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Saccharomyces cerevisiae Thg1 Uses 5′-Pyrophosphate Removal To Control Addition of Nucleotides to tRNA^{His}

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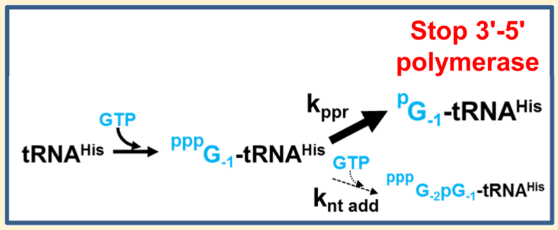
Supporting Information

ABSTRACT: In eukaryotes, the tRNA^{His} guanylyltransferase (Thg1) catalyzes 3′−5′ addition of a single guanosine residue to the −1 position (G_{-1}) of tRNA^{His}, across from a highly conserved adenosine at position 73 (A_{73}). 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 tRNA^{His} 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 tRNA^{His} variants. For 3′−5′ polymerization, Thg1 uses the 3′-end of the tRNA^{His} 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′ polymerase 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 tRNA^{His} substrates in which the identity of the N_{-1}:N_{73} 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 tRNA^{His} is proposed.



Canonical 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.[1,2] Enzymes of the tRNA^{His} 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.[3,5] In eukaryotes, Thg1 enzymes use the 3′−5′ addition reaction to add a single G residue (G_{-1}) to the 5′-end of tRNA^{His},[3,6,7] 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.[4,8−10] 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′-monophosporylated eukaryotic (A_{73}-containing) tRNA^{His} 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[3] and also occurs during the 3′−5′ polymerase reaction, once the first nucleotide has been added to the 5′-end of the tRNA[5] (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

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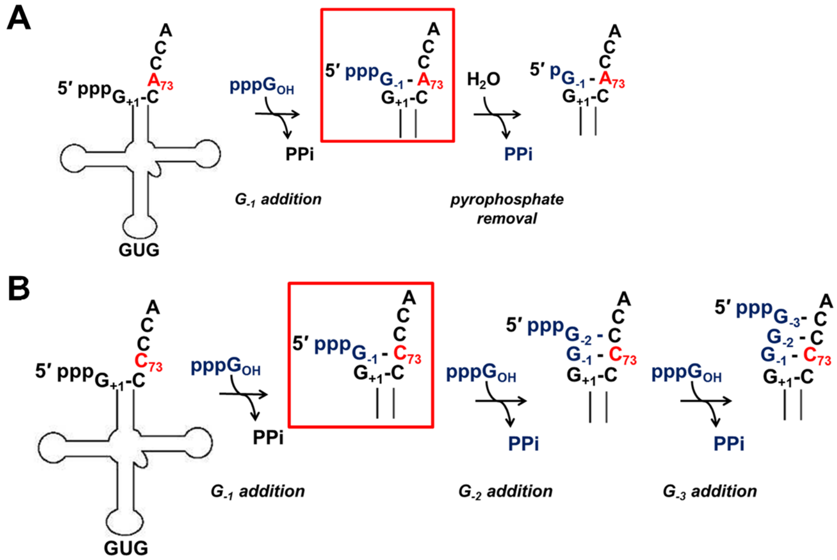

Figure 1. Alternative biochemical reactions catalyzed by SceThg1. (A) SceThg1 catalyzes non-Watson−Crick addition of G_{-1} to wild-type (A_{73}-containing) $tRNA^{His}$. After addition of the G_{-1} residue, SceThg1 removes pyrophosphate from the 5′-end of the wild-type tRNA. (B) SceThg1 catalyzes Watson−Crick-dependent 3′−5′ polymerization with C_{73}-containing $tRNA^{His}$ variant substrates. For this reaction, SceThg1 is presumed to use the 5′-triphosphate generated after each nucleotide is added as the activated end for the next nucleotide addition. Red boxes indicate the 5′- and 3′-termini of tRNA transcripts used as substrates to study pyrophosphate removal vs nucleotide addition in this work.

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_{-1} 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 archaeal TLP counterparts. Eukaryotic Thg1 enzymes such as *Saccharomyces cerevisiae* Thg1 (SceThg1, formerly termed yThg1) catalyze two different types of 3′−5′ addition reactions with similar kinetic efficiency, depending on the $tRNA^{His}$ substrate (Figure 1).[5] The first reaction is non-WC base-paired addition of G_{-1} opposite A_{73} observed with wild-type $tRNA^{His}$ (Figure 1A), and the second is WC-templated 3′−5′ polymerization of multiple G-C or C-G base pairs observed with $tRNA^{His}$ 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_{-1} nucleotide.[9,10] Moreover, SceThg1 does not polymerize multiple A-U or U-A base pairs with $tRNA^{His}$, while TLPs catalyze polymerization of all four WC base-paired nucleotides.[5,10] A key feature of the eukaryote-specific G_{-1} addition activity is the ability of the enzyme to terminate addition after only a single G_{-1} nucleotide is added to wild-type (A_{73}-containing) $tRNA^{His}$, despite the presence of the downstream C_{74} and C_{75} residues in the tRNA, which are used as the template for addition of multiple G residues in the C_{73}-containing $tRNA^{His}$ 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_{-1} 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_{-1} 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 $tRNA^{His}$ 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_{-1}:N_{73} base pairs, in the presence of various NTPs. Here we demonstrate that the varied abilities of SceThg1 to remove the 5′-pyrophosphate from each $tRNA^{His}$ 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 $tRNA^{His}$.

■ MATERIALS AND METHODS

Nucleotides and Reagents. NTPs (100 mM LiCl salts) used for enzyme assays were purchased from Roche; [γ-^{32}P]GTP (6000 Ci/mmol), [α-^{32}P]CTP (3000 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), and [α-^{32}P]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_6-tagged enzyme.[14,15] Briefly,

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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 of Labeled and Unlabeled tRNA.** *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-^{32}P-labeled tRNA (p*pp-tRNA), tRNAs were transcribed in the presence of [γ-^{32}P]GTP, according to published methods.[14] To generate the α-labeled tRNA (ppp*-tRNA) species used for some assays (see below), unlabeled 5′-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-^{32}P]G_{-1}-tRNA^{His} Assays. Kinetic parameters for turnover of the γ-^{32}P-labeled p*ppG_{-1}tRNA^{His} substrates were determined at room temperature by reacting 5′-p*pp-G_{-1}:A_{73}tRNA^{His} or 5′-p*pp-G_{-1}:C_{73}tRNA^{His} 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 $MgCl_2$, 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_i) from unreacted labeled tRNA. For the determination of $K_{D,app,GTP}$, reaction mixtures contained varied concentrations of GTP (0.01−1 mM) and a saturating concentration of enzyme (15 μM). For the determination of $K_{D,app,tRNA}$, 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)

$$P_{obs} = P_{max}[1 - \exp(-k_{obs}t)] \quad (1)$$

where P_{obs} is the percent product formed at each time and P_{max} is the maximal percent of product formation observed during each time course. P_{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_{obs} values determined for each concentration of GTP or SceThg1 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_{obs} values derived from fits to data obtained from at least two independent experiments unless otherwise indicated, and reported errors in k_{max} and $K_{D,app}$ are the least-squares estimate of the standard error derived from a fit to the combined data from all independent experiments using Kaleidagraph.

$$k_{obs} = k_{max} \times [\mathrm{NTP}]/(K_{D,app,NTP} + [\mathrm{NTP}]) \quad (2)$$

$$k_{obs} = k_{max} \times [\mathrm{SceThg1}]/(K_{D,app,tRNA} + [\mathrm{SceThg1}]) \quad (3)$$

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

$$k_{obs} = \nu_o/P_{max} \quad (4)$$

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

Construction of [α-^{32}P]N_{-1}tRNA^{His} Substrates. α-^{32}P-labeled tRNA^{His} substrates containing ppp*G_{-1} or ppp*C_{-1} were generated by reacting 15 μM SceThg1 with 20 pmol of 5′-triphosphorylated tRNA^{His} transcripts initiated at the +1 position and containing the desired 3′-terminal sequence and 20 pmol of [α-^{32}P]GTP or [α-^{32}P]CTP, respectively, in Thg1 reaction buffer at room temperature for 5−30 min. The ppp*U_{-1}:A_{73}-tRNA^{His} substrate was generated by reacting 20 μM purified TLP4 from *Dictyostelium discoideum*[8] with 80 pmol of 5′-triphosphorylated tRNA^{His} transcript initiated at the +1 position and 80 pmol of [α-^{32}P]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 ppp*N_{-1}-tRNA. The fraction of each purified substrate that retained the ppp*N_{-1} 5′-end (as opposed to p*N_{-1} that results from pyrophosphate removal) was determined by P1 nuclease digestion, which generates 5′-phosphorylated mononucleotides (5′-p*N_{-1} and 5′-ppp*N_{-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% 5′-ppp*N_{-1} using this method (see Figure S3 of the Supporting Information).

[α-^{32}P]N_{-1}tRNA^{His} Assays. *Intrinsic (without NTP) Rate of Pyrophosphate Removal.* Kinetic parameters for the reaction of the α-^{32}P-labeled (ppp*N_{-1}-containing) tRNA^{His} 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 p*N_{-1} (from pyrophosphate removal) or ppp*N_{-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

Scheme 1

$$\underset{(ppp^*N_{-1})}{ppp^*N_{-1}\text{-tRNA}} \xrightarrow{k_{ppr}} \underset{(p^*N_{-1})}{p^*N_{-1}\text{-tRNA}}$$

reactions (eq 4) to yield the k_{obs} and P_{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 μM (saturating) SceThg1 and the indicated NTP at 1 mM were taken at various time points and split into two separate digestion reaction mixtures.

Scheme 2

$$ppp^*N_{-1}\text{-tRNA} \xrightarrow{k_{ppr}} p^*N_{-1}\text{-tRNA}$$
$$ppp^*N_{-1}\text{-tRNA} \xrightarrow{k_{add}} pppN_{-2}p^*N_{-1}\text{-tRNA (and further addition)}$$

To directly measure time courses of nucleotide addition, aliquots (5 μL) 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^*_i), which is derived from two possible sources: unreacted tRNA substrate or generation of p*tRNA by pyrophosphate removal. The labeled oligonucleotides and P^*_i are resolved as described previously by silica TLC in a 1-propanol/NH_4OH/H_2O [55:35:10 (v:v:v)] solvent system.[10] Addition of ATP or GTP yields labeled oligonucleotide $A_{-2}p^*N_{-1}pGpC$ or $G_{-2}p^*N_{-1}pGpC$, respectively, after RNase A/CIP treatment, and addition of CTP or UTP yields oligonucleotide $C_{-2}p^*N_{-1}pG_{+1}$ or $U_{-2}p^*N_{-1}pG_{+1}$, respectively, after RNase T1/CIP treatment. The percent of total products due to addition (P_{add}) can be determined directly from the amount of labeled oligonucleotide(s) compared to the total amount of radioactivity at each time point.

To simultaneously measure the total rate of reaction by both pathways (see Scheme 2), a second digestion with nuclease P1 (as described above for the reactions without NTP) was performed on 10 μL aliquots taken at the same time points as for the RNase A/T1 digests described above. In these reactions, p*N is produced either directly by pyrophosphate removal or as a consequence of nucleotide addition (because nuclease P1 also cleaves the addition products shown in Scheme 2 to yield a labeled p^*N_{-1}). p^*N_{-1} and ppp^*N_{-1} (corresponding to unreacted substrate) were resolved by PEI-cellulose TLC and quantified as described above for the reactions without NTP. These reactions also provide an indirect measurement of the percent of product produced specifically by pyrophosphate removal (P_{PPr}), as indicated in eq 5

$$P_{total} - P_{add} = P_{PPr} \qquad (5)$$

where P_{total} corresponds to the observed p*G from the nuclease P1 digest and P_{add} corresponds to the observed addition products from the RNase/CIP digest.

The k_{obs} and P_{max} for pyrophosphate removal versus nucleotide addition reactions were determined either by a fit of the time courses of each product formation (P_{add} measured directly and P_{PPr} 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 N_{-1}–N_{73} 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_{PPr}) reaction products. The fits of these P_{PPr} data to eq 1 yield k_{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_{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 N_{-2} addition reaction ($P_{add,max}$) divided by the total reaction amplitude (P_{total}). Thus, using the k_{obs} for the addition reaction (k_{add}) that was directly measured by the RNase/CIP digest, the k_{obs} for pyrophosphate removal (k_{ppr}) could be calculated from eq 6.

$$P_{add,max}/P_{total} = k_{add}/(k_{add} + k_{ppr}) \qquad (6)$$

RESULTS

The Intrinsic Rate of Removal of Pyrophosphate from the G_{-1} Residue of Base-Paired tRNA^{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 5′-γ-^{32}P-triphosphorylated tRNA^{His} (p*pp-tRNA^{His}) transcripts as substrates for the pyrophosphate removal reaction, to mimic the G_{-1}-containing products after the nucleotidyl 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).[13] Because a 5′-triphosphorylated end on the tRNA is generated regardless of whether the G_{-1} nucleotide is added by the adenylylation-dependent or adenylylation-independent pathway of 3′–5′ addition (Figure S1 of the

Table 1. Kinetics of 5′-Pyrophosphate Removal Measured with γ-Labeled (p*ppG$_{-1}$) tRNA^{His}

	without NTP		1 mM GTP: enzyme titration		1 mM GTP: GTP titration	
tRNA^{His}	k_{ppr} (min^{-1})	$K_{D,tRNA}$ (μM)	k_{ppr} (min^{-1})	$K_{D,tRNA}$ (μM)	k_{ppr} (min^{-1})	$K_{D,GTP}$ (μM)
G_{-1}:A_{73}	0.030 ± 0.003	1.6 ± 0.7	0.77 ± 0.04	2.5 ± 0.4	0.19 ± 0.01	92 ± 15
G_{-1}:C_{73}	0.0006[a]	ND[b]	0.43 ± 0.04	16 ± 4	0.43 ± 0.05	106 ± 30

[a] k_{ppr} estimate determined using the method of initial rates. [b] Parameter not determined.

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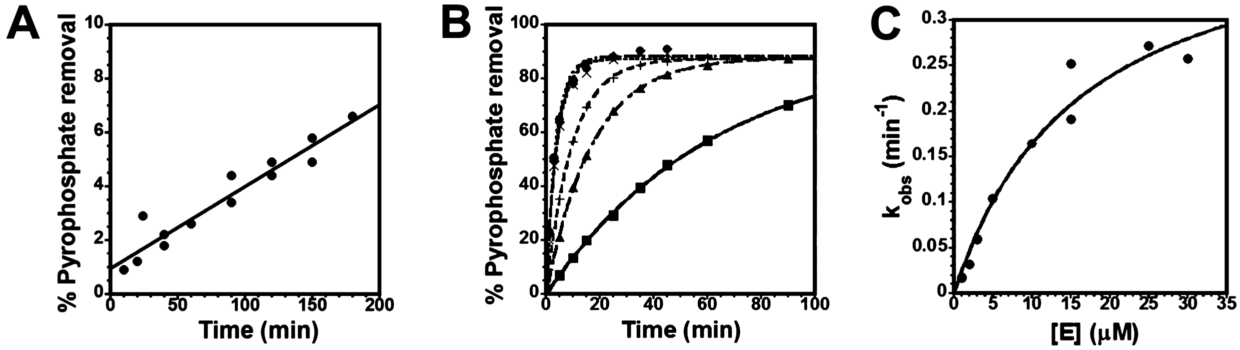

Figure 2. Pyrophosphate release measured with γ-^{32}P-labeled p*ppG$_{-1}$:C$_{73}$-tRNA^{His}. (A) Plot of percent pyrophosphate removal reaction products vs time with p*ppG$_{-1}$:C$_{73}$-tRNA^{His} and 15 μM SceThg1 in the absence of any added nucleotide cofactor. The slope of the line fit to the data was used to determine k_{obs} by the method of initial rates (eq 4). (B) Plot of percent pyrophosphate removal reaction products vs time with p*ppG$_{-1}$:C$_{73}$-tRNA^{His} in the presence of 1 mM GTP and 1 (■), 3 (▲), 5 (+), 15 (×), 25 (●), and 30 μM (◆) SceThg1. Lines represent fits of each time course to a single-exponential equation (eq 1) to yield k_{obs}. (C) Observed rates of pyrophosphate removal plotted as a function of SceThg1 concentration and fit to eq 3 to determine the maximal rate constant (k_{ppr}) and apparent dissociation constant ($K_{D,app}$) for pppG$_{-1}$:C$_{73}$-tRNA^{His}.

Supporting Information), assays that measure reactivity with ppp-tRNA species (generated by various methods as described below) are appropriate for studying the kinetics of the reaction with the intermediate generated subsequent to N$_{-1}$ addition.

The rate constant for pyrophosphate removal activity (k_{ppr}) of SceThg1 with wild-type (p*ppG$_{-1}$:A$_{73}$) tRNA^{His} was determined by incubating labeled tRNA with excess enzyme in the absence of any added nucleotide under single-turnover conditions. In this assay, SceThg1-catalyzed hydrolysis of the 5′-triphosphate causes the release of labeled pyrophosphate, which was quantified to determine the pseudo-first-order rate constant (k_{ppr}) of 0.030 ± 0.003 min^{-1} under standard SceThg1 reaction conditions (Table 1).[13] To determine whether the presence of the C$_{73}$ nucleotide in the tRNA^{His} variant that is a substrate for multiple-G addition alters the inherent ability of SceThg1 to remove the 5′-pyrophosphate, the same reactions were performed with 5′-γ-^{32}P-triphosphorylated, G$_{-1}$-containing C$_{73}$-tRNA^{His} (i.e., the termini of the substrate are as indicated by the red box in Figure 1B). With this substrate (p*ppG$_{-1}$:C$_{73}$), even in the presence of high concentrations of SceThg1 (15 μM, which is significantly greater than the measured $K_{D,app}$ for the pppG$_{-1}$:A$_{73}$-tRNA^{His} 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$_{-1}$:C$_{73}$ 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 5′-pyrophosphate from the C$_{73}$-containing tRNA, as compared to the robust rate of removal of 5′-pyrophosphate from the wild-type substrate.

Previously, however, we observed that addition of 1 mM GTP stimulated the k_{ppr} with wild-type tRNA^{His}, despite the fact that GTP does not appear to compulsorily participate in the chemistry of the 5′-pyrosphosphate removal reaction (see Figure 1A).[13] 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$_{-1}$:C$_{73}$ 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$_{-1}$:C$_{73}$ 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 k_{ppr} and $K_{D,app,tRNA}$ for the release of pyrophosphate from the p*pp-G$_{-1}$:C$_{73}$ substrate based on the hyperbolic dependence of the observed rates of nucleotide addition on the concentration of SceThg1 (Figure 2C and Table 1). Importantly, however, this assay with γ-^{32}P-labeled tRNA cannot distinguish whether the stimulated release of PP$_i$ observed in the presence of GTP is due to pyrophosphate hydrolysis from the 5′-triphosphorylated G$_{-1}$ nucleotide (i.e., pyrophosphate removal as in Figure 1A) or the addition of the next (G$_{-2}$) nucleotide to the substrate (as in Figure 1B), because labeled P*P$_i$ would be released as a consequence of either of these reactions. Repeating the single-turnover measurements to determine k_{obs} as a function of varied GTP concentration yielded similar kinetic parameters (for k_{ppr} and $K_{D,app,GTP}$) for both G$_{-1}$:A$_{73}$ and G$_{-1}$:C$_{73}$ substrates (Figure S2 of the Supporting Information and Table 1). Therefore, existing assays using γ-^{32}P-labeled tRNA are not sufficient to distinguish between the products of the pyrophosphate removal and nucleotide addition activities of SceThg1.

A New Assay for Distinguishing between Removal of 5′-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 tRNA^{His} variants that contain other combinations of N$_{-1}$:N$_{73}$ nucleotides, because the identity of the −1:73 base pair is known to affect the number of nucleotides added by SceThg1.[5] Because transcripts that are initiated with 5′-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′−5′ addition activity of Thg1 to generate labeled tRNAs to be used as substrates in the assays was developed.

The assay developed here utilizes 5′-[α-^{32}P]tRNA^{His} (ppp*-tRNA^{His}) substrates generated by incubating Thg1 with various combinations of [α-^{32}P]NTPs and tRNA^{His} 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 5′-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

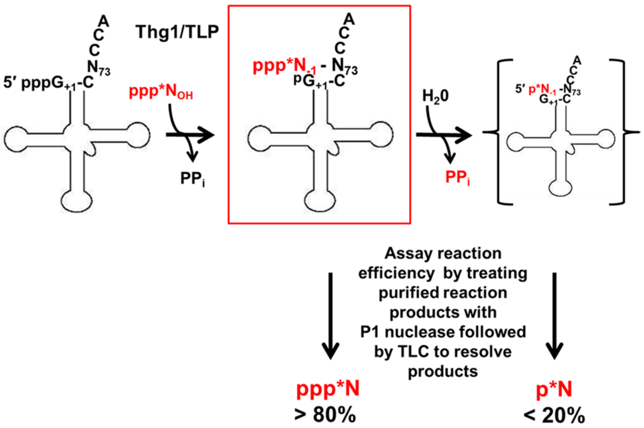

Figure 3. Labeling scheme for generating α-^{32}P-labeled tRNA^{His} substrates. 5′-ppp*N$_{-1}$-tRNA^{His} substrates were generated by incubating excess Thg1 (from various sources, as indicated in Materials and Methods) with the desired [α-^{32}P]NTP and tRNA^{His} transcripts that are initiated with G$_{+1}$. 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 5′-triphosphate yields ppp*N; if the 5′-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 5′-nucleotide of each substrate is incorporated by Thg1, and not by T7 RNA polymerase, substrates that are initiated uniquely with α-labeled 5′-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*N$_{-1}$:N$_{73}$ base pair was varied by including different tRNA^{His} transcripts and labeled NTPs in the Thg1 labeling reactions. All substrates used for assays described below were demonstrated to contain a minimum of 80% 5′-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 C$_{73}$-tRNA^{His} Variants. Rates of reaction were measured with ppp*G$_{-1}$:A$_{73}$- and ppp*G$_{-1}$:C$_{73}$-tRNA^{His} [for which rates had been determined previously using γ-^{32}P-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 5′-pyrophosphate, generating p*G$_{-1}$ after nuclease digestion, which is readily resolved from ppp*G$_{-1}$ 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*G$_{-1}$:A$_{73}$- and ppp*G$_{-1}$:C$_{73}$-tRNA substrates were in excellent agreement with the k_{ppr} obtained with γ-^{32}P-labeled substrates described above (compare Tables 1 and 2).

Next, assays were performed with the same substrates (ppp*G$_{-1}$:A$_{73}$ and ppp*G$_{-1}$:C$_{73}$) 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/phosphatase treatment and resolution of an oligonucleotide corresponding to the nucleotide addition product (i.e., G$_{-2}$p*G$_{-1}$pGpC) from labeled inorganic phosphate (P*$_i$), which is derived from either unreacted tRNA substrate or 5′-p*tRNA generated by pyrophosphate removal (Figure 4C,D). The total reaction progress (total product generated by both pyrophosphate removal and nucleotide addition) was determined by nuclease P1 digestion, as described above for the reactions without NTP (Figure 4E,F). Single-turnover rate constants and maximal product formation were determined from plots of the percent of each product observed as a function of time, as described in Materials and Methods (Figure 5 and Table 2).

As expected, but now explicitly shown by the direct enzyme assay, the 5′-p-tRNA product of pyrophosphate removal is the major product (84%) generated from the wild-type (G$_{-1}$:A$_{73}$) tRNA^{His} in the presence of GTP (Table 2 and Figure 5). Likewise, nucleotide addition products are the major products (83%) generated from the reactions under the same conditions with G$_{-1}$:C$_{73}$-tRNA^{His} (Table 2 and Figure 5). For G$_{-1}$:A$_{73}$-tRNA^{His}, the k_{obs} for pyrophosphate removal measured with the α-labeled assay was 0.31 ± 0.03 min^{-1}, which agrees well with the average k_{ppr} measured with the γ-labeled assay (0.5 ± 0.3 min^{-1}) (Tables 1 and 2). For G$_{-1}$:C$_{73}$-tRNA^{His}, the k_{obs} for nucleotide addition measured with the α-labeled assay was 0.25 ± 0.01 min^{-1}, which again compares well with the value of 0.43 ± 0.05 min^{-1} measured with the previous assay.

A Kinetic Mechanism Accounts for the Termination of Addition after a Single Nucleotide Is Added to Mismatch-Terminating tRNA^{His}. We used the α-labeled assay to measure activities with two tRNA^{His} variant substrates containing other non-WC N$_{-1}$:N$_{73}$ base pairs (G$_{-1}$:G$_{73}$ and G$_{-1}$:U$_{73}$), to determine whether the fact that the rate of pyrophosphate removal is faster than that of nucleotide addition is a unique property of the universally conserved eukaryotic G$_{-1}$:A$_{73}$ terminal base pair or whether the same outcome would be observed for other mismatched tRNA termini. The α-^{32}P-labeled (ppp*-tRNA) versions of each substrate were generated by SceThg1-catalyzed addition of [α-^{32}P]GTP to *in vitro* tRNA^{His} transcripts containing G$_{73}$ or U$_{73}$ (Figure S3 of the Supporting Information). Rates were first measured in the absence of added GTP (as in Figure 4) where the intrinsic rate of pyrophosphate removal for each substrate was readily measurable; time courses fit well to a single-exponential equation, yielding k_{obs} values for both substrates that were almost identical to that observed for the G$_{-1}$:A$_{73}$-tRNA (Table 2).

In the presence of GTP, where either pyrophosphate removal or nucleotide addition is a possible outcome of the reaction (Figure 1 and Scheme 2), the k_{obs} values for pyrophosphate removal were stimulated ~10-fold for both G$_{-1}$:G$_{73}$ and G$_{-1}$:U$_{73}$ substrates, similar to the observed stimulation of G$_{-1}$:A$_{73}$-tRNA (Table 2). For rates of nucleotide addition, however, there were some differences between the two non-WC variants. Reaction with the G$_{-1}$:G$_{73}$ mismatched substrate was similar to that with G$_{-1}$:A$_{73}$-tRNA^{His}, with only slightly larger amounts of G$_{-2}$ added (P_{max} of 16% for G$_{-1}$:G$_{73}$ vs 7% for G$_{-1}$:A$_{73}$). For the G$_{-1}$:U$_{73}$ substrate, the k_{obs} for G$_{-2}$ addition was measurably higher and similar to k_{obs} for pyrophosphate removal, leading to roughly equivalent amounts of reaction products that can be attributed to the addition of G$_{-2}$ versus pyrophosphate removal (Table 2). Thus, a kinetic partitioning mechanism in which the relative rates of

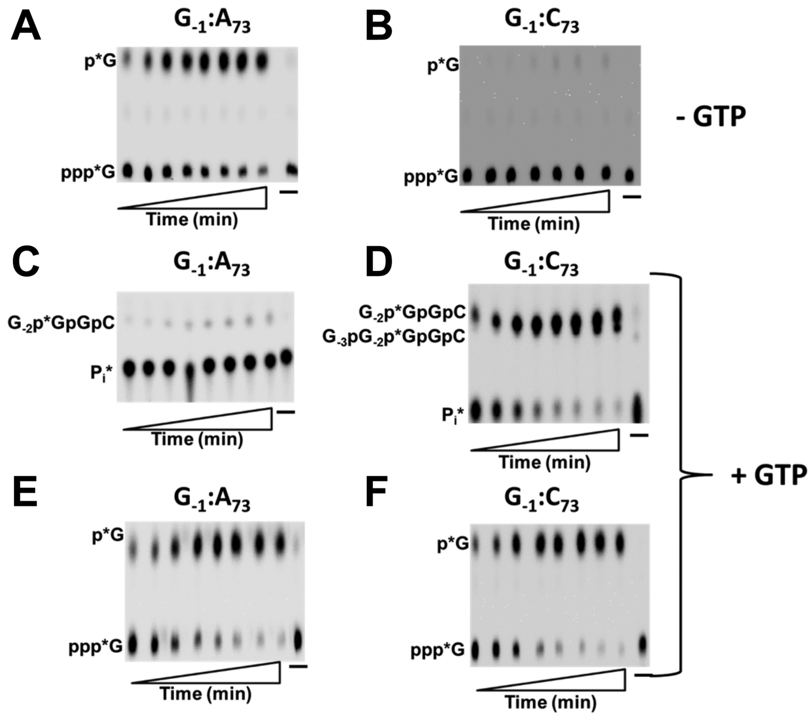

Figure 4. α-Labeled digestion assays for simultaneously measuring the kinetics of pyrophosphate removal and nucleotide addition reactions with 5′-ppp*-tRNA^{His}. (A and B) Representative single-turnover assays for determining k_{obs} for pyrophosphate removal in the absence of added GTP with (A) ppp*G_{-1}:A_{73}-tRNA^{His} or (B) ppp*G_{-1}:C_{73}-tRNA^{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_{max}. (C and D) Representative single-turnover assays for determining k_{obs} and P_{max} for addition of a nucleotide to (C) ppp*G_{-1}:A_{73}-tRNA^{His} or (D) ppp*G_{-1}:C_{73}-tRNA^{His}. Reactions shown are time courses of activity with 15 μM SceThg1 in excess over tRNA substrate and in the presence of 1 mM GTP; aliquots at indicated time points were digested with RNase A, followed by treatment with calf intestinal phosphatase, and products were resolved by silica TLC. Plots of addition product (P_{add}) vs time as quantified from these data were fit to eq 1 to yield k_{obs} and P_{max}. (E and F) Representative single-turnover assays for determining the GTP-stimulated k_{obs} for the removal of pyrophosphate from (E) ppp*G_{-1}:A_{73}-tRNA^{His} or (F) ppp*G_{-1}:C_{73}-tRNA^{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_{total}) from either G_{-2} addition 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_{add} measured in panels C and D) from P_{total} yields the apparent product specifically arising from pyrophosphate removal (P_{PPr}) at each time point (eq 5). For substrates with non-WC base-paired ends, P_{PPr} is fit directly to eq 1 to yield k_{obs} and P_{max}. For the WC base-paired substrates in the presence of a base-pairing NTP (such as the substrate assayed in panels B, D, and F), a kinetic partitioning mechanism (eq 6) is used to calculate k_{obs} for the removal of pyrophosphate (k_{ppr}) from the measured k_{add}, P_{add}, and P_{total}.

Table 2. Kinetics of 5′-Pyrophosphate Removal vs Nucleotide Addition Measured with α-Labeled (ppp*N_{-1}) tRNA^{His}

	without NTP		1 mM GTP			
	pyrophosphate removal		pyrophosphate removal		nucleotide (G_{-2}) addition	
tRNA^{His}	k_{obs} (min^{-1})	P_{max} (%)	k_{obs} (min^{-1})	P_{max} (%)	k_{obs} (min^{-1})	P_{max} (%)
G_{-1}:A_{73}	0.025 ± 0.001	75 ± 1	0.31 ± 0.03	84 ± 2	0.05 ± 0.01	7 ± 1
G_{-1}:G_{73}	0.021 ± 0.003	48 ± 2	0.20 ± 0.03	67 ± 2	0.05 ± 0.02	16 ± 2
G_{-1}:U_{73}	0.022 ± 0.003	59 ± 2	0.26 ± 0.02	38 ± 1	0.17 ± 0.03	43 ± 2
G_{-1}:C_{73}	0.0002^{a}	5^{b}	0.02 ± 0.01^{c}	N/A	0.25 ± 0.01	83 ± 1
C_{-1}:G_{73}	0.0006^{a}	12^{b}	0.02 ± 0.01^{c}	N/A	0.21 ± 0.02	74 ± 2
U_{-1}:A_{73}	0.017 ± 0.001	69 ± 2	1.2 ± 0.5^{c}	N/A	0.4 ± 0.1	19 ± 1

$^{a}k_{obs}$ estimate derived using the method of 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. The P_{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 α 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^{His}, multiple additions of U or A nucleotides by SceThg1 are not

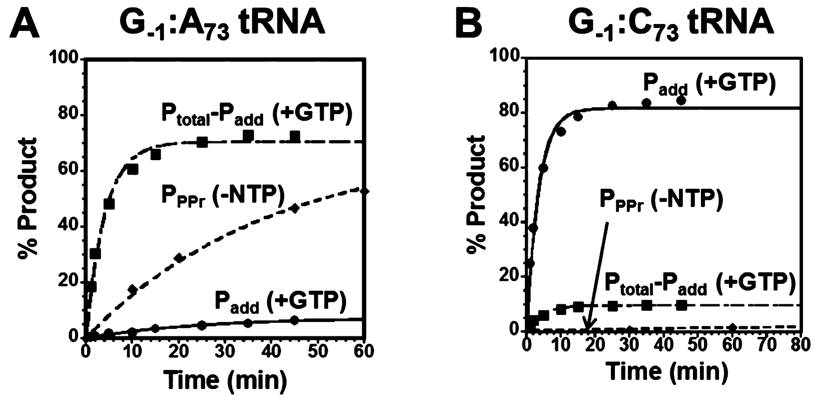

Figure 5. Determination of kinetic parameters for pyrophosphate removal vs nucleotide addition with ppp*G_{-1}:A_{73}- and ppp*G_{-1}:C_{73}-$tRNA^{His}$. Time courses of product formation by pyrophosphate removal for reactions without any added NTP [P_{PPr}, −NTP (◆)], for nucleotide (G_{-2}/G_{-3}) addition observed in the presence of 1 mM GTP [P_{add}, +GTP (●)], and for pyrophosphate removal products in the presence of 1 mM GTP calculated according to eq 5 [P_{total}-P_{add}, +GTP (■)] were all measured as described with (A) ppp*G_{-1}:A_{73}-$tRNA^{His}$ or (B) ppp*G_{-1}:C_{73}-$tRNA^{His}$. Reactions were the same reactions analyzed in Figure 4; fits to derive k_{obs} and P_{max} for each reaction are as indicated in Materials and Methods.

Table 3. Kinetics of Pyrophosphate Removal Measured with α-Labeled (ppp*N-1) $tRNA^{His}$ in the Presence of NTPs (1 mM each)

$tRNA^{His}$	k_{obs} (min^{-1})				
	without NTP[a]	GTP[a]	ATP	CTP	UTP
G_{-1}:A_{73}	0.025 ± 0.001	0.31 ± 0.03	0.12 ± 0.03	0.8 ± 0.3[d]	0.4 ± 0.1[d]
G_{-1}:G_{73}	0.021 ± 0.003	0.20 ± 0.03	0.16 ± 0.02	0.8 ± 0.2	0.3 ± 0.1
G_{-1}:U_{73}	0.022 ± 0.003	0.26 ± 0.02	0.13 ± 0.01	0.7 ± 0.1	0.4 ± 0.1
G_{-1}:C_{73}	0.0002[b]	0.02 ± 0.01[c]	0.003[b]	0.026 ± 0.002	0.014 ± 0.002
C_{-1}:G_{73}	0.0006[b]	0.02 ± 0.01[c]	0.05 ± 0.01	0.17 ± 0.05	0.05 ± 0.01
U_{-1}:A_{73}	0.017 ± 0.001	1.2 ± 0.5[c]	0.18 ± 0.01	0.4 ± 0.1	0.26 ± 0.04

[a]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. [c]k_{obs} for pyrophosphate removal calculated by partitioning from eq 6, as described in Materials and Methods. [d]k_{obs} derived from a fit to data obtained from a single experiment with this tRNA/NTP combination.

observed.[5] Variant $tRNA^{His}$ substrates ppp*C_{-1}:G_{73} and ppp*U_{-1}:A_{73} 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 G_{-1}:C_{73}-tRNA, the intrinsic (without GTP) rate of pyrophosphate removal with the ppp*C_{-1}:G_{73} 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, k_{obs} and end point P_{max} calculated for nucleotide addition and pyrophosphate removal reactions were similar to those observed for the G_{-1}:C_{73} substrate (Table 2) and suggest that a faster rate of nucleotide addition than of pyrophosphate removal leads to the prevalence of nucleotide addition products observed with these tRNAs.

We hypothesized that the U_{-1}:A_{73} variant might exhibit behavior different from that of substrates with G:C/C:G base-paired termini because SceThg1 did not polymerize multiple U:A or A:U base pairs in earlier primer extension assays.[5] Consistent with this observation, the maximal product observed (P_{max}) due to addition of a nucleotide to the U:A base-paired ppp*tRNA was only 19% of the total substrate in the reactions (Figure S4 of the Supporting Information and Table 2). Interestingly, the k_{obs} for addition was relatively fast, similar to that observed other WC base-paired substrates (Table 2), but this was accompanied by a correspondingly higher rate of pyrophosphate removal, as calculated according to the observed partitioning between addition and pyrophosphate removal reaction products (eq 6).

Rates of Pyrophosphate Removal Depend on the Identity of the NTP Included in Reactions. The substrates described above contain different N_{-1}:N_{73} base pairs, but all of the tested tRNAs contain a canonical 3′-C_{74}CA end; thus, assays performed so far in the presence of GTP reflect the addition of a WC-templated G_{-2} to form G_{-2}:C_{74} (and possibly additional base pairs as shown in Figure 1B). We sought to determine whether the identity of the potential N_{-2} nucleotide affects the relative rates of SceThg1-catalyzed reactions. Kinetic parameters for pyrophosphate removal versus addition of N_{-2} to the N_{-1}:N_{73}-tRNA substrates described above were measured in the presence of each of the other three NTPs (ATP, UTP, and CTP) so that all possible interactions of N_{-2} with the C_{74} nucleotide could be tested. Assays with ppp*-tRNA were performed as described earlier (Figure 4), except that to detect addition of U_{-2} or C_{-2}, reaction mixtures were digested with RNase T1 instead of RNase A (see Materials and Methods).

The NTP dependence of the observed reaction kinetics was nearly identical with all three substrates that terminate in non-WC (G_{-1}:A_{73}, G_{-1}:G_{73}, and G_{-1}:U_{73}) base pairs. For all three substrates, as with GTP, the majorities (60−80%) of products observed in the presence of ATP, UTP, or CTP were pyrophosphate removal products, and k_{obs} values were readily determined from these time courses (Table 3). Little or no

Table 4. Kinetics of Pyrophosphate Removal versus Nucleotide Addition Measured with α-Labeled (ppp*N_{-1}) $tRNA^{His}$ Substrates with Variations at N_{74}

$tRNA^{His}$	GTP		ATP		CTP		UTP	
	k_{obs} (min^{-1})	P_{max} (%)	k_{obs} (min^{-1})	P_{max} (%)	k_{obs} (min^{-1})	P_{max} (%)	k_{obs} (min^{-1})	P_{max} (%)
Pyrophosphate Removal								
G:C$\underline{C}_{74}$CA	0.02 ± 0.01[c]	N/A	0.003[a]	34[b]	0.026 ± 0.002	82 ± 3	0.014 ± 0.002	72 ± 4
G:C$\underline{A}_{74}$CA	0.08 ± 0.02[c]	N/A	0.001[a]	13[b]	0.012 ± 0.001	88 ± 2	0.05 ± 0.01[c]	N/A
C:G$\underline{C}_{74}$CA	0.02 ± 0.01[c]	N/A	0.05 ± 0.01	59 ± 6	0.17 ± 0.05	78 ± 4	0.05 ± 0.01	79 ± 5
C:G$\underline{G}_{74}$GA	0.06 ± 0.01[c]	N/A	0.006 ± 0.001	60 ± 10	0.08 ± 0.01[c]	N/A	0.014 ± 0.001	78 ± 1
Nucleotide Addition								
G:C$\underline{C}_{74}$CA	0.25 ± 0.01	83 ± 1	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]
G:C$\underline{A}_{74}$CA	0.03 ± 0.01	22 ± 3	ND[d]	ND[d]	ND[d]	ND[d]	0.016 ± 0.001	22 ± 1
C:G$\underline{C}_{74}$CA	0.21 ± 0.02	74 ± 2	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]
C:G$\underline{G}_{74}$GA	0.013 ± 0.001	12 ± 1	ND[d]	ND[d]	0.120 ± 0.012	54 ± 1	ND[d]	ND[d]

[a] k_{max} estimate derived from observed rates determined using the method of initial rates. [b] Maximal amount of product observed after reaction for 3–4 h. [c] k_{obs} for pyrophosphate removal calculated by partitioning from eq 6, as described in Materials and Methods; P_{max} for these products not applicable (N/A) because rate constants were not derived from a fit to eq 1. [d] Not determined because of the <3% product detected in assays.

detectable N_{-2} addition 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 slowest 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_{73} position (G_{-1}:C_{73}, C_{-1}:G_{73}, and U_{-1}:A_{73}), 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 5) 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_{-1}:C_{73}/C_{-1}:G_{73}-tRNAs versus U_{-1}:A_{73}-tRNA was clear. The k_{obs} values for the removal of pyrophosphate from the ppp*U_{-1}:A_{73} 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_{-1}:C_{73}- and ppp*C_{-1}:G_{73}-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_{74} (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_{74} or G_{74}/G_{75}), and thus template the incorporation of U_{-2} or C_{-2}, 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-^{32}P-labeled (ppp*-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_{74}-containing tRNA (G:C$\underline{A}$CA) revealed two salient features. First, introduction of A_{74} in place of C_{74} caused a significant increase in the extent and rate of addition of U_{-2}, with a corresponding decrease in the extent of addition of G_{-2} (Table 4). Interestingly, despite the apparent ability of SceThg1 to recognize and form a U_{-2}:A_{74} base pair, the U_{-2} 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_{-2} addition products (Table 4).

For the $tRNA^{His}$ variant containing G_{74}/G_{75}, the extent of product formation by the two competing pathways is also largely consistent with WC base pairing requirements. Addition of C_{-2} to G_{74}-$tRNA^{His}$ accounted for 54% of the total products (as compared with no detectable C_{-2} addition to the analogous C_{74}-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_{74}-tRNA compared with 74% when the GTP can make a base pair with C_{74} (Table 4)]. While SceThg1 catalyzed little to no detectable non-WC addition of ATP, CTP, or UTP, a low level (10–20%) of G_{-2} addition was observed with both N_{74} variant substrates. This limited, but detectable, ability of SceThg1 to add G_{-2} to create non-WC base pairs (in this case, G_{-2}:A_{74} or G_{-2}:G_{74}) may reflect features of the eukaryotic Thg1 active site that evolved to specifically allow addition of a non-WC G_{-1} 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_{-1}:A_{73}) $tRNA^{His}$. Time courses of pyrophosphate 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′-ddGTP (Figure 6 and Table S2 of the Supporting Information).

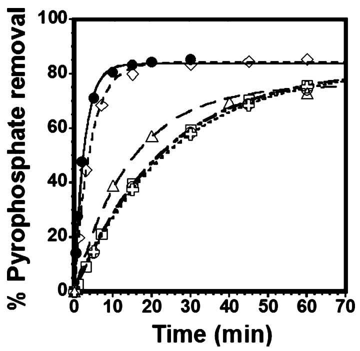

Figure 6. Rates of pyrophosphate removal with p*ppG$_{-1}$:A$_{73}$-tRNA^{His} in the presence of GTP nucleotide analogues. Single-turnover measurements of k_{obs} for pyrophosphate removal with limiting pppG$_{-1}$:A$_{73}$tRNA^{His} substrate determined in the presence of 15 μM SceThg1 and 1 mM GTP (●), 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}, a kinetic mechanism in which the rate of removal of pyrophosphate from the 5′ pppG$_{-1}$:A$_{73}$-containing tRNA^{His} is faster than the rate of addition of G$_{-2}$ to this same tRNA effectively terminates the reaction after the single G$_{-1}$ 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$_{-1}$:A$_{73}$-containing tRNA^{His} 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.[9] Thus, the 5′-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} 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.[6,16,17] The pressure to evolve a mechanism to limit the addition of nucleotides to tRNA^{His} 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.[18] Interestingly, the presence of additional (G$_{-2}$ and G$_{-3}$) nucleotides on tRNA^{His} does not interfere with histidylation of tRNA^{His} *in vivo* in yeast[19] and, in fact, enhances the reaction of the HisRS slightly, suggesting that avoiding defects in aminoacylation 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.[20] 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 identity 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.[13] 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$_{-1}$: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_{-2} over other nucleotides to the A_{74}-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.[9,10,21,22]

An added advantage to the α-labeled enzyme assays developed here is the ability to investigate the apparent kinetics of addition of the N_{-2} 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_{-1} addition reaction catalyzed by SceThg1),[13] the k_{obs} and P_{max} for adding a WC base-paired G_{-2} or C_{-2} nucleotide are significantly greater than the corresponding values for formation of non-WC base pairs (Table 4), yet the overall rate constants for G_{-2} addition are slower than the k_{obs} for G_{-1} addition (~0.25 min^{-1} for G_{-2} measured here vs 3.0 min^{-1} for G_{-1} 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 castellanii* and *Spizellomyces punctatus* do not appear to efficiently remove the 5′-pyrophosphate from nucleotide addition products generated by their 3′−5′ polymerase activity, as judged by the accumulation of 5′-triphosphorylated 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 5′-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′-triphosphate by TLPs implies that a different mechanism may be used by these enzymes to terminate 3′−5′ addition and that the pyrophosphate removal activity is a unique adaptation of eukaryotic Thg1 enzymes involved specifically in $tRNA^{His}$ maturation. It remains possible that the activated 5′-end of the fully repaired tRNA is removed by TLPs under reaction conditions that have not yet been identified, or by other unidentified enzyme(s) in the cell. Interestingly, removal of the 5′-pyrophosphate from the G_{-1} residue is not necessarily a ubiquitous reaction even in eukaryotes. In chicken mitochondria, the presence of a 5′-triphosphorylated G_{-1} on $tRNA^{His}$ was inferred by the ability of the *in vivo*-isolated tRNA to be capped by the capping guanylyltransferase, which requires a free 5′-triphosphate.[23] A more complete investigation of the 5′-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_{-1}:N_{73}-$tRNA^{His}$ 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_{73}-$tRNA^{His}$ versus G_{-1}:C_{73}-$tRNA^{His}$ (Figure S2), assessment of the 5′-phosphorylation status of ppp*-$tRNA^{His}$ substrates for α-labeled digestion assays (Figure S3), and assays for measuring the kinetics of reactions with ppp*U_{-1}:A_{73}-$tRNA^{His}$ (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

HisRS, histidyl-tRNA synthetase; Thg1, $tRNA^{His}$ guanylyltransferase; TLPs, Thg1-like proteins; SceThg1, *S. cerevisiae* Thg1 (formerly yThg1); p-$tRNA^{His}$, 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; ppr, pyrophosphate removal.

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