**Saccharomyces cerevisiae** Thg1 Uses 5′-Pyrophosphate Removal To Control Addition of Nucleotides to tRNA⁰⁰¹

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**Supporting Information**

ABSTRACT: In eukaryotes, the tRNA⁰⁰¹ guanylyltransferase (Thg1) catalyzes 3′-5′ addition of a single guanosine residue to the −1 position (G₋₁) of tRNA⁰⁰¹, across from a highly conserved adenosine at position 73 (A73). After addition of G₋₁, Thg1 removes pyrophosphate from the tRNA 5′-end, generating 5′-monophosphorylated G₋₁-containing tRNA. The presence of the 5′-monophosphorylated G₋₁ residue is important for recognition of tRNA⁰⁰¹ by its cognate histidyl-tRNA synthetase. In addition to the single-G₋₁ addition reaction, Thg1 polymerizes multiple G residues to the 5′-end of tRNA⁰⁰¹ variants. For 3′-5′ polymerization, Thg1 uses the 3′-end of the tRNA⁰⁰¹ acceptor stem as a template. The mechanism of reverse polymerization is presumed to involve nucleophilic attack of the 3′-OH from each incoming NTP on the intact 5′-triphosphate created by the preceding nucleotide addition. The potential exists for competition between 5′-pyrophosphate removal and 3′-5′ reverse polymerization reactions that could define the outcome of Thg1-catalyzed addition, yet the interplay between these competing reactions has not been investigated for any Thg1 enzyme. Here we establish transient kinetic assays to characterize the pyrophosphate removal versus nucleotide addition activities of yeast Thg1 with a set of tRNA⁰⁰¹ substrates in which the identity of the N₋₁:N₋₂ base pair was varied to mimic various products of the N₋₁ addition reaction catalyzed by Thg1. We demonstrate that retention of the 5′-triphosphate is correlated with efficient 3′-5′ reverse polymerization. A kinetic partitioning mechanism that acts to prevent addition of nucleotides beyond the −1 position with wild-type tRNA⁰⁰¹ is proposed.

Canalonic DNA and RNA polymerases all synthesize nucleic acids in the 5′-3′ direction by virtue of the attack of a 3′-hydroxyl from the growing polynucleotide chain on the 5′-triphosphate of an incoming NTP.⁴⁻⁵ Enzymes of the tRNA⁰⁰¹ guanylyltransferase (Thg1)/Thg1-like protein (TLP) superfamily are the only known exceptions to this rule.³⁻⁴ Thg1/TLP enzymes use the reverse chemistry, promoting the 3′-hydroxyl of an incoming NTP to attack a 5′-triphosphate (or similarly activated 5′-end) on the growing polynucleotide chain, thus achieving nucleotide addition in the opposite (3′-5′) direction compared to all other known polymerases.⁵⁻⁶ In eukaryotes, Thg1 enzymes use the 3′-5′ addition reaction to add a single G residue (G₋₁) to the 5′-end of tRNA⁰⁰¹,⁵⁻⁶ while TLPs utilize 3′-5′ polymerase chemistry to repair 5′-truncated tRNAs, which is a required reaction during mitochondrial 5′-tRNA editing and is likely involved in additional physiological processes.⁶⁻⁹ Despite the fact that Thg1 enzymes share no obvious sequence similarity with any other known enzyme family, the crystal structures of human Thg1 and bacterial TLP revealed that Thg1 family enzymes utilize a conserved active site that is strikingly similar to that of canonical 5′-3′ DNA/RNA polymerases to catalyze the 3′-5′ addition reaction.¹¹⁻¹² This observation raises new questions about the relationship between 5′-3′ and 3′-5′ polymerase enzymes and the possibility of more widespread use of the 3′-5′ polymerase active site in biology.

Transient kinetic assays have been developed to characterize each of the three chemical steps that comprise the single-nucleotide (G₋₁) addition reaction, which is observed with 5′-monophosphorylated eukaryotic (A73-containing) tRNA⁰⁰¹ substrates. These chemical steps are (1) activation of the 5′-monophosphorylated tRNA by adenylylation, (2) nucleotidyl transfer to add the incoming NTP to the activated 5′-end of the tRNA, and (3) removal of the 5′-pyrophosphate from the added G₋₁ nucleotide (Figure S1 of the Supporting Information).¹³ Thg1 enzymes can also catalyze 3′-5′ addition via an ATP-independent mechanism that bypasses the first of these three chemical steps and utilizes a 5′-triphosphorylated tRNA as the activated substrate for nucleotidyl transfer (Figure S1 of the Supporting Information). This ATP-independent reaction was first observed in vitro with 5′-triphosphorylated transcripts and also occurs during the 3′-5′ polymerase reaction, once the first nucleotide has been added to the 5′-end of the tRNA (Figure 1B). The transient kinetic assays, in combination with alterations of Thg1 active site residues, revealed information about the molecular function of two nucleotides visualized in the Thg1 structure.¹¹,¹³ Despite this information, many questions about the molecular mechanism...
of Thg1 catalysis remain unanswered, including the positions of the bound tRNA substrate and the incoming NTP that is added to the 5′-end, the basis for selection of a non-WC templated G<sub>−1</sub> versus Watson–Crick (WC) base-pairing nucleotides during tRNA repair, and the roles of multiple conformational changes that are likely to occur during the reaction.

One unanswered question is the molecular basis for distinct biochemical properties associated with eukaryotic Thg1-type enzymes compared with their largely bacterial and archaean TLP counterparts. Eukaryotic Thg1 enzymes such as SceThg1 (formerly termed γThg1) catalyze two different types of 3′–5′ addition reactions with similar kinetic efficiency, depending on the tRNA<sup>His</sup> substrate (Figure 1).<sup>5</sup> The first reaction is non-WC base-paired addition of G<sub>−1</sub> opposite A<sub>−1</sub> observed with wild-type tRNA<sup>His</sup> (Figure 1A), and the second is WC-templated 3′–5′ polymerization of multiple G-C or C-G base pairs observed with tRNA<sup>His</sup> variants (Figure 1B). TLPs, on the other hand, are exclusively template-dependent 3′–5′ polymerases and do not efficiently add the non-WC paired G<sub>−1</sub> nucleotide.<sup>9,10</sup> Moreover, SceThg1 does not polymerize multiple A-U or U-A base pairs with tRNA<sup>His</sup>, while TLPs catalyze polymerization of all four WC base-paired nucleotides.<sup>5,10</sup> A key feature of the eukaryote-specific G<sub>−1</sub> addition activity is the ability of the enzyme to terminate addition after only a single G<sub>−1</sub> nucleotide is added to wild-type (A<sub>−1</sub>-containing) tRNA<sup>His</sup>, despite the presence of the downstream C<sub>−4</sub> and C<sub>−3</sub> residues in the tRNA, which are used as the template for addition of multiple G residues in the C<sub>−7</sub>-containing tRNA<sup>His</sup> variants. This property of SceThg1 is the focus of this study.

According to the proposed mechanism of 3′–5′ addition, the presence of a 5′-triphosphate could be a controlling factor for the ability of Thg1 to add more than one nucleotide to the 5′-end of an RNA. When Thg1 adds multiple nucleotides, it is proposed to use the 5′-triphosphorylated end from the previous nucleotide addition as the activated end for each subsequent nucleotide addition (Figure 1B); however, SceThg1 also catalyzes removal of the 5′-pyrophosphate from the added G<sub>−1</sub> nucleotide, and hydrolysis of this activated end could consequently prevent further nucleotide addition (Figure 1A). Thus, the pyrophosphate removal and nucleotide addition activities of SceThg1 could compete for available 5′-triphosphorylated ends generated by G<sub>−1</sub> addition, impacting the balance between single- and multiple-nucleotide addition reactions.

To test whether differences in the efficiency of pyrophosphate removal versus nucleotide addition steps catalyzed by SceThg1 explain the differences between single- and multiple-nucleotide addition reactions observed with different tRNA<sup>His</sup> substrates, we developed transient kinetic assays to directly measure pyrophosphate removal and polymerization activities with a series of substrates that contain different combinations of N<sub>−1</sub>·N<sub>−2</sub> base pairs, in the presence of various NTPs. Here we demonstrate that the varied abilities of SceThg1 to remove the 5′-pyrophosphate from each tRNA<sup>His</sup> substrate correlate well with the observed addition of multiple nucleotides to some tRNA substrates, but single nucleotides to others. Thus, the existence of the 5′-pyrophosphate removal activity catalyzed by SceThg1 is an important trait that is required for control of the 3′–5′ addition reaction with tRNA<sup>His</sup>.

## MATERIALS AND METHODS

### Nucleotides and Reagents.

NTPs (100 mM LiCl salts) used for enzyme assays were purchased from Roche; [γ<sup>−32P</sup>]GTP (6000 Ci/mmol), [α<sup>−32P</sup>]CTP (3000 Ci/mmol), and [α<sup>−32P</sup>]UTP (3000 Ci/mmol) were purchased from Perkin-Elmer. For Thg1 transient kinetic assays, ribonuclease A (RNase A) and ribonuclease T1 (RNase T1) were purchased from Ambion; calf alkaline intestinal phosphatase (CIP) was purchased from Invitrogen, and P1 nuclease was purchased from Sigma.

### SceThg1 Expression and Purification.

Escherichia coli strain BL21-DE3(pLysS) was used for overexpression and purification of SceThg1, which were performed as previously described for the N-terminally His<sub>6</sub>-tagged enzyme.<sup>14,15</sup> Briefly,
SceThg1 was purified from a 0.5 L culture using immobilized metal ion affinity chromatography, dialyzed into buffer containing 50% glycerol for storage at −20 °C, and assessed for purity (≥90% as judged by visual inspection of the purified enzyme preparation) using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In vitro transcription reactions were performed with T7 RNA polymerase using runoff transcription from digested plasmids that encode the various tRNA genes downstream of the T7 RNA polymerase promoter, as previously described.9,14 To create γ-32P-labeled tRNA (p*pp-tRNA), tRNAs were transcribed in the presence of [γ-32P]GTP, according to published methods.14 To generate the α-labeled tRNA (ppp*-tRNA) species used for some assays (see below), unlabeled S’-triphosphorylated tRNA variants were similarly transcribed by T7 RNA polymerase in the presence of NTPs (2 mM each). All tRNA transcripts (labeled or unlabeled) were gel-purified after electrophoresis on 10% polyacrylamide, 4 M urea gels, followed by phenol/chloroform extraction and ethanol precipitation. The resulting purified tRNAs were resuspended in 10 mM Tris-HCl (pH 7.5) and stored at −20 °C.

[γ-32P]GTP-tRNA Assays. Kinetic parameters for turnover of the γ-32P-labeled p*pp-g-tRNA substrates were determined at room temperature by reacting S’-p*pp-G−1-tRNA or S’-p*pp-G−1:C73tRNAHis with an at least 10-fold excess of purified enzyme in the presence or absence of the indicated concentrations of GTP. Thg1 reaction buffer (used for all assays) contained 10 mM MgCl₂, 3 mM dithiothreitol (DTT), 125 mM NaCl, 0.2 mg/mL BSA, and 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) (pH 7.5). At desired time points, aliquots (3 μL) were removed and added to a new tube containing 1 μL of 250 mM EDTA to quench the reactions; then 2 μL of each quenched time point was spotted on PEI-cellulose TLC plates (EM Science). Plates were washed in 100% methanol, air-dried, and resolved using a 0.5 M potassium phosphate (pH 6.3)/methanol [80:20 (v:v)] solvent system to separate the labeled pyrophosphate (P*P) from unreacted labeled tRNA. For the determination of KD,pp,GTP reaction mixtures contained varied concentrations of GTP (0.01–1 mM) and a saturating concentration of enzyme (15 μM). For the determination of KD,pp, RNA observed rates were measured from reaction mixtures containing labeled tRNA with or without 1 mM GTP, initiated by the addition of varied enzyme concentrations (1.0–30 μM).

Time courses of product formation were plotted and fit to a single-exponential rate equation (eq 1) using Kaleidagraph (Synergy Software)

\[
P_{\text{obs}} = P_{\text{max}} \left[1 - \exp(-k_{\text{obs}}t)\right]
\]

where \(P_{\text{obs}}\) is the percent product formed at each time and \(P_{\text{max}}\) is the maximal percent of product formation observed during each time course. \(P_{\text{max}}\) values of 80–90% were routinely observed, with the amount of unreacted substrate remaining in each assay likely due to minor amounts of incorrectly folded tRNA that are typically observed in enzyme assays with in vitro-transcribed RNAs.

The resulting \(k_{\text{obs}}\) values determined for each concentration of GTP or Sccthgl were plotted and fit to eq 2 or 3, respectively, as described in the text to yield pseudo-first-order maximal rate constants and dissociation constants. All reported kinetic parameters were determined using \(k_{\text{obs}}\) values derived from fits to data obtained from at least two independent experiments unless otherwise indicated, and reported errors in \(k_{\text{max}}\) and KD,pp,GTP are the least-squares estimate of the standard error derived from a fit to the combined data from all independent experiments using Kaleidagraph.

\[
k_{\text{obs}} = k_{\text{max}} \times \left[\frac{[\text{NTP}]}{[K_{\text{D,pp,GTP}} + [\text{NTP}]})\right]
\]

\[
k_{\text{obs}} = k_{\text{max}} \times \left[\frac{[\text{SceThg1}]}{[K_{\text{D,pp,tRNA}} + [\text{SceThg1}])}\right]
\]

For time courses that continued to exhibit linear rates of conversion to product even after reaction for 3 h, \(k_{\text{obs}}\) was estimated using the method of initial rates, according to eq 4

\[
k_{\text{obs}} = \frac{v}{P_{\text{max}}}
\]

where \(v\) is the linear initial rate derived from the slope of the fraction product versus time plots and \(P_{\text{max}}\) is the maximal fraction of product conversion observed in NTP-stimulated reactions.

Construction of [α-32P]N15tRNAHis Substrates. α-32P-labeled tRNAHis substrates containing pppG−1 or pppC−1 were generated by reacting 15 μM SceThg1 with 20 pmol of S’-triphosphorylated tRNAHis transcripts initiated at the +1 position and containing the desired 3’-terminal sequence and 20 pmol of [α-32P]CTP, respectively, in Thg1 reaction buffer at room temperature for 5–30 min. The pppU−1:A−2−tRNAHis substrate was generated by reacting 20 μM purified TLP4 from Dictostelium discoideum8 with 80 pmol of S’-triphosphorylated tRNAHis transcript initiated at the +1 position and 80 pmol of [α-32P]UTP in reaction buffer at room temperature for 3–4 h. The resulting labeled tRNA species were isolated after electrophoresis (10% polyacrylamide, 4 M urea gel) and purified by phenol/chloroform extraction followed by ethanol precipitation. Conditions (time and enzyme concentration) for labeling each substrate were chosen to minimize the amount of pyrophosphate removed from the added N−1 nucleotide and thus to maximize the yield of the desired pppN−1-tRNA. The fraction of each purified substrate that retained the pppN−1 5’-end (as opposed to pN−1 that results from pyrophosphate removal) was determined by P1 nuclease digestion, which generates S’-phosphorylated mononucleotides (5’-pN−1 and S’-pppN−1) that can be resolved using PEI-cellulose TLC in a 1 M LiCl solvent system. All substrates utilized for this work were obtained with a minimum of 80% S’-pppN−1 using this method (see Figure S3 of the Supporting Information).

[α-32P]N15,tRNAHis Assays. Intrinsic (without NTP) Rate of Pyrophosphate Removal. Kinetic parameters for the reaction of the α-32P-labeled (pppN−1-containing) tRNAHis substrates were determined at room temperature by reacting tRNA substrates (≤40 nM each) in the presence of 15 μM SceThg1 in Thg1 reaction buffer as described above. At various time points, aliquots (10 μL) were quenched by removal and addition to a new tube containing a phenol/chloroform/isoamyl alcohol mixture [25:24:1 (v:v:v)] and purified by extraction followed by ethanol precipitation. The purified concentrated reaction mixtures were then digested with P1 nuclease to generate pN−1 (from pyrophosphate removal) or pppN−1 (resulting from unreacted tRNA) (Scheme 1; P1 digestion products indicated in parentheses below each species). These digestion products were resolved using PEI-cellulose TLC in a 1 M LiCl solvent system, and time courses of product formation were fit to a single-exponential rate equation (eq 1) or by the method of linear initial rates for slow...
reaction (eq 4) to yield the \( k_{\text{obs}} \) and \( P_{\text{max}} \) for pyrophosphate removal in the absence of added NTP.

**Rates of Nucleotide Addition and Pyrophosphate Removal in the Presence of NTPs.** For reaction mixtures containing added NTP (where either nucleotide addition or pyrophosphate removal could occur (see Scheme 2)), aliquots from reaction mixtures containing 15 \( \mu M \) (saturating) SceThg1 and the indicated NTP at 1 \( \mathrm{mM} \) were taken at various time points and split into two separate digestion reaction mixtures.

To directly measure time courses of nucleotide addition, aliquots (5 \( \mu L \)) were digested with nucleases (RNase A for reaction mixtures containing GTP or ATP or RNase T1 for reaction mixtures containing CTP or UTP) and then treated with CIP to remove terminal phosphates from the labeled nucleotide or oligonucleotide products. This treatment yields two products, either labeled oligonucleotides corresponding to the relevant nucleotide addition products (as indicated below) or inorganic phosphate (\( P^* \)), which is derived from two possible sources: unreacted tRNA substrate or generation of \( \text{p}^*\text{tRNA} \) by pyrophosphate removal. The labeled oligonucleotides and \( P^* \) are resolved as described previously by silica TLC in a 1-propanol/NH_4OH/H_2O [55:35:10 (v:v:v)] solvent system. Addition of ATP or GTP yields labeled oligonucleotides and \( P^* \) from reaction mixtures containing \( 15 \mathrm{pGpC} \) or \( 1\mu M \) \( 	ext{GTP} \) to remove \( P^*\text{tRNA} \) or pyrophosphate (\( P^*\text{tRNA} \)) by pyrophosphate removal. The labeled oligonucleotides and \( P^* \) are produced either directly by pyrophosphate removal or inorganic phosphate of \( 1\text{pGpC} \) or \( 1\mu M \) \( 	ext{GTP} \) to remove \( P^*\text{tRNA} \) or pyrophosphate (\( P^*\text{tRNA} \)) by pyrophosphate removal (\( k_{\text{obs}} \)) for PPR (eq 6).

**Scheme 2**

\[
\text{Scheme 1}
\]

\[
\begin{align*}
\text{ppp}^*\text{N}_1\text{tRNA} & \xrightarrow{k_{\text{ppr}}} \text{p}^*\text{N}_1\text{tRNA} \\
\text{(ppp}^*\text{N}_1) & \xrightarrow{k_{\text{ppr}}} \text{p}^*\text{N}_1\text{tRNA} \text{(and further addition)}
\end{align*}
\]

The Intrinsic Rate of Removal of Pyrophosphate from the G-1 Residue of Base-Paired tRNA\textsubscript{His} Substrates Is Slow. Previously, we developed transient kinetic assays to measure the pyrophosphate removal step of the G-1 addition reaction (Figure S1 of the Supporting Information); these assays used \( \text{S}^* \cdot \gamma^* \cdot \text{P-triphosphorylated tRNA}^{\text{His}} \) (\( \text{p}^*\text{pp}^*\text{tRNA}^{\text{His}} \)) transcripts as substrates for the pyrophosphate removal reaction, to mimic the G-1-containing products after the nucleotidy transfer (G-1 addition) step had been catalyzed (i.e., the termini of the substrate are as indicated by the red box in Figure 1A). Because a \( \text{S}^* \)-triphosphorylated end on the tRNA is generated regardless of whether the G-1 nucleotide is added by the adenylation-dependent or adenylation-independent pathway of \( 3' \to 5' \) addition (Figure S1 of the

**Table 1. Kinetics of 5'-Pyrophosphate Removal Measured with \( \gamma \)-Labeled (\( \text{p}^*\text{pG}_{-1} \)) tRNA\textsubscript{His}**

| \( \text{tRNA}^{\text{His}} \) | \( k_{\text{ppr}} \) (min\(^{-1}\)) | \( k_{\text{D,RTA}} \) (\( \mu M \)) | \( k_{\text{ppr}} \) (min\(^{-1}\)) | \( k_{\text{D,RTA}} \) (\( \mu M \)) |
|----------------|----------------|----------------|----------------|----------------|
| \( G-1 \cdot \text{A}_{-2} \) | 0.030 \( \pm \) 0.003 | 1.6 \( \pm \) 0.7 | 0.77 \( \pm \) 0.04 | 2.5 \( \pm \) 0.4 |
| \( G-1 \cdot \text{C}_{-2} \) | 0.0006\(^a\) | ND\(^b\) | 0.43 \( \pm \) 0.04 | 16 \( \pm \) 4 |

\(^a\)\( k_{\text{ppr}} \) estimate determined using the method of initial rates. \(^b\)Parameter not determined.

\( P_{\text{total}} \) corresponds to the observed \( \text{p}^*\text{G} \) from the nuclease P1 digest and \( P_{\text{add}} \) corresponds to the observed addition products from the RNase/CIP digest. The \( k_{\text{obs}} \) and \( P_{\text{max}} \) for pyrophosphate removal versus nucleotide addition reactions were determined either by a fit of the time courses of each product formation (\( P_{\text{add}} \) measured directly and \( P_{\text{PPK}} \) calculated from eq 5) to the single-exponential rate equation (eq 1) or by the method of linear initial rates for slow reactions (eq 4), as indicated.

For tRNA substrates with WC base-paired \( \text{N}_{-1} \cdot \text{N}_{-2} \) ends assayed in the presence of the correct WC base pairing NTP, such that nucleotide addition is an abundantly observed reaction product, the indirect subtraction method (eq 5) generates correspondingly small values for the percent of pyrophosphate removal (\( P_{\text{PPK}} \)) reaction products. The fits of these \( P_{\text{PPK}} \) data to eq 1 yield \( k_{\text{obs}} \) values for pyrophosphate removal that are incompatible with the observed reaction amplitudes. Therefore, a kinetic partitioning method was used for these reactions, as indicated in each table, to calculate the \( k_{\text{obs}} \) for PPR (eq 6). For this approach, the fraction of total reaction products due specifically to nucleotide addition at the completion of the reaction (i.e., when [ES] is maximal) was determined from the maximal amplitude of the \( \text{N}_{-1} \) addition reaction (\( P_{\text{add,max}} \)) divided by the total reaction amplitude (\( P_{\text{total}} \)). Thus, using the \( k_{\text{obs}} \) for the addition reaction (\( k_{\text{add}} \)) that was directly measured by the RNase/CIP digest, the \( k_{\text{obs}} \) for pyrophosphate removal (\( k_{\text{ppr}} \)) could be calculated from eq 6.

\[
P_{\text{add,max}}/P_{\text{total}} = k_{\text{add}}/(k_{\text{add}} + k_{\text{ppr}})
\]
In this assay, SceThg1-catalyzed hydrolysis of the in the absence of any added nucleotide under single-turnover determined by incubating labeled tRNA with excess enzyme were performed with 5′SceThg1 to remove the 5′ing C73-tRNAHis (i.e., the termini of the substrate are as estimated from these data to be *fi.* Thus, SceThg1 exhibits a very limited intrinsic ability to remove (p′-triphosphate) causes the release of labeled pyrophosphate, which was quantified to determine the pseudo-first-order rate constant (k(pppG−1:C73) of 0.030 ± 0.003 min−1 under standard SceThg1 reaction conditions (Table 1). To determine whether the presence of the C73 nucleotide in the tRNAHis variant that is a substrate for multiplet addition alters the inherent ability of SceThg1 to remove the S′-pyrophosphate, the same reactions were performed with S′-p′-triphosphorylated, G−1-containing C73-tRNAHis (i.e., the termini of the substrate are as indicated by the red box in Figure 1B). With this substrate (p′ppG−1:C73), even in the presence of high concentrations of SceThg1 (15 μM, which is significantly greater than the measured Kapp for the pppG−1:A73-tRNAHis substrate), time courses of product formation remained linear after 3 h, and less than 10% of the tRNA was converted to product (Figure 2A). Using the method of initial rates (eq 4), the observed rate of pyrophosphate removal for the G−1:C73 variant substrate was estimated from these data to be ~0.0006 min−1 (Table 1). Thus, SceThg1 exhibits a very limited intrinsic ability to remove the S′-pyrophosphate from the C73-containing tRNA, as compared to the robust rate of removal of S′-pyrophosphate from the wild-type substrate.

Previously, however, we observed that addition of 1 mM GTP stimulated the kppp and with wild-type tRNAHis, despite the fact that GTP does not appear to compulsorily participate in the chemistry of the S′-pyrophosphate removal reaction (see Figure 1A). Because GTP is necessarily included in standard G addition assays (and is also present in the cell at concentrations similar to those employed in these assays), the ability to remove pyrophosphate from the base-paired G−1:C73 variant tRNA might also be enhanced in the presence of GTP. Indeed, time courses of release of labeled pyrophosphate from the reactions with p′pp-G−1:C73 substrate in the presence of GTP were readily observed and fit well to the single-exponential equation (eq 1) (Figure 2B). From these assays, we determined the kppp and KappRNA for the release of pyrophosphate from the p′pp-G−1:C73 substrate based on the pyrophosphate removal and nucleotide addition activity of SceThg1.

A New Assay for Distinguishing between Removal of S′-Pyrophosphate and Addition of G−2. We developed a new enzyme assay that allows simultaneous detection of both pyrophosphate removal and nucleotide addition products. In doing so, we also sought to develop an assay that would permit characterization of tRNAHis variants that contain other nucleotides, because the identity of the −1:73 base pair is known to affect the number of nucleotides added by SceThg1. Because transcripts that are initiated with S′-triphosphorylated nucleotides other than G−1 are not efficiently produced by wild-type T7 RNA polymerase, an alternative strategy that takes advantage of the inherent 3′− S′ addition activity of Thg1 to generate labeled tRNAs to be used as substrates in the assays was developed.

The assay developed here utilizes S′-[α-32P]tRNAHis (ppp−1 tRNAHis) substrates generated by incubating Thg1 with various combinations of [α-32P]NTPs and tRNAHis transcripts engineered to lack a −1 nucleotide (as described in Materials and Methods). In this labeling scheme, Thg1 catalyzes the addition of the desired α-labeled NTP to the −1 position of the tRNA, thus generating a S′-triphosphorylated tRNA (shown in the red box in Figure 3) similar to the earlier substrates generated by in vitro transcription, but with two important advantages. First, the substrates are labeled at the α-phosphate, allowing for separate quantification of both pyrophosphate
Figure 3. Labeling scheme for generating α-32P-labeled tRNAHis substrates. S′-ppp*N1:N3-tRNAHis substrates were generated by incubating in excess Thg1 (from various sources, as indicated in Materials and Methods) with the desired [α-32P]NTP and tRNAHis transcripts that are initiated with G1. Reaction conditions were optimized to yield major reaction products as indicated in the red box, while minimizing the amount of subsequent removal of pyrophosphate from the tRNA (indicated by the tRNA in brackets). The purity of the resulting labeled substrates was determined by P1 nuclease digestion of the purified labeled tRNA and subsequent TLC analysis (see Figure S3 of the Supporting Information). P1 cleavage of the substrate with an intact S′-triphosphate yields ppp*N if the S′-pyrophosphate was removed during the preparation of the substrate, this is visualized as p*N.

removal and nucleotide addition products, as described below. Second, because the S′-nucleotide of each substrate is incorporated by Thg1, and not by T7 RNA polymerase, substrates that are initiated uniquely with α-labeled S′-triphosphorylated nucleotides other than G can be generated by this approach. A series of substrates were generated in which the identity of the terminal ppp*N1:N3 base pair was varied by including different tRNAHis transcripts and labeled NTPs in the Thg1 labeling reactions. All substrates used for assays described below were demonstrated to contain a minimum of 80% S′-triphosphate after purification by the P1 nuclease analysis described above (Figure S3 of the Supporting Information).

Validation of the α-Labeled Assay with the Wild Type and C32-tRNAHis Variants. Rates of reaction were measured with ppp*G1:A73 and ppp*G1:C73-tRNAHis [for which rates had been determined previously using γ-32P-labeled tRNA (Table 1)] as described in Materials and Methods. In the absence of any added NTP, the only possible reaction outcome is the removal of the S′-pyrophosphate, generating p*G1 after nuclelease digestion, which is readily resolved from ppp*G1 derived from unreacted tRNA (Scheme 1). These products were quantified for both tRNA substrates (Figure 4A,B). The observed intrinsic (without NTP) rates of pyrophosphate removal for both ppp*G1:A73 and ppp*G1:C73-tRNA substrates were in excellent agreement with the kobs obtained with γ-32P-labeled substrates described above (compare Tables 1 and 2).

Next, assays were performed with the same substrates (ppp*G1:A73 and ppp*G1:C73) in the presence of added GTP, and separate digestions were used to simultaneously measure the rates of nucleotide addition and pyrophosphate removal, as described in Materials and Methods. Nucleotide addition products were measured directly by RNase A/
these data were the apparent product speciﬁed by $k_{obs}$ for pyrophosphate removal in the absence of added GTP with (A) $\text{ppp}^*\text{G}_{12-13}\text{tRNA}^{\text{His}}$ or (B) $\text{ppp}^*\text{G}_{12-25}\text{tRNA}^{\text{His}}$. Reactions shown are time courses of activity with 15 μM SceThg1 in excess over tRNA substrate; aliquots from each time point were digested with P1 nuclease followed by resolution on PEI-cellulose TLC plates. Plots of product vs time as quantiﬁed from these data were ﬁt to eq 1 to yield $k_{obs}$ and $P_{\text{max}}$ (C and D) Representative single-turnover assays for determining the GTP-stimulated $k_{obs}$ for the removal of pyrophosphate from (E) $\text{ppp}^*\text{G}_{12-13}\text{tRNA}^{\text{His}}$ or (F) $\text{ppp}^*\text{G}_{12-25}\text{tRNA}^{\text{His}}$. Aliquots from the same reactions analyzed in panels C and D were removed at each time point, digested with nuclease P1, and subsequently resolved by PEI-cellulose TLC. The total reaction products ($P_{\text{total}}$) from either G:A or pyrophosphate removal were quantiﬁed as the percent of $P^*G$ (which is produced by either reaction product (see Scheme 2)) observed as a function of time. Subtraction of the percent product due to addition ($P_{\text{add}}$ measured in panels C and D) from $P_{\text{total}}$ yields the apparent product speciﬁcally arising from pyrophosphate removal ($P_{\text{ppr}}$) at each time point (eq 5). For substrates with non-WC base-paired ends, $P_{\text{ppr}}$ is ﬁt directly to eq 1 to yield $k_{obs}$ and $P_{\text{max}}$. For the WC base-paired substrates in the presence of GTP (eq 6) is used to calculate $k_{obs}$ for the removal of pyrophosphate ($k_{\text{ppr}}$) from the measured $k_{\text{add}}$, $P_{\text{add}}$, and $P_{\text{total}}$.

Table 2. Kinetics of $5'-\text{Pyrophosphate Removal vs Nucleotide Addition Measured with } \alpha\text{-Labeled (\text{ppp}^*\text{N}_{\text{sub}}) tRNA}^{\text{His}}$

| tRNA$^{\text{His}}$ | without NTP | 1 mM GTP |
|---------------------|-------------|----------|
|                     | $k_{obs}$ (min$^{-1}$) | $P_{\text{max}}$ (%) | $k_{obs}$ (min$^{-1}$) | $P_{\text{max}}$ (%) | $k_{obs}$ (min$^{-1}$) | $P_{\text{max}}$ (%) |
| $\text{G}_{12-13}\text{tRNA}^{\text{His}}$ | 0.025 ± 0.001 | 75 ± 1 | 0.31 ± 0.03 | 84 ± 2 | 0.05 ± 0.01 | 7 ± 1 |
| $\text{G}_{12-25}\text{tRNA}^{\text{His}}$ | 0.021 ± 0.003 | 48 ± 2 | 0.20 ± 0.03 | 67 ± 2 | 0.05 ± 0.02 | 16 ± 2 |
| $\text{G}_{12-13}\text{tRNA}^{\text{His}}$ | 0.022 ± 0.003 | 59 ± 2 | 0.26 ± 0.02 | 38 ± 1 | 0.17 ± 0.03 | 43 ± 2 |
| $\text{G}_{12-25}\text{tRNA}^{\text{His}}$ | 0.0002$^a$ | 31$^b$ | 0.02 ± 0.01$^c$ | N/A | 0.25 ± 0.01 | 83 ± 1 |
| $\text{C}_{12-13}\text{tRNA}^{\text{His}}$ | 0.0006$^a$ | 12$^b$ | 0.02 ± 0.01$^c$ | N/A | 0.21 ± 0.02 | 74 ± 2 |
| $\text{U}_{12-13}\text{tRNA}^{\text{His}}$ | 0.017 ± 0.001 | 69 ± 2 | 1.2 ± 0.5$^c$ | N/A | 0.04 ± 0.1 | 19 ± 1 |

$^a$k$_{obs}$ estimated derived using the method of the initial rates. $^b$Maximal amount of product observed after reaction for 3–4 h. $^c$k$_{obs}$ for the removal of pyrophosphate from WC base-paired substrates calculated by partitioning from eq 6, as described in Materials and Methods. $P_{\text{max}}$ for these products is not applicable (N/A) because rates were not derived from the fit to eq 1.

pyrophosphate removal versus nucleotide addition reactions determine the reaction outcome seems to apply to these tRNAs.

**Mechanistic Rationale for Differences with G:C versus A:U Base Pairs.** As described above, an advantage of the $\alpha$ labeling scheme is the ability to introduce labeled triphosphorylated nucleotides other than G to the 5’-end of the tRNA substrates. We took advantage of this feature to investigate an earlier observation that SceThg1, unlike TLPs, exhibits a preference for G:C or C:G base pairs. Even when a poly(A) or poly(U) sequence is engineered into the 3’-end of tRNA$^{\text{His}}$, multiple additions of U or A nucleotides by SceThg1 are not
observed. Variant tRNAHis substrates ppp*G1:A73 and ppp*U1:A73 were created (Figure S3 of the Supporting Information) and tested to determine whether the identity of the −1:73 base pair affects the kinetic behavior of SceThg1.

As observed for G1:C73-tRNA, the intrinsic (without GTP) rate of pyrophosphate removal with the ppp*C1−G2−variant was extremely slow; time courses revealed only ∼12% removal of pyrophosphate even after long times, and the $k_{obs}$ was estimated to be ∼0.0006 min⁻¹ using the method of initial rates (Table 2). Likewise, for reactions performed in the presence of GTP where partitioning between the two pathways is possible, the $k_{obs}$ for pyrophosphate removal calculated by partitioning from eq 6, as described in Materials and Methods. $k_{obs}$ for pyrophosphate removal calculated by partitioning from eq 6, as described in Materials and Methods.

Table 3. Kinetics of Pyrophosphate Removal Measured with α-Labeled (ppp*N−1) tRNAHis in the Presence of NTPs (1 mM each)

| tRNAHis | without NTPa | GTPb | ATP | CTP | UTP |
|---------|-------------|------|-----|-----|-----|
| G1:A73  | 0.25 ± 0.001| 0.31 ± 0.03| 0.12 ± 0.03| 0.8 ± 0.3c| 0.4 ± 0.1c|
| G1:G73  | 0.21 ± 0.003| 0.20 ± 0.03| 0.16 ± 0.02| 0.8 ± 0.2| 0.3 ± 0.1|
| G1:U73  | 0.22 ± 0.003| 0.26 ± 0.02| 0.13 ± 0.01| 0.7 ± 0.1| 0.4 ± 0.1|
| G2:C73  | 0.0002b, 0.02 ± 0.01c, 0.005c, 0.026 ± 0.002, 0.014 ± 0.002 |
| C1:G73  | 0.0006b, 0.02 ± 0.01c, 0.05 ± 0.01, 0.17 ± 0.05, 0.05 ± 0.01 |
| U1:A73  | 0.017 ± 0.001| 1.2 ± 0.3, 0.18 ± 0.01, 0.4 ± 0.1, 0.26 ± 0.04 |

“Values as reported previously in Table 2 and shown for comparison. b$ k_{obs}$ estimate derived from observed rates determined using the method of initial rates. $ k_{obs}$ for pyrophosphate removal calculated by partitioning from eq 6, as described in Materials and Methods. “$ k_{obs}$ derived from a fit to data obtained from a single experiment with this tRNA/NTP combination.
detectable N-2 addtion was observed for any of the other NTPs, and thus, the k_{obs} for addition could not be accurately measured for most reactions (Table S1 of the Supporting Information). For the few combinations in which a small amount of observed N-2 addition allowed rates to be determined, the observed rates of pyrophosphate removal (Table 3) were nevertheless faster than the k_{obs} for addition, consistent with the observed abundance of pyrophosphate removal products. The k_{obs} values for pyrophosphate removal were consistently slower in the presence of ATP (by ~2-fold relative to those with GTP) and fastest in the presence of CTP (~2–3-fold higher than rates in the presence of GTP), suggesting specific interactions between SceThg1 and the NTP bound in the active site that affect the ability of each NTP to promote the rate of pyrophosphate removal.

For substrates containing WC base pairs at the N-1:N-2 position (G_{134}:C_{735}, C_{134}:G_{735}, and U_{134}:A_{735}), the same kinetic comparison revealed two important features. First, efficient N-2 addition of any NTP other than GTP was not observed (Table S1 of the Supporting Information), and therefore, the k_{obs} for pyrophosphate removal in the presence of ATP, CTP, or UTP was determined by the subtraction method (eq S) and fitting the time courses of calculated pyrophosphate removal products to eq 1 (Table 3). Second, as with reactions measured in the presence of GTP, the distinct behavior of SceThg1 with G_{134}:C_{735}/C_{134}:G_{735}tRNAs versus U_{134}:A_{735}tRNA was clear. The k_{obs} values for the removal of pyrophosphate from the ppp*N_{134}tRNA_{32} substrate in the presence of each NTP were strikingly similar to the k_{obs} values with the mismatch-containing tRNAs (Table 3). In contrast, for ppp*G_{134}:C_{735} and ppp*G_{134}:G_{735}tRNA, although the k_{obs} values for the pyrophosphate removal reaction in the presence of other NTPs were all stimulated to various extents above the intrinsic (without NTP) k_{obs}, there was no obvious trend correlating k_{obs} with the identity of the NTP included in the reaction (Table 3).

**Template Dependence of N-2 Addition.** Efficient addition of N-2 by SceThg1 in the preceding experiments appears to require two components. First, the tRNA substrate must contain a G:C/C:G WC base-paired end, and second, the correct NTP to make a WC base pair with N-2 (GTP in the previous examples) must be included in the reaction. To further probe the apparently special preference of SceThg1 for synthesis of G/C base pairs, two variant tRNA substrates were generated that similarly contain WC terminal G:C/C:G base pairs but have alterations in the 3’-end template bases of the acceptor stem (to either A_{34} or G_{34}/G_{35}), and thus template the incorporation of U_{20} or C_{20}, respectively (Figure S2 of the Supporting Information). Assays were performed to measure the rates and amplitudes for pyrophosphate removal versus nucleotide addition reactions with these α-32P-labeled (ppp*N_{134}tRNA) substrates in the presence of each of the four NTPs (Table 4).

Comparison of k_{obs} and P_{max} for pyrophosphate removal versus N-2 addition with the A_{20}-containing tRNA (G-C\_ACA) revealed two salient features. First, introduction of A_{34} in place of C_{34} caused a significant increase in the extent and rate of addition of U_{20}, with a corresponding decrease in the extent of addition of G_{20} (Table 4). Interestingly, despite the apparent ability of SceThg1 to recognize and form a U_{20}:A_{34} base pair, the U_{20} addition products constitute only a minor fraction (~20%) of the total products. Second, the calculated k_{obs} for pyrophosphate removal with this substrate (0.049 min^{-1}) remains faster than the rate of subsequent addition (0.016 min^{-1}), consistent with the minority of U_{20} addition products (Table 4).

For the tRNA^{His} variant containing G_{20}/G_{35}, the extent of product formation by the two competing pathways is also largely consistent with WC base pairing requirements. Addition of C_{34} to G_{20}-tRNA^{His} accounted for 54% of the total products (as compared with no detectable C_{20} addition to the analogous C_{35}-containing tRNA) (Table 4). There was a correspondingly dramatic decrease in the P_{max} due to nucleotide addition observed in the presence of GTP [12% for G_{20}-tRNA compared with 74% when the GTP can make a base pair with C_{34} (Table 4)]. While SceThg1 catalyzed little to no detectable non-WC addition of ATP, CTP, or UTP, a low level (10–20%) of G_{20} addition was observed with both N_{20} variant substrates. This limited, but detectable, ability of SceThg1 to add G_{20} to create non-WC base pairs (in this case, G_{20}:A_{34} or G_{20}:G_{34}) may reflect features of the eukaryotic Thg1 active site that evolved to specifically allow addition of a non-WC G_{20} nucleotide during tRNA^{His} maturation.

**The GTP 3’-OH Is Required To Stimulate Pyrophosphate Removal.** The role of the nucleotide cofactor in enhancing pyrophosphate removal was investigated further by testing the effects of GTP nucleotide analogues on the kinetics of the pyrophosphate removal reaction with wild-type (p**ppG_{134}:A_{735}) tRNA^{His}. Time courses of pyrophosphate

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**Table 4. Kinetics of Pyrophosphate Removal versus Nucleotide Addition Measured with α-Labeled (ppp*N_{134}) tRNA^{His} Substrates with Variations at N-2**
removal for reaction mixtures containing 15 μM enzyme and GTP analogues (1 mM each) revealed that 2′-dGTP stimulated the $k_{obs}$ 6-fold, comparable to the 10-fold stimulation of the rate observed with the standard GTP nucleotide. While 3′-methoxy-GTP exerted only a modest stimulatory effect (2-fold) on the rate of the reaction, no stimulation was observed with either 3′-dGTP or 2′,3′-d-dGTP (Figure 6 and Table S2 of the Supporting Information).

Figure 6. Rates of pyrophosphate removal with $^{3}p$ppG$_{1-2}$:A$_{73}$-tRNA$^{His}_{S}$ in the presence of GTP nucleotide analogues. Single-turnover measurements of $k_{obs}$ for GTP removal with limiting pppG$_{1-2}$:tRNA$^{His}_{S}$ substrate determined in the presence of 15 μM SceThg1 and 1 mM GTP (●), 3′-dGTP (□), 2′-dGTP (○), 2′,3′-ddGTP (○), and 3′-methoxy-GTP (△) or without NTP (open +). Product formation was measured by time courses of labeled pyrophosphate formation visualized on PEI-cellulose TLC, plotted as a function of time and fit to a single-exponential equation (eq 1) to yield the observed rate ($k_{obs}$) for reaction in the presence of each NTP analogue (Table S2 of the Supporting Information).

■ DISCUSSION

Here, we used kinetic assays to investigate the interplay between two competing reactions: pyrophosphate removal and nucleotide addition catalyzed by SceThg1. Taken together, the data suggest that with wild-type tRNA$^{His}_{S}$, a kinetic mechanism in which the rate of removal of pyrophosphate from the 3′ pppG$_{1-2}$:tRNA$^{His}_{S}$ substrate is faster than the rate of addition of G$_{3}$ to this same tRNA effectively terminates the reaction after the single G$_{3}$ nucleotide is added by SceThg1 (Tables 1 and 2). The data also reveal a complex role for additional NTPs in both reactions. Rate constants and the maximal amplitude of product formation for the pyrophosphate removal versus nucleotide addition reactions depend on the identity of the base pair at the −1:73 terminus, as well as on the identity of the potential base pair to be formed between the incoming NTP and the N$_{74}$ nucleotide (Tables 3 and 4). Finally, we reveal that the kinetic preference for catalyzing the removal of pyrophosphate from U$_{1-2}$:A$_{73}$-tRNA$^{His}_{S}$ effectively limits the ability of SceThg1 to efficiently add subsequent nucleotides to substrates that terminate with a U:A base pair, thus rationalizing the previously observed lack of multiple U or A additions by the 3′−5′ polymerase activity (Tables 2−4).

On the basis of the shared ability of all Thg1/TLP enzymes to catalyze 3′−5′ polymerase activity, this WC-dependent polymerization reaction is suggested to be the ancestral activity of Thg1 family enzymes. Thus, the 3′−pyrophosphate removal reaction may have been exploited by eukaryotes as a means of limiting this ancestral 3′−5′ polymerase activity for the purposes of tRNA$^{His}_{S}$ maturation and thus could provide a rationale for the acquisition of the A$_{73}$ discriminator nucleotide, as opposed to the C$_{73}$ found nearly universally in bacteria and archaea. The pressure to evolve a mechanism to limit the addition of nucleotides to tRNA$^{His}_{S}$ may have arisen from the need to avoid perturbation of the structure of the 3′-CCA end, where base-paired interactions between extra 5′-nucleotides and the 3′-terminal CCA could interfere with the optimal function of the translation machinery. Interestingly, the presence of additional (G$_{-2}$ and G$_{-3}$) nucleotides on tRNA$^{His}_{S}$ does not interfere with histidylation of tRNA$^{His}_{S}$ in vivo in yeast and, in fact, enhances the reaction of the HisRS slightly, suggesting that avoiding defects in aminocyclization was not specifically the critical driving force for evolution of 5′-pyrophosphate removal activity.

The use of a kinetic mechanism to control relative rates, and therefore outcomes, of competing reactions is well-documented in biology. Canonical 5′−3′ DNA polymerases employ a similar kinetic partitioning mechanism whereby the rate of nucleotide addition is dramatically decreased once a mismatched (non-WC) base pair is formed during a preceding 5′−3′ addition reaction. This permits the proofreading exonuclease activity to compete more effectively with polymerase activity at the site of a nucleotide misincorporation. The molecular basis for the faster rate of removal of 5′-pyrophosphate from non-WC base-paired substrates than from base-paired termini by SceThg1 is not yet apparent. Although we previously used site-directed mutagenesis combined with structural data to identify residues that play roles in the adenylylation and nucleotidyl transfer steps of the G$_{-1}$ addition reaction, protein residues that participate uniquely in the 5′-pyrophosphate removal step have not been identified. Identification of residues that are important for this step, such as a general base that could deprotonate a nucleophilic water to enhance attack on the α-β phosphodiester bond, could help to rationalize the distinct outcomes of different substrates in terms of 5′-pyrophosphate removal. It is also possible that the nucleophilic water is coordinated solely by either of the two essential active site metals and/or the NTP or tRNA substrates, and that unique protein residues that participate directly in the chemistry of this step may not be identified. The role of the stimulating NTP in the pyrophosphate removal step, despite the fact that no NTP is formally required for the chemistry of pyrophosphate removal (Figure 1A), is particularly enigmatic. The kinetic studies reported here demonstrate that the identity of the NTP included in the reactions has a significant effect on the observed rates of pyrophosphate removal, and in particular that the 3′-hydroxyl is required for this stimulation (Table 3 and Figure 6). Under conditions that favor pyrophosphate removal, the 3′-hydroxyl of the stimulating NTP might stabilize the catalytic metal ions, might help to optimally position the tRNA and/or nucleophilic water molecule, or might induce a conformation of the [ES] complex that increases the accessibility of the active site to water.

Through this work, we provide a kinetic basis for the previously unexplained observation that SceThg1 efficiently catalyzes the formation of G:C and C:G WC base pairs but does not efficiently polymerize A−U base pairs with any substrate. We observed that the pyrophosphate removal activity under all conditions preferentially acts upon tRNAs containing a U$_{1-2}$:A$_{73}$ terminating base pair (Tables 2 and 3). Thus, although SceThg1 can add UTP to create a U:A base pair, it efficiently removes the activated 5′-end from this added
nucleotide and effectively terminates subsequent addition reactions. We note that this is not due to an inability of SceThg1 to distinguish a U:A base pair from a mismatched base-pairing combination, because the preference for addition of U− to other nucleotides to the A− containing tRNA^His variant was readily observed in our assays (Table 4). This distinct behavior with G-C versus U-A base pairs is a significant way that eukaryotic Thg1-type enzymes differ from TLPs that catalyze tRNA repair, because TLPs readily polymerize all four WC base pairs with various truncated tRNA substrates.21,22

An added advantage to the α-labeled enzyme assays developed here is the ability to investigate the apparent kinetics of addition of the N− nucleotide to tRNA^His substrates catalyzed by SceThg1. Under the conditions tested here (1 mM NTP, which is well above the K_{D,GTP} of 25 μM for the G− addition reaction catalyzed by SceThg1), the k_{obs} and P_{max} for adding a WC base-paired G− or C− nucleotide are significantly greater than the corresponding values for formation of non-WC base pairs (Table 4), yet the overall rate constants for G− addition are slower than the k_{obs} for G− measured vs 3.0 min−1 for G− measured previously.13 These results suggest that although SceThg1 exhibits 3′−5′ polymerization with some tRNA substrates, addition of a nucleotide at the −1 position is the preferred reaction of SceThg1.

Interestingly, TLPs from Acanthamoeba castellani and Spizellomyces punctatus do not appear to efficiently remove the 3′′pyrophosphate from nucleotide addition products generated by their 3′−5′ polymerase activity, as judged by the accumulation of 3′−5′ phosphorylated tRNA on the ends of tRNA species repaired by these enzymes;21,22 however, the editing reaction catalyzed by TLPs also requires formation of a discrete 3′−end, and these enzymes must therefore possess a mechanism for terminating addition after completing repair of the tRNA aminoacyl-acceptor stem. The retention of the 5′′capped by the capping guanylyltransferase, which requires a free 5′′−phosphorylate the G−1 residue is not necessarily a ubiquitous reaction even in eukaryotes. In chicken mitochondria, the presence of a 3′−5′phosphorylated G−1 on tRNA^His was inferred by the absence of the in vivo-isolated tRNA to be capped by the capping guanylyltransferase, which requires a free 3′−5′phosphorylated G−1 residue. A more complete investigation of the 3′−phosphorylation status of Thg1 and TLP reaction products in vivo may provide important evidence for how these reactions are controlled in various members of the Thg1 enzyme superfamily.

ASSOCIATED CONTENT

Supporting Information
Parameters for nucleotide addition kinetics with N−:N7− tRNA^His variants (Table S1), observed rates of pyrophosphate release in the presence of GTP analogues (Table S2), alternative reaction pathways for addition of G−1 to yeast tRNA^His (Figure S1), GTP dependence of pyrophosphate release kinetics for G−1:A− tRNA^His versus G−1:C− tRNA^His (Figure S2), assessment of the 3′−phosphorylation status of pp+p−tRNA^His substrates for α-labeled digestion assays (Figure S3), and assays for measuring the kinetics of reactions with pp+p−U−1:A−2 tRNA^His (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

HisRS, histidyl-tRNA synthetase; Thg1, tRNA^His guanylyltransferase; TLPs, Thg1-like proteins; SceThg1, S. cerevisiae Thg1 (formerly yThg1); p-tRNAHis, 5′−monophosphorylated tRNA^His; ppp−tRNA^His, 5′−triphosphorylated tRNA^His; pppG−1− tRNA^His 5′−triphosphorylated G−1−containing tRNA^His; WC, Watson−Crick; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4−2-hydroxyethyl-1-piperazineethanesulfonic acid; RNase A, ribonuclease A; ppp, pyrophosphate removal.

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