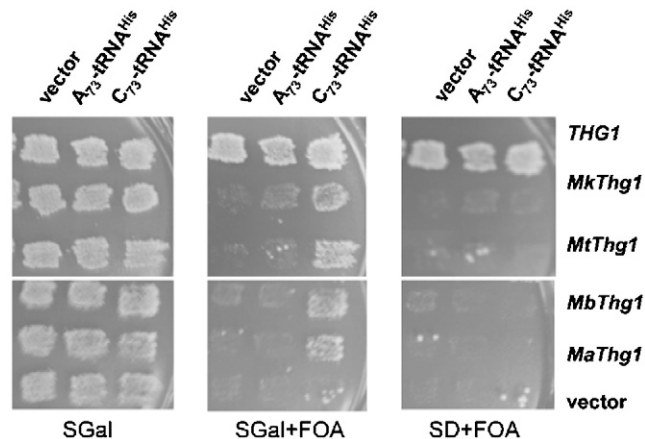
Archaeal Thg1 Homologs Complement the Essential Function of Yeast Thg1 in Yeast Cells When Provided with an Appropriate tRNA Substrate for Templated $3'-5'$ Addition.

To test in vivo function of the archaeal enzymes, we performed a variation of a previously described plasmid shuffle assay (13, 23). Archaeal Thg1 homologs (CEN LEU2) were introduced into the yeast *thg1Δ* strain JJY20 [relevant genotype: *thg1Δ leu2Δ ura3Δ (CEN URA3 PTHG1-THG1)*], and subsequently grown on 5-fluoroorotic acid (FOA)-containing media that selects against the yeast *THG1 URA3* covering plasmid, resulting in a strain in which the archaeal Thg1 enzymes are the only potential source of Thg1 activity in the cells. Consistent with the lack of G$_{-1}$ addition activity exhibited by archaeal Thg1 in vitro with wild-type yeast tRNA^{His} (Fig. 5), each archaeal Thg1-expressing strain was unable to grow on FOA (Fig. 5, compare growth of strains spotted in the first column for each of the three media shown), indicating that these archaeal Thg1 enzymes are not able to replace the essential function of yeast Thg1 in vivo in yeast.

We then tested whether the ability of the archaeal homologs to add G$_{-1}$ opposite C$_{73}$ could be exploited to support growth of the yeast deletion mutant. To the *thg1Δ* strains created above, we simultaneously introduced a third plasmid, containing either an additional copy of wild-type (A$_{73}$-containing) or variant (C$_{73}$-containing) tRNA^{His}, and again tested the ability of the resulting strains to grow on media containing FOA (Fig. 5, second panel). In each strain containing archaeal Thg1, significant growth was observed only in strains that also expressed C$_{73}$-tRNA^{His}, and not in those expressing wild-type tRNA^{His} or a vector control. The ability to complement the *thg1Δ* growth phenotype was dependent on expression of archaeal Thg1, because the complementation was only observed on galactose-containing media (inducing conditions for expression from the P$_{GAL}$ *thg1* promoter) and not in dextrose-containing media (repressing conditions) (Fig. 5, compare second and third panels). Because the presence of G$_{-1}$ is required for histidylation and thus growth in yeast (4), these results indicate that archaeal Thg1 homologs add G$_{-1}$ to C$_{73}$-containing tRNA^{His} in vivo. The complementation observed in the presence of C$_{73}$-tRNA^{His} also demonstrates that the lack of growth observed in strains containing only wild-type (A$_{73}$) tRNA^{His} is not due to limiting expression of the archaeal enzymes in yeast.

Archaeal Thg1 Homologs Catalyze Template-Dependent $3'-5'$ Addition with Wild-Type tRNA^{His}.

In light of the strong preference for

Fig. 4. Archaeal Thg1 homologs efficiently catalyze G-addition opposite C$_{73}$ with a tRNA^{His} variant substrate. Assay of purified yeast and archaeal Thg1 homologs (1 μL each) with 5'-^{32}P labeled C$_{73}$-tRNA^{His} variant. Lane-, buffer only. Positions of G$_{-1}$, G$_{-2}$, and G$_{-3}$ reaction products are indicated to the right of the figure.

Fig. 5. Archaeal Thg1 homologs support growth of the yeast *thg1Δ* strain in the presence of C$_{73}$-tRNA^{His}. Replica plating results with yeast *thg1Δ* strains transformed with *LEU2* plasmids containing various Thg1 homologs (indicated to the right of the figure) and *HIS3* plasmids containing various tRNA^{His} genes (indicated on top of each panel). Growth media are indicated below each panel; images were taken after 3–4 days of growth at 30 °C.

addition of G_{-1} opposite C_{73} exhibited by the archaeal Thg1 enzymes, we revisited the lack of G_{-1} addition previously observed with the wild-type (A_{73}-containing) tRNA^{His} substrate. We tested the ability of archaeal Thg1 homologs to add U_{-1} to wild-type tRNA^{His}, to test the hypothesis that these enzymes might require the ability to form a Watson–Crick base pair for nucleotide addition to occur. Unlike G_{-1} addition to A_{73}-yeast tRNA^{His} that is catalyzed by yeast Thg1 but not the archaeal homologs, the addition of U_{-1} opposite A_{73} is observed with both yeast and archaeal enzymes (Fig. 6A, representative assay for MaThg1 activity is shown). Therefore, the inability of the archaeal enzymes to add G_{-1} to wild-type tRNA^{His} does not reflect an inherent inability to recognize and use this tRNA substrate, but instead reflects an apparent preference of archaeal Thg1 for template-dependent $3'-5'$ addition.

To further probe the template-dependent nature of the reaction catalyzed by MaThg1 with yeast tRNA^{His}, steady-state initial rates of $3'-5'$ nucleotide addition were measured using each of the four possible NTPs and ppp-tRNA^{His} with yeast and MaThg1. For yeast Thg1, it was previously demonstrated that any nucleotide can be added to the $5'$ end of wild-type tRNA^{His} after prolonged incubation at high concentrations of Thg1 (15), although, consistent with the physiological role of Thg1 in yeast, the rate of G_{-1} addition under steady-state conditions is significantly faster than the rate of addition of any other N_{-1} residue (Fig. 6B). In contrast, for MaThg1 the NTP preference was essentially reversed, with UTP added most efficiently to the A_{73}-containing tRNA^{His} substrate, consistent with a requirement for formation of a Watson–Crick base pair for catalysis of $3'-5'$ nucleotide addition (Fig. 6B). Thus, template-dependent $3'-5'$ addition activity of archaeal Thg1 is not limited to formation of G-C base pairs. The rate of addition of U_{-1}($v_o/[E] = 0.04$ hr^{-1}) is nearly 10-fold slower than the k_{cat} for G_{-1} addition to C_{73}-tRNA^{His} by MaThg1 (Table 1), suggesting an additional effect of the identity of the base pair being formed on templated activity with tRNA^{His} substrates. The slower rate of MaThg1-catalyzed U-A vs. G-C addition likely contributes to the lack of complementation of the yeast $thg1\Delta$ phenotype observed by archaeal Thg1 homologs in the presence of A_{73}-tRNA^{His} (Fig. 5).

Discussion

In this study, we demonstrated that archaeal homologs of yeast Thg1 catalyze solely template-dependent $3'-5'$ nucleotide addition to tRNA^{His} substrates, and do not catalyze the nontemplated G opposite A addition reaction that is the hallmark reaction of eukaryal Thg1 enzymes. Purified Thg1 homologs from four different archaea do not efficiently add G_{-1} to wild-type yeast tRNA^{His} (Fig. 3), but instead add G_{-1} to a tRNA^{His} variant that contains C_{73} and is thus a substrate for template-dependent G_{-1} addition (Fig. 4). Moreover, archaeal Thg1 homologs substitute

for the essential G_{-1} addition function of yeast Thg1 in vivo, as long as they are provided with the C_{73}-containing tRNA^{His} substrate that reflects the preference of these enzymes to catalyze templated $3'-5'$ nucleotide addition (Fig. 5). $3'-5'$ addition catalyzed by archaeal Thg1 is not restricted to forming G-C base pairs, but also forms U-A base pairs, as evidenced by the preference for MaThg1 to efficiently add U_{-1} over other non-Watson–Crick pairing N_{-1} residues to wild-type (A_{73}-containing) tRNA^{His}, thus distinguishing these enzymes from yeast Thg1 that prefers to add the nontemplated G_{-1} to A_{73}-tRNA^{His} (Fig. 6). However, because yeast Thg1 is also capable of a previously described template-dependent $3'-5'$ addition activity (15), these results demonstrate that the ability to catalyze Watson–Crick base pair-dependent nucleotide addition is a universal property of Thg1 family members from multiple domains of life. In contrast, the ability to add G_{-1} opposite A_{73} with eukaryal tRNA^{His} is so far a unique feature of eukaryal Thg1 enzymes.

Biological Function for Template-Dependent Thg1 Addition Activity. Although template-dependent $3'-5'$ nucleotide addition was originally discovered as a property of yeast Thg1, the physiological relevance of this activity in yeast remains unknown (15). Nonetheless, unexplained interactions of yeast Thg1 with the Orc2 component of the origin recognition complex (24) and cell-cycle progression defects associated with decreased Thg1 expression in yeast and human cells (24, 25) suggest that the role of the enzyme in eukarya may be more complicated than is currently understood. However, this work provides an opportunity to explore possible physiological roles for template-directed nucleotide addition activity in other organisms. Certain archaea (represented here by *M. thermoautotrophicus* and *M. kandleri*) lack a genomically encoded G-residue at the −1 position of the tRNA^{His} gene (Fig. 2), and thus can not incorporate G_{-1} via an RNase P-dependent pathway like the one used in bacteria. Because the discriminator nucleotide in archaeal tRNA^{His} is universally a C_{73}, our results are consistent with a role for archaeal Thg1 in this G_{-1} addition reaction, thus this work provides unique evidence for a likely physiological role for Thg1-catalyzed template-dependent $3'-5'$ nucleotide addition in any organism.

We note that, whereas the measured k_{cat} values for archaeal Thg1 activity are quite low (0.3–1.1 hr^{-1}) (Table 1), the ability of archaeal enzymes to support growth of the yeast $thg1\Delta$ strain in the presence of C_{73}-tRNA^{His} (Fig. 5) demonstrates that these enzymes catalyze G_{-1} addition on a biologically relevant timescale. Because the tRNA substrates used here are derived from yeast tRNA^{His} sequences, it is possible that the in vitro activities of the archaeal enzymes could be improved using tRNA^{His} substrates derived from archaea. However, because the k_{cat}/K_M values measured for yeast Thg1 activity are similarly low (Table 1), it is possible that the measured in vitro activities of both yeast and archaeal enzymes do not accurately reflect the in vivo rates. Such a discrepancy could be caused by the use of nonoptimal in vitro reaction conditions, lack of important tRNA modifications on the in vitro transcribed substrates, or lack of additional components that stimulate Thg1 activity in vivo. While this remains a possibility, investigations of alternative assay conditions have not yet yielded increased rates with the yeast enzyme, and Thg1 purified from *E. coli* in the absence of any other yeast proteins is catalytically active and not stimulated by inclusion of yeast crude extracts in the assay.

A role for archaeal Thg1 homologs in G_{-1} addition to C_{73}-containing tRNA^{His} in archaea accounts for the presence of the enzyme in the majority of archaea in which it has been identified, including *M. thermoautotrophicus* and *M. kandleri*. However, a second group of archaea, including *M. barkeri* and *M. acetivorans*, is particularly intriguing in that, whereas we have shown here that enzymes from these species catalyze G_{-1} addition in vitro and in vivo, these organisms also contain a genomically encoded G_{-1}
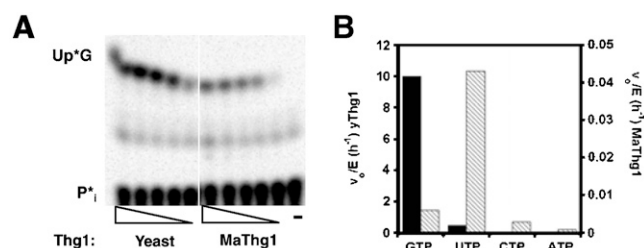
Fig. 6. Archaeal Thg1 catalyzes template-dependent U_{-1} addition activity with wild-type tRNA^{His}. (A) U_{-1} addition assay with $5'$-^{32}P labeled tRNA^{His} and titrations (5-fold serial dilutions) of yeast or MaThg1. Lane-, buffer only. Identity of observed reaction products indicated to the left of the panel. (B) Initial rates of N_{-1} addition to wild-type tRNA^{His} (2 μM) with yeast Thg1 (0.2 μM) (solid bars) or MaThg1 (0.15 μM) (hatched bars), and 1 mM of each NTP, as indicated. Note separate scale on Y-axis for each enzyme's activity.

Table 1. Steady-state kinetic parameters for G_{-1} addition to $pppC_{73}$-tRNA^{His}.

Substrate	Enzyme	k_{cat} (hr^{-1})	K_M (µM)	k_{cat}/K_M (M^{-1} s^{-1})	%k_{cat}/K_M (relative to yeast Thg1)
A$_{73}$-tRNA^{His}	yeast Thg1	8.4*±0.9	0.42*±0.13	5500*±1200	—
C$_{73}$-tRNA^{His}	yeast Thg1	20.4 ± 2.4	0.99 ± 0.29	5670 ± 1200	100
C$_{73}$-tRNA^{His}	MaThg1	0.30 ± 0.03	0.12 ± 0.05	700 ± 230	12
C$_{73}$-tRNA^{His}	MtThg1	1.1†	1.1±0.85‡	300	5

*Values taken from ref (21), errors are ± SEM.
†k_{cat} taken from the [tRNA]-independent v_o/[E] at the peak temperature for MtThg1 activity (55 °C) (*SI Text*).
‡K_M measured at 25 °C (*SI Text*).

residue in tRNA^{His} (Fig. 2). This raises the possibility that, in some archaea, G_{-1} addition could occur by either or both of the two known G_{-1} incorporation pathways. It is also possible that the presence of Thg1 in these organisms is required for another activity that is independent of a role for the enzyme in maturation of tRNA^{His}.

Alternative Biological Roles for Template-Dependent 3′–5′ Nucleotide Addition. Possible biological roles for templated nucleotide addition activity in the 3′–5′ direction, opposite to that of all known DNA and RNA polymerases, are not limited to tRNA^{His} maturation. For example, templated 3′–5′ addition is required for a tRNA editing activity that functions in the mitochondria of certain lower eukarya, where it is used to repair up to three mismatches found in many of the mitochondrial tRNA genes (18, 19). The editing reaction requires stepwise 3′–5′ addition, using the nucleotides of the 3′ half of the aminoacyl-acceptor stem as a template (19, 26–28). Similarities between the enzymatic activities required for G_{-1} addition, now known to be catalyzed by Thg1, and 5′-tRNA editing, for which the identity of the enzyme(s) involved remains a mystery, were noted at the time of the discovery of the editing activity (18, 19). It is tempting to speculate that Thg1 enzymes from archaea that do not require enzymatic addition of G_{-1} to tRNA^{His} may participate in a similar tRNA editing or repair activity in their respective organisms, which would necessarily take advantage of their ability to catalyze template-dependent 3′–5′ addition. Although 5′-editing or repair of tRNA has not been identified in archaea, the existence of such activities may mirror tRNA 3′-end surveillance mechanisms that are found in multiple domains of life (29–31). The example of the ubiquitous 3′-CCA addition enzyme is particularly intriguing in this respect, because it catalyzes the essential de novo addition of the 3′-CCA end to the majority of eukaryal tRNAs, but in bacteria and archaea, predominantly functions in 3′-end repair pathways, because the 3′-CCA sequence is normally encoded in the genome (32, 33).

Implications for Thg1 3′–5′ Nucleotide Addition Mechanism. As the only known enzyme family that catalyzes nucleotide addition in the opposite direction to all known DNA and RNA polymerases, the Thg1 molecular mechanism is of great interest, but is currently unknown. The results presented here add an additional dimension to this issue, raising the question of how enzymes from archaea and yeast exhibit distinct biochemical properties despite sharing a significant degree of overall sequence similarity (even the most distantly related archaeal enzymes share approximately 30% identity with yeast Thg1), including many residues that play critical roles in yeast Thg1 activity (23). A second issue to be explained is the apparently inefficient addition of multiple G nucleotides to C$_{73}$-tRNA^{His} observed with most archaeal Thg1 enzymes. Although consistent with the presumed biological need for only a single G_{-1} residue at the 5′ end of tRNA^{His}, this suggests the existence of additional mechanism(s) used by some archaeal Thg1 enzymes to limit the addition of multiple nucleotides to the 5′ end of tRNA^{His}. It remains to be seen whether multiple nucleotide additions analogous to yeast reverse poly-

merase activity are catalyzed by these archaeal Thg1 homologs with other substrates.

Implications for Evolution of 3′–5′ Addition Activities. Despite the fact that eukaryal G_{-1} addition required for tRNA^{His} maturation was the first Thg1 activity to be described, these results indicate that this is not likely the ancestral activity of Thg1 family members. Instead, template-dependent nucleotide addition is catalyzed by diverse Thg1 family members from multiple domains of life, and therefore these results suggest a scenario in which template-dependent 3′–5′ addition was a property of the earliest Thg1 family members. Further investigation of Thg1 homologs from other organisms, including >20 bacteria in which Thg1 homologs can now be found, will be important for understanding the evolution of 3′–5′ nucleotide addition activities. Interestingly, Thg1-containing bacteria all contain a genomically encoded G_{-1} residue, suggesting that they are similar to the 7 archaea that do not require enzymatic G_{-1} addition to tRNA^{His} (Fig. 2), and therefore the function of bacterial Thg1 may be the same as in these archaea. Particularly, it will be interesting to determine the evolutionary basis for the apparent acquisition of the ability to catalyze nontemplated G_{-1} addition opposite A$_{73}$ by eukaryal Thg1 family members.

Methods

Expression and Purification of Thg1 Homologs. Cloned archaeal Thg1 enzymes were expressed in and affinity-purified from *E. coli*, essentially as previously described for yeast Thg1. For detailed descriptions of cloning, expression and purification protocols, see *SI Text*.

3′–5′ Nucleotide Addition Assays with p-tRNA Substrates. Assays contained approximately 1 nM 5′-^{32}P-tRNA (specific activity 6,000 Ci/mmol, prepared as described in *SI Text*) in 25 mM Hepes, 10 mM MgCl$_2$, 3 mM DTT, 125 mM NaCl, 0.2 mg/mL BSA, pH 7.5 buffer, 0.1 mM ATP and 1 mM GTP, unless otherwise indicated, according to the previously published protocol (21). Reactions were initiated by addition of either 1 µl enzyme (final concentration 0.1–1 µL depending on concentration of the purified stock), or 5-fold serial dilutions of enzyme starting with the same purified stock, as indicated, and were performed for 2 h at 25 °C.

U$_{-1}$ addition activity was tested using a variation of the previously described nuclease protection assay with 5′-^{32}P-labeled tRNA^{His}. Assays were performed identically to those described above, except that reactions were treated with RNase T1 (Ambion) in place of RNase A, to release a labeled U$_{-1}$p∗G$_{+1}$ dimer product if U$_{-1}$ is added, but still yield labeled phosphate if no nucleotide has been added. The identity of the Up*G reaction product was confirmed by further subjecting the reaction products to RNase T2 digestion that released Up* after reaction of the tRNA with Thg1.

Steady-State Kinetic Parameters for Thg1 Activity. Triphosphorylated ^{32}PpppC$_{73}$-tRNA^{His} (*SI Text*) was assayed as described previously (21) in Thg1 assay buffer, in the presence of 1 mM GTP at room temperature, unless otherwise indicated. Steady-state reactions contained 0.05–8 µM C$_{73}$-tRNA^{His} and 1–500 nM of each purified Thg1 such that at least 5-fold excess tRNA was maintained in each reaction. Time courses measured linear initial rates (<10%) of the release of labeled ^{32}PP$_i$ due to G_{-1} addition to the ^{32}Ppp-tRNA for each [tRNA]. Steady-state kinetic parameters k_{cat}, K_M, and k_{cat}/K_M for each enzyme were determined by a fit of the initial velocities obtained as a function of [C$_{73}$-tRNA^{His}] to the Michaelis–Menten equation using

KaleidaGraph (Synergy). Initial rates of N_{-1} addition to A_{73}-tRNA^{His} (Fig. 6) were measured in the same way, with ppp-tRNA^{His} substrate.

Analysis of In Vivo Thg1 Function of Archaeal Thg1 Homologs. Plasmids for galactose-inducible expression of archaeal Thg1 [*CEN LEU2 P$_{GAL}$ – thg1**] (*SI Text*), along with analogous yeast *THG1*-containing and empty vector control plasmids, were transformed into yeast strain JJY20 [relevant genotype *MATα thg1Δ::kanMX his3–1 leu2Δ met15Δ ura3Δ (CEN URA3 P$_{THG1}$ – THGI)*] (34). Positive (Leu$^+$) transformants were selected at 30 °C on synthetic dextrose (SD) media (35), and tested by replica plating at 30 °C to synthetic galactose (SGal) media containing FOA, to induce expression of the various Thg1 homologs and select against the wild-type yeast *THG1 URA3* plasmid. To test the tRNA requirement for Thg1 function, *CEN HIS3* plasmids containing either wild-type (A_{73})-tRNA^{His}, C_{73}-tRNA^{His}, or no tRNA were transformed into each of the Leu$^+$ strains using the same method. Positive transformants were selected on SD media lacking leucine and histidine, and further subjected to replica plating as described above.

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