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Biochemical analysis of Human $tRNA^{His}$ guanylyltransferase in mitochondrial $tRNA^{His}$ maturation

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ABSTRACT

Mitochondria contain their own protein synthesis machinery, which includes mitochondrial tRNA maturation. It has been suggested that mammalian mitochondrial $tRNA^{His}$ ($mtRNA^{His}$) is matured by post-transcriptional addition of guanosine at the −1 position (G_{-1}), which serves as an identity element for mitochondrial histidyl-tRNA synthetase. However, the exact maturation process of mammalian $mtRNA^{His}$ remains unclear. In cytoplasmic $tRNA^{His}$ ($ctRNA^{His}$) maturation, $tRNA^{His}$ guanylyltransferase (Thg1) adds a GTP onto the 5′-terminal of $ctRNA^{His}$ and then removes the 5′-pyrophosphate to yield the mature 5′-monophospholylated G_{-1}-$ctRNA^{His}$ (pG_{-1}-$ctRNA^{His}$). Although mammalian Thg1 is localized to both the cytoplasm and mitochondria, it remains unclear whether mammalian Thg1 plays a role in $mtRNA^{His}$ maturation in mitochondria. Here, we demonstrated that human Thg1 (hThg1) catalyzes the G_{-1} addition reaction for both human $ctRNA^{His}$ and $mtRNA^{His}$ through recognition of the anticodon. While hThg1 catalyzed consecutive GTP additions to $mtRNA^{His}$ *in vitro*, it did not exhibit any activity toward mature pG_{-1}-$mtRNA^{His}$. We further found that hThg1 could add a GMP directly to the 5′-terminal of $mtRNA^{His}$ in a template-dependent manner, but fungal Thg1 could not. Therefore, we hypothesized that acceleration of the pyrophosphate removal activity before or after the G_{-1} addition reaction is a key feature of hThg1 for maintaining a normal 5′-terminal of $mtRNA^{His}$ in human mitochondria. This study provided a new insight into the differences between $tRNA^{His}$ maturation in the cytoplasm and mitochondria of humans.

Keywords: transfer RNA; RNA modification; Mitochondria; Guanylyltransferase

1. Introduction

Mitochondria are organelles found in most eukaryotic cells that contain their own genomic DNA and protein synthesis machinery, comprising mitochondrial ribosomes, tRNAs, and several translational factors [1,2]. RNA components necessary for mitochondrial translation are produced by the mitochondria, whereas all protein components, such as ribosomal proteins, translation factors, aminoacyl tRNA synthetases, and RNA modification enzymes, are encoded by the nuclear genome. Many of their genes contain an additional 5′-extension encoding a mitochondrial targeting sequence (MTS), which is required to transport their protein products into the mitochondria following synthesis in the cytoplasm.

As cytoplasmic tRNA (ctRNA) maturation, mitochondrial tRNAs (mtRNAs) are matured via several enzymatic reactions, such as 5′- and 3′-processing, nucleotide addition, and chemical modification [1,3]. In eukaryote cytoplasm, only $tRNA^{His}$ possesses an additional guanosine at position −1 (G_{-1}), which is a major recognition element for histidyl-tRNA synthetase (HisRS) [4,5]. This G_{-1} of $tRNA^{His}$ is post transcriptionally added by $tRNA^{His}$ guanylyltransferase (Thg1) [6,7,8]. Thg1 recognizes a His anticodon (GUG) in a 5′-monophosphorylated $tRNA^{His}$ (p-$tRNA^{His}$), which is cleaved by RNase P from pre-$tRNA^{His}$ [9,10]. The Thg1-catalyzed G_{-1} addition reaction involves three steps [6,11] (Fig. 1A): 1) adenylylation, the p-$tRNA^{His}$ is activated by ATP to produce a 5′-adenylylated-$tRNA^{His}$ intermediate; 2) guanylylation, the 3′-OH of an incoming GTP attacks the 5′-5′ phosphate linkage of the intermediate to produce 5′-triphospholylated G_{-1}-$tRNA^{His}$ intermediate (pppG_{-1}-$tRNA^{His}$); 3) pyrophosphate removal, the pyrophosphate group is removed from the intermediate pppG_{-1}-$tRNA^{His}$ and mature 5′-monophospholylated G_{-1}-$tRNA^{His}$ (pG_{-1}-$tRNA^{His}$) is generated.

It has been recently reported that G_{-1} is also found in the $tRNA^{His}$ of mammalian mitochondria based on mass spectrometric analysis of the post-transcriptional modifications in each mitochondrial tRNA species [12]. Additionally, human mitochondrial HisRS requires the G_{-1} of $mtRNA^{His}$ for efficient aminoacylation [13]. Because the 5′-nucleotide of the mammalian $mtRNA^{His}$ gene is T, and not G, it is possible that G_{-1} is added enzymatically after 5′-terminal processing by mitochondrial RNase P [12]. However, the exact maturation process of mammalian $mtRNA^{His}$ remains unclear.

Only one *thg1* gene is encoded in human genome and is predicted to possess an MTS at the N-terminus [8]. Human Thg1 (hThg1) is likely targeted to both cytosol and mitochondria through the alternative translation initiation sites. A localization analysis of hThg1 revealed that the full-length protein, containing the MTS, is localized to mitochondria, whereas hThg1 lacking the MTS accumulates in the cytoplasm [14]. hThg1 is also known as IHG-1 (induced in high glucose-1) and plays an important regulatory role in mitochondrial biogenesis and is associated with the development of fibrosis in diabetic nephropathy [14]. However, its biological function in mitochondrial translation remains unknown.

We demonstrated that hThg1 catalyzes G_{-1} addition reactions to both human cytoplasmic and mitochondrial $tRNA^{His}$ (hctRNA^{His} and hmtRNA^{His}, respectively) in a His anticodon-dependent manner. Additionally, hThg1 added multiple GTPs to hmtRNA^{His} *in vitro*, although a single G_{-1} was observed at the 5′-end of hmtRNA^{His} in mitochondria. We also confirmed that hThg1 does not exhibit any adenylylation nor guanylylation activity toward mature pG_{-1}-tRNA^{His}, suggesting that multiple GTP addition is dependent on the 5′-triphosphate group of the $pppG_{-1}$-tRNA^{His} intermediate prior to pyrophosphate removal. Interestingly, we found that hThg1 can add a guanosine monophosphate (GMP) to the hmtRNA^{His} to directly produce mature pG_{-1}-hmtRNA^{His}; however, *Candita albicans* Thg1 (CaThg1) cannot. Therefore, we propose a hypothesis whereby the acceleration of pyrophosphate removal before or after the guanylylation reaction is a key feature of hThg1 that maintain the 5′-terminal of mature hmtRNA^{His} in human mitochondria.

2. Materials and methods

2.1 Preparation of hThg1 and CaThg1

The gene encoding hThg1, lacking the MTS, was amplified by PCR from pCMV6-XL5, inculding ORF of hThg1 (SC125936; ORIGENE) as a template, and inserted into the *Nde*1/*Xho*1 sites of a modified pET28b vector. The primer sequences are described in the Supplementary Tables S1. hThg1 and CaThg1 were overexpressed in *E. coli* BL21 (DE3)-pRARE2, and purified as previously described [10]. Purified Thg1 enzymes were dialyzed against 25 mM HEPES-Na pH 7.5, 500 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, 50% (v/v) glycerol. Dialyzed samples were concentrated to a final concentration of 170–190 μM, and stored at −30°C

2.2 Preparation of substate tRNAs

Double-stranded DNAs encoding the T7 promoter and *human* mitochondrial and cytoplasmic tRNA^{His} variants were amplified by PCR with three overlapping primers, and cloned into the *BamHI*/*HindIII* site of pUC19. The inserted sequences were verified by DNA sequencing. Transcription templates were obtained by PCR from the constructed plasmids. Details of primers used in tRNA preparations are described in Supplementary Tables S1. The *in vitro* transcription was performed with T7 RNA polymerase at 37°C for 3 h. 5′-monophospholylated tRNA^{His} variants (p-tRNA^{His}) were generated by inclusion of 20 mM GMP during *in vitro* transcription. 5′-triphospholylated tRNA^{His} variants (ppp-tRNA^{His}) were transcribed without GMP. The reaction mixture was treated with DNase I at 37°C for 30 min, and purified using 10% denaturing urea-polyacrylamide gel electrophoresis (urea-PAGE). tRNAs were extracted from gel slices and refolded simultaneously in H_2O at 4°C for 18 h. The extracted tRNAs were precipitated with ethanol, dissolved in TE buffer pH8.0, and stored at −80°C.

2.3 Adenylylation and guanylylation assay

All $tRNA^{His}$ variants were refolded in a mixture of 1 μM of $tRNA^{His}$ in a reaction buffer (25 mM HEPES-Na pH 7.5, 125 mM NaCl, 10 mM $MgCl_2$, and 2 mM spermidin-HCl) at 90°C for 1 min, and then gradually cooled to 25°C. Adenylylation assay of Thg1 enzymes were performed with 1 μM of p-$tRNA^{His}$ and 1 mM ATP in the reaction buffer, and intiated by addition of a saturating concentration of enzyme (10 μM). Guanylylation reactions of Thg1 enzymes were preformed in the reaction buffer containing 1 μM of ppp-$tRNA^{His}$ and 1 mM GTP or 1 μM of p-$tRNA^{His}$, 0.1 mM ATP and 1 mM GTP. At various time points, aliquots (1 μL) were mixed with an equal volume of loading buffer (10 M urea, 50 mM EDTA, and 0.1% Xylene cyanol), and analyzed by 10% Urea-PAGE. The gels were stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific), and visualized and quantified using Typhoon and Image Quant software (GE Healthsciences). Single-turnover rate constants (k_{obs}) were determined as previously reported [15]. Time courses of product formation were plotted and fit to a single-exponential rate equation: $Pt = P_{max}[1 - \exp(-k_{obs}t)]$ (eq. 1), where Pt is the fraction of product formed at each time, and P_{max} is the maximum amount of product conversion observed during each time course. k_{obs} values for multiple GTP addition reactions with mitochondrial $tRNA^{His}$ variants were determined from the fraction of all reaction products. All reported parameters were determined from at least two independent experiments.

2.4 Gel-Shift Assay

The gel-shift assay was performed in a binding buffer containing 25 mM HEPES-Na pH 7.5, 125 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 10 μM p-tRNAs, and varying amounts of hThg1 (5-40 μM) at room temperature for 20 min. After incubation, the mixture were loaded on a native PAGE gel (5% acrylamide, 1× TB, 5 mM MgOAc, 5% glycerol). The gel was electrophoresed at 3 mA per gel for 3–4 h at 4°C in 1× TB and 5 mM MgOAc. After electrophoresis, the reaction products were visualized on Typhoon (GE Healthsciences) after staining with ethidium bromide.

3. Results and Discussion

3.1. Human Thg1 catalyzes G_{-1} addition to both cytoplasmic and mitochondrial $tRNA^{His}$

hThg1 contains a conserved N-terminal MTS, which would be cleaved at the same position as the N-terminus of cytoplasmic hThg1 (Met 30) after transporting into mitochondria (Fig. S1A). Therefore mitochondrial and cytoplasmic forms have identical amino acid sequences. Alternatively, nucleotide sequence of $hmtRNA^{His}$ and $hctRNA^{His}$ differ from each other except for the His anticodon GUG (Fig. 1B). In addition, both predicted secondary structures are also divergent, especially with respect to the size of D-loop, implying that the $hmtRNA^{His}$ forms a different structure from the $hctRNA^{His}$, which adopts a canonical L-shaped form [1].

To test whether hThg1 can catalyze both $hmtRNA^{His}$ and $hctRNA^{His}$ for G_{-1} addition, we prepared

hThg1 without the MTS sequence (Fig. S1B). First, we investigated whether hThg1 catalyzes the adenylylation reactions of both hmtRNA^{His} and hctRNA^{His}, which is the first step of tRNA^{His} maturation. Observed rate constants (k_{obs}) of the adenylylation reactions were determined under-single-turnover conditions using both types of p-tRNAs in the presence of only ATP. Reaction products were differentiated by their molecular weights using urea-PAGE. The adenylylated products of both p-hmtRNA^{His} and p-hctRNA^{His} were detected (Fig. 1C and D). hThg1 catalyzed the adenylylation reaction of p-hmtRNA^{His} with a k_{obs} of 4.7×10^{-3} min^{-1} (Fig. 1E), whereas the k_{obs} for hctRNA^{His} was 14 times faster (64×10^{-3} min^{-1}; Fig. 1E). This suggests that hThg1 has distinct adenylylation specificities for the two different tRNA^{His} species.

Biochemical characterization of four kinds of *Dictyostelium discoideum* Thg1-like proteins (DdiTLPs) revealed that DdiTLP2, which is localized only to mitochondria, catalyzes a mitochondrial-specific tRNA^{His} maturation reaction that is His anticodon-independent, whereas the cytoplasmic tRNA^{His} maturation that is typically catalyzed by DdiThg1 is anticodon-dependent [17]. To compare the hThg1 tRNA recognition mechanism between the tRNAs, we created anticodon variants of hmtRNA^{His} and hctRNA^{His}, in which the GUG anticodon were replaced with the GAA anticodon (hmtRNA$^{His}_{GAA}$ and hctRNA$^{His}_{GAA}$, respectively; Fig. 1B). Consistent with previous characterization of *S. cerevisiae* Thg1 (ScThg1) [9], the GUG to GAA conversion completely abolished the ability of hThg1 to catalyze the adenylylation of both tRNAs (Fig. S2A and B), suggesting that hThg1 recognizes both of them in a His anticodon-dependent manner. These data suggest that while hThg1 distinguishes hmtRNA^{His} and hctRNA^{His} from other tRNAs in an anticodon-dependent manner, the overall tRNA binding affinity and mechanism may be different between the two types of tRNA^{His} species, which may affect adenylylation activity though tRNA binding.

Next, we evaluated the interaction between hThg1 and both types of tRNA^{His} were analyzed using a gel-shift assay (Fig. 1F). The mobility of hmtRNA^{His} was significantly faster than that of hctRNA^{His} on a native-PAGE gel, further suggesting a structural difference between them that is consistent with secondary structure predictions (Fig. 1B). Both bands gradually shifted upwards with increasing concentration of hThg1, indicating that hThg1 can bind to both tRNAs. The gel-shift assay also revealed two distinct up-shifted bands in the case of hmtRNA^{His}, suggesting that hThg1 formed a tetrameric structure that could bind one or two hmtRNA^{His} molecules to produce 4:1 and 4:2 complexes, as has been previously reported [10,16]. In contrast, two bands were not observed upon hThg1 binding to hctRNA^{His}. Thus, these results indicate that hThg1 exhibits two distinct tRNA binding modes to hmtRNA^{His} or hctRNA^{His}.

Finally, we investigated guanylylation activity, which is the second step of the G_{-1} addition reaction, by using a ppp-tRNA^{His} in the presence of GTP, which can be incorporated without the adenylylation step, as previously reported [18]. Guanylylation assay was performed in the same manner as adenylylation assay. The guanylylated products were detected in the both reactions with

ppp-hmtRNA^{HIs} and ppp-hctRNA^{His} (Fig. 1G, H and I), indicating that hThg1 can add GTP into both types of tRNA^{His}. hThg1 can add a single GTP to hctRNA^{His}, whose structure contains an adenosine residue opposite to G_{-1} that is conserved in eukaryotes, as previously reported [16,19] (Fig. 1H). Interestingly, three GTPs (G_{-1}, G_{-2} and G_{-3}) were consecutively incorporated into ppp-hmtRNA^{His} (Fig. 1G). In a previous study of ScThg1 [18,20], switching the opposite ctRNA^{His} adenosine to a cytosine induced consecutive GTP addition using the conserved CCA 3′-end of the tRNA as a template. In hmtRNA^{His} the opposite nucleotide is naturally a cytosine and is encoded in mitochondrial DNA. These results demonstrate that consecutive GTP addition to ppp-hmtRNA^{His} is catalyzed in a template-dependent manner because of the genomically encoded cytosine opposite to G_{-1}. Additionally, hThg1 could also catalyze the consecutive GTP addition to p-hmtRNA^{His} in the presence of both ATP and GTP (Fig. S2C). p-hmtRNA^{His} is identical to the reaction product after 5'-processing of pre-tRNA^{His} by mitochondrial RNase P, suggesting that hThg1 could potentially catalyze consecutive GTP addition to p-hmtRNA^{His} in mitochondria. However, only single G_{-1} modification was observed at the 5'-terminal of mature hmtRNA^{His} in a previous report [12]. Thus, these results imply that there may be an additional mechanism during hmtRNA^{His} maturation that prevents consecutive hThg1-catalyzed GTP addition in mitochondria.

3.2. Pyrophosphate removal from the 5'-terminal of hmtRNA^{His} is important in preventing consecutive GTP addition to hmtRNA^{His}

To further investigate our proposed mechanism for the maturation of hmtRNA^{His} in mitochondria, we assessed whether hThg1 catalyzes re-activation with ATP and subsequent consecutive GTP addition by using matured hmtRNA^{His} containing a 5'-monophosphalylated G_{-1} (pG_{-1}-hmtRNA^{His}) as a substrate.

pG_{-1}-hmtRNA^{His} was not a substrate for the adenylylation reaction of hThg1 (Fig. 2A) and no guanylylated product was observed in the presence of both ATP and GTP (Fig. 2B), indicating that hThg1 exhibit no activity toward mature hmtRNA^{His}. In contrast, hThg1 catalyzed consecutive G_{-2} and G_{-3} addition to pppG_{-1}-hmtRNA^{His}, which is an intermediate prior to pyrophosphate removal (Fig. 2C). Biochemical analysis of ScThg1 previously suggested that consecutive nucleotide addition terminates before the next nucleotide addition when ScThg1 removes the pyrophosphate from the 5'-terminal of tRNA^{His} [20]. Consistent with this, our results suggested that the 5'-triphosphate of hmtRNA^{His} intermediate is necessary for continuous GTP addition on hmtRNA^{His}. Therefore, we hypothesize that an acceleration of pyrophosphate removal activity could be an additional mechanism to maintain the precise 5'-terminal maturation of hmtRNA^{His} in human mitochondria.

We further tested whether hThg1 can utilize a guanosine monophosphate (GMP), a GTP from which the 5'-pyrophosohate group has been removed, as a substrate for G_{-1} addition to hmtRNA^{His}. Surprisingly, although the 5'-triphosphate group of GTP was essential for the guanylylation of

hctRNA^{His}, GMP could be directly incorporated into hmtRNA^{His} (Fig. 3A and B). Furthermore, only a single G_{-1} addition was observed when using a GMP as a substrate. However, it has been reported that the concentration of GMP is significantly lower than that of GTP in human cells [21]. In addition, the k_{obs} value for GMP addition (7.8×10^{-3} min^{-1}; Fig. 3C) was 22 times slower than that of GTP addition (169×10^{-3} min^{-1}; Fig. 1H), indicating that hThg1 strongly prefers GTP over GMP for guanylylation. Thus, it is unlikely that hThg1 utilizes GMP for guanylylation of hmtRNA^{His} in human mitochondria to prevent consecutive addition. However, it is possible that hThg1 could remove pyrophosphate from incoming GTP and then use the resulting GMP for G_{-1} addition to hmtRNA^{His}. It has not yet been reported that hThg1 exhibits such a GTP pyrophosphate hydrolysis activity *in vitro*, implying that the specific conditions in mitochondria (pH, nucleotide and ion concentration, etc.) [22,23] might act to enhance such an activity either before or after the guanylylation reaction.

Human mitochondrial RNase P is comprises three protein subunits: mitochondrial RNase P proteins 1, 2 and 3 (MRPP1, MRPP2 and MRPP3, respectively) [24]. Interestingly, it has been recently reported that MRPP1/2 remains bound to the tRNA product after the 5'-processing step and enhance the subsequent 3'-processing activity by presenting the nascent tRNA to the mitochondrial CCA-adding enzyme [25]. Thus MRPP1/2 serves as a processing platform for several down-stream tRNA maturation steps in human mitochondria. This suggests that MRPP1/2 may remain bound to the hmtRNA^{His} after 5'- and 3'-processing and may participate with hThg1 in G_{-1} addition. For instance, MRPP1/2 may enhance the pyrophosphate removal activity of hThg1 discussed above or may recruit MRPP3 again and catalyze re-processing of the elongated G_{-2} and G_{-3} of hmtRNA^{His} after consecutive GTP addition by hThg1.

3.3. Template-dependent GMP addition activity of hThg1

We found that hThg1 can use GMP as a substrate for the guanylylation of hmtRNA^{His} but not hctRNA^{His}. Comparison of the nucleotide sequences of hmtRNA^{His} and hctRNA^{His} revealed that the residue opposite to G_{-1} in the structure of hmtRNA^{His} is C whereas in hctRNA^{His} it is A (Fig. 1B). To assess the relationship between the tRNA specificity for GMP addition and the opposite residue, we prepared two substitutions in opposite residues of hmtRNA^{His} and hctRNA^{His} (hmtRNA^{His}-A and hctRNA^{His}-C, respectively), and measured the hThg1 GMP addition activities toward these substituted variants.

GMP addition activity was completely abolished in hmtRNA^{His}-A (Fig. 3D) whereas a single GMP addition was observed in hctRNA^{His}-C (Fig. 3E). Furthermore, the k_{obs} for GMP addition to hctRNA^{His}-C was almost the same as for hmtRNA^{His} (Fig. 3C), suggesting that the structural differences between these tRNA^{His}s do not affect the GMP addition activity of hThg1. These results strongly indicate that the GMP addition activity of hThg1 is specific to hmtRNA^{His} because of dependence on Watson-Crick base pairing between the incoming GMP and the opposite C on

hmtRNA^{His}.

ScThg1 requires the triphosphate group of GTP for G_{-1} addition to cytoplasmic tRNA^{His} containing an opposite A [11]. Consistent with this, no guanylation activity of hctRNA^{His} using GMP was observed with CaThg1, which is highly homologous to ScThg1 (Fig. S3A). Interestingly, CaThg1 could also not catalyze GMP addition to hmtRNA^{His} (Fig. S3B), whereas multiple GTP addition activity for hmtRNA^{His} was observed (Fig. S3C); the k_{obs} of the reaction by CaThg1 was even faster than that of hThg1 (Fig. S3D). These results suggest that CaThg1 recognizes hmtRNA^{His} as a substrate for guanylylation but, unlike hThg1, is unable to use GMP for the reaction. Cumulatively, our results demonstrate that template-dependent GMP addition is a characteristic feature of hThg1, but not fungal Thg1 enzymes from *C. albicans* and *S. cervisieae*, which may not require Thg1 activity for tRNA^{His} maturation in their mitochondria [26].

In crystal structures of hThg1-dGTP and CaThg1-GTP complexes [10,16], the 5'-triphosphophate of the incoming GTP is coordinated with a Mg^{2+} ion and interacts with several conserved residues whose substitutions significantly reduce the G_{-1} addition activity by Thg1 to ctRNA^{His}. Therefore, the tight binding between the 5'-triphosphophate of the incoming GTP and the active site of Thg1 might be conserved in eukaryotic Thg1 enzymes to facilitate the mismatched G:A pairing that is a characteristic feature of ctRNA^{His} maturation in eukaryotes. In contrast, in the case of GMP addition there is no interaction through a 5'-triphosphate group and thus Watson-Crick base pairing between the base moiety of GMP and the opposite C is the main interaction for recognizing GMP in the guanylylation reaction. Because hThg1, and not CaThg1, recognizes GMP as a substrate for the reaction, it exhibited the ability to form stronger G:C base pairing during the guanylylation reaction than CaThg1. In other words, CaThg1 may be specialized in G:A pair formation by weakening the G:C base pair formation in the reaction because it works only to mature the ctRNA^{His} containing the opposite A. In contrast, hThg1 can mature both hctRNA^{His} and hmtRNA^{His}, and thus may possess both activities with similar efficiencies to form either the G:A mismatch or the canonical G:C base pair during the guanylylation reaction.

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Figure legends

Figure 1: The adenylylation and guanylylation activity of hThg1 for mitochondrial and cytoplasmic $tRNA^{His}$.

(A) The reaction scheme for G_{-1} addition to $tRNA^{His}$ by Thg1. First, the 5′-monophosphorylated pre-$tRNA^{His}$ formed by cleavage by RNase P is activated by adenylylation using ATP. Second, GTP is transferred to the activated 5′-end of pre-$tRNA^{His}$ in the guanylylation step. Finally, the 5′-pyrophosphate is removed from the G_{-1} nucleotide to yield the monophosphorylated G_{-1}-$tRNA^{His}$. Thg1 can use 5′-triphospholylated pre-$tRNA^{His}$ as a substrate for the guanylylaiton reaction without the adenylylation step.

(B) Human mitochondrial and cytoplasmic $tRNA^{His}$ ($hmtRNA^{His}$ (left) and $hctRNA^{His}$ (right), respectively) are drawn as cloverleaf structures. $hmtRNA^{His}$ and $hctRNA^{His}$ contain a His-specific anticodon GUG, but a different residue (C_{69} or A_{73}) opposite the -1 position.

(C-D) Gel based assays of adenylylation reaction by hThg1 with $hmtRNA^{His}$ **(C)** or $hctRNA^{His}$ **(D)**. Representative single-turnover assays for determining k_{obs} for adenylylation in the presence of p-$tRNA^{His}$ with ATP. The reactions shown are time courses of activity with hThg1 in excess $tRNA^{His}$; aliquots from each time point were applied on urea-PAGE gel to separate reaction product from an unreacted substrate. RNA fragments were detected by SybrGold staining.

(E) Time course of adenylylation activity for $tRNA^{His}$ variants; $hmtRNA^{His}$ (red, ●), and $hctRNA^{His}$ (blue, ■). Lines represent fits of each time course to a single-exponential equation (eq. 1) to yield k_{obs}. The bars in the graphs are SD of more than two independent experiments.

(F) Results of the gel-shift assay. $tRNA^{His}$ (50 pmol) was incubated with 25, 50, 100 and 200 pmol of hThg1. Reaction mixtures were loaded on a 5% native-PAGE gel. The gel was stained with ethidium bromide.

(G-H) Guanylylation assay of hThg1 with $hmtRNA^{His}$ **(F)** or $hctRNA^{His}$ **(G)** in the presence of ppp-$tRNA^{His}$ with GTP.

(I) Time course of guanylylation activity for $tRNA^{His}$ variants; $hmtRNA^{His}$ (red, ●), and $hctRNA^{His}$ (blue, ■).

Figure 2: The guanylylation activity of hThg1 toward mature pG_{-1}-$mtRNA^{His}$.

(A-C) The adenylylation and guanylylation reactions with the mature pG_{-1}-$hmtRNA^{His}$ were analyzed by urea-PAGE. pG_{-1}-$hmtRNA^{His}$ was incubated with hThg1 in the presence of only ATP **(A)** or both ATP and GTP **(B)**. $pppG_{-1}$-$hmtRNA^{His}$, the intermediate prior to the pyrophosphate removal, was incubated with hThg1 and GTP **(C)**.

Figure 3: The GMP addition of hThg1 for $tRNA^{His}$ variants.

(A-D) Guanylylation reactions using GMP as a substrate were examined with several $tRNA^{His}$ variants: ppp-$hmtRNA^{His}$ **(A)**, ppp-$hctRNA^{His}$ **(B)**, and two substitutions of the opposite residue in $hmtRNA^{His}$ and $hctRNA^{His}$ ($hmtRNA^{His}$-C **(C)** and $hctRNA^{His}$-A **(D)**, respectively).

(E) Time courses of GMP addition to $tRNA^{His}$ variants; $hmtRNA^{His}$ (red, ●) and $hctRNA^{His}$-A (blue, ■). Lines represent fits of each time course to a single-exponential equation (eq. 1) to yield k_{obs}. The bars in the graphs are SD of more than two independent experiments.

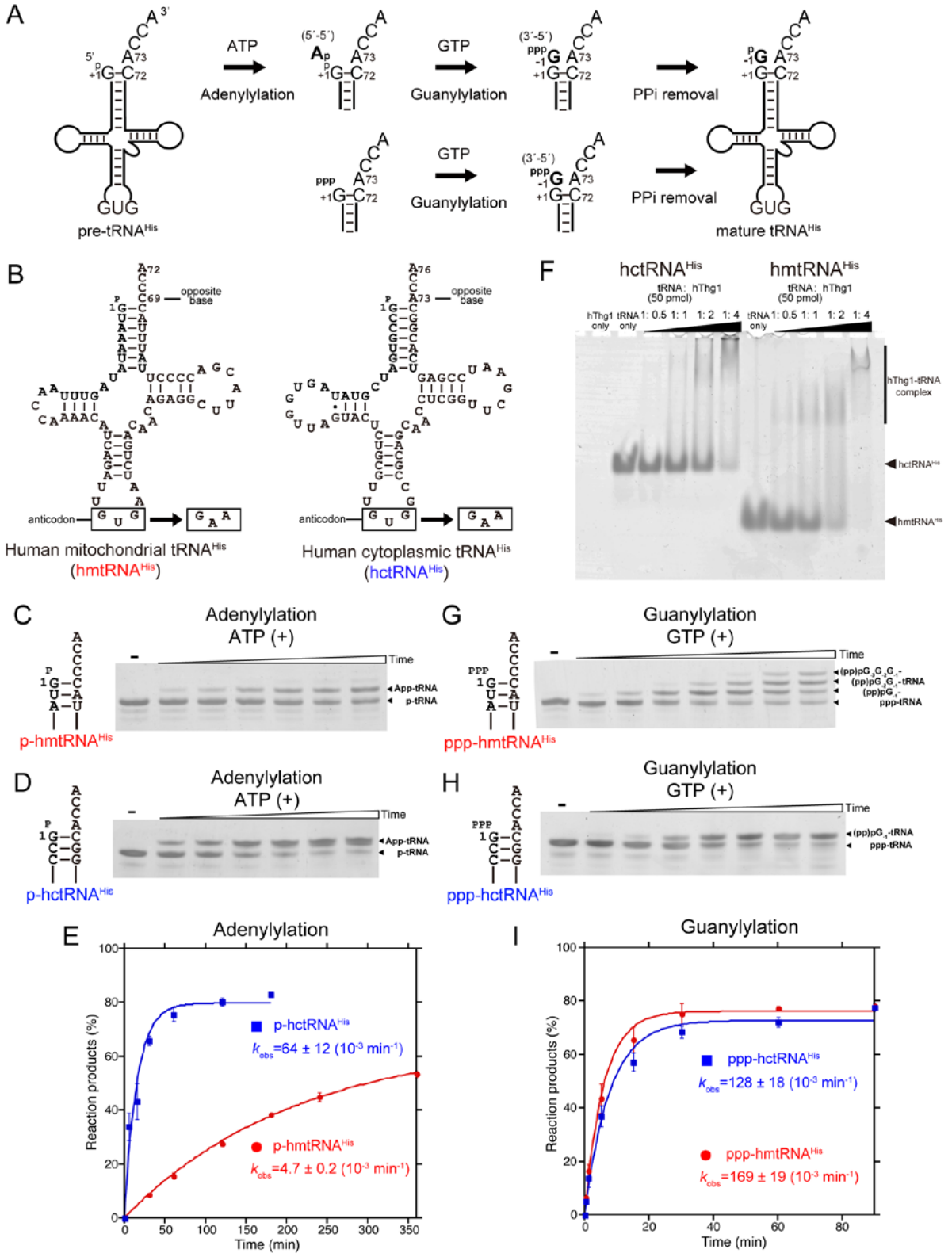

A
ATP
Adenylylation
GTP
Guanylylation
PPi removal
pre-tRNA^His
mature tRNA^His
B
opposite base
anticodon
Human mitochondrial tRNA^His
(hmtRNA^His)
Human cytoplasmic tRNA^His
(hctRNA^His)
F
hctRNA^His
hmtRNA^His
tRNA: hThg1 (50 pmol)
hThg1 only
tRNA only
1: 0.5
1: 1
1: 2
1: 4
hThg1-tRNA complex
C
Adenylylation
ATP (+)
Time
App-tRNA
p-tRNA
p-hmtRNA^His
G
Guanylylation
GTP (+)
ppp-hmtRNA^His
D
p-hctRNA^His
H
ppp-hctRNA^His
E
Reaction products (%)
p-hctRNA^His
k_obs=64 ± 12 (10^-3 min^-1)
p-hmtRNA^His
k_obs=4.7 ± 0.2 (10^-3 min^-1)
Time (min)
I
ppp-hctRNA^His
k_obs=128 ± 18 (10^-3 min^-1)
ppp-hmtRNA^His
k_obs=169 ± 19 (10^-3 min^-1)

Figure1

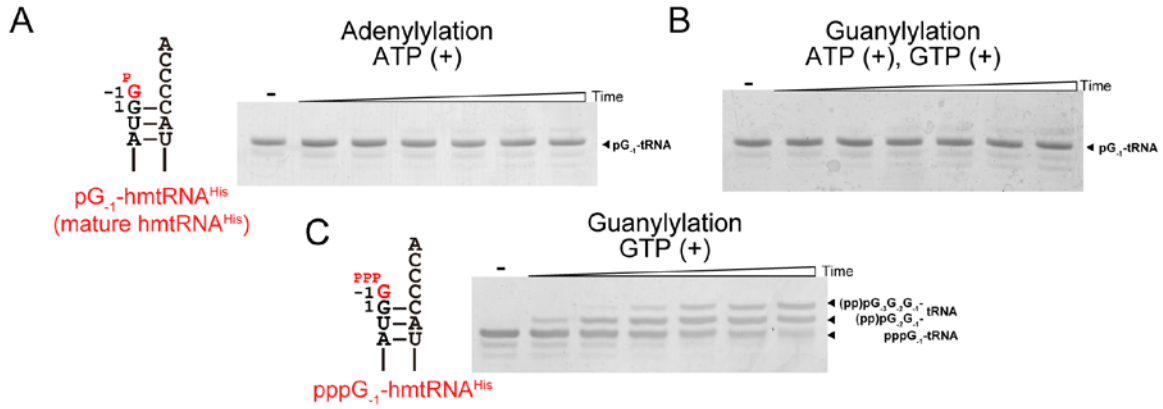

A
Adenylylation
ATP (+)
Time
pG-1-tRNA
pG-1-hmtRNAHis
(mature hmtRNAHis)
B
Guanylylation
ATP (+), GTP (+)
Time
pG-1-tRNA
C
Guanylylation
GTP (+)
Time
(pp)pG-3G-2G-1- tRNA
(pp)pG-2G-1-
pppG-1-tRNA
pppG-1-hmtRNAHis

Figure 2

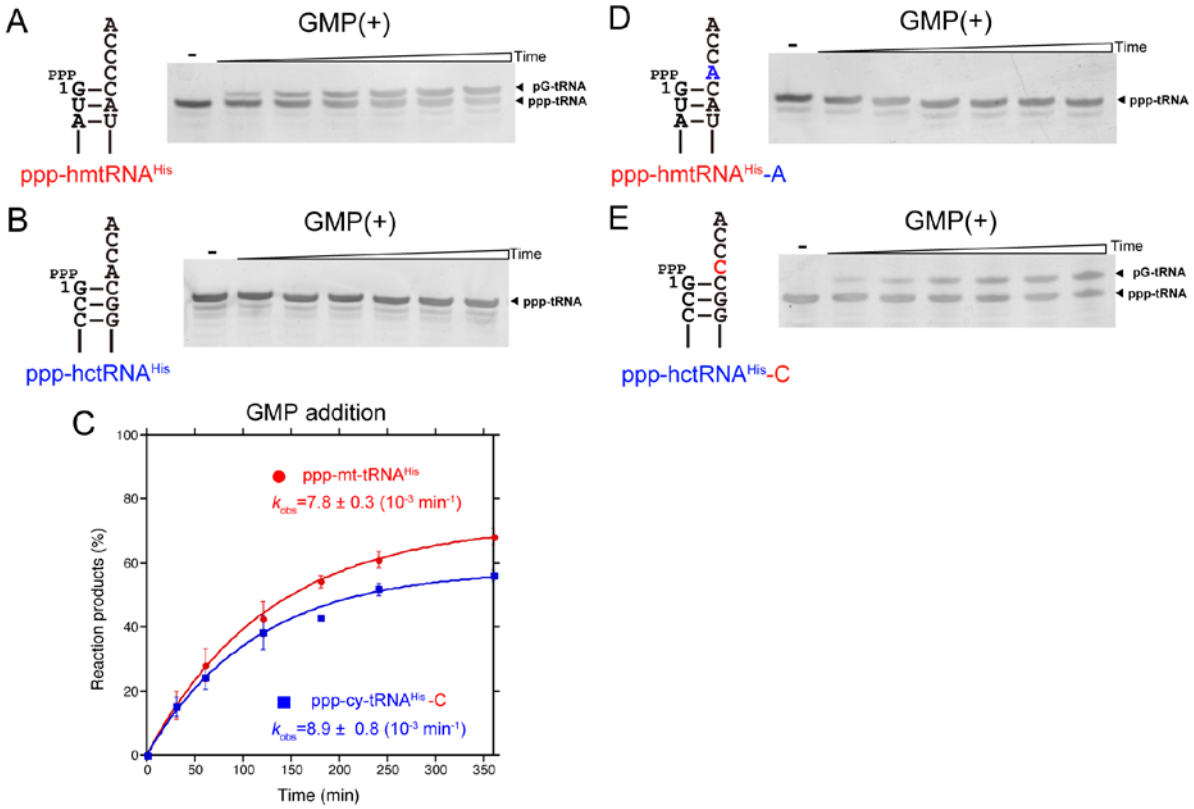

A
GMP(+)
Time
pG-tRNA
ppp-tRNA
ppp-hmtRNA^{His}
B
GMP(+)
Time
ppp-tRNA
ppp-hctRNA^{His}
C
GMP addition
ppp-mt-tRNA^{His}
k_{obs}=7.8 ± 0.3 (10^{-3} min^{-1})
ppp-cy-tRNA^{His}-C
k_{obs}=8.9 ± 0.8 (10^{-3} min^{-1})
Reaction products (%)
Time (min)
D
GMP(+)
Time
ppp-tRNA
ppp-hmtRNA^{His}-A
E
GMP(+)
Time
pG-tRNA
ppp-tRNA
ppp-hctRNA^{His}-C

Figure 3