Life without post-transcriptional addition of G₋₁: two alternatives for tRNA^{His} identity in Eukarya

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ABSTRACT

The identity of tRNA^{His} is strongly associated with the presence of an additional 5'-guanosine residue (G₋₁) in all three domains of life. The critical nature of the G₋₁ residue is underscored by the fact that two entirely distinct mechanisms for its acquisition are observed, with cotranscriptional incorporation observed in Bacteria, while post-transcriptional addition of G₋₁ occurs in Eukarya. Here, through our investigation of eukaryotes that lack obvious homologs of the post-transcriptional G₋₁-addition enzyme Thg1, we identify alternative pathways to tRNA^{His} identity that controvert these well-established rules. We demonstrate that *Trypanosoma brucei*, like *Acanthamoeba castellanii*, lacks the G₋₁ identity element on tRNA^{His} and utilizes a noncanonical G₋₁-independent histidyl-tRNA synthetase (HisRS). Purified HisRS enzymes from *A. castellanii* and *T. brucei* exhibit a mechanism of tRNA^{His} recognition that is distinct from canonical G₋₁-dependent synthetases. Moreover, noncanonical HisRS enzymes genetically complement the loss of *THG1* in *Saccharomyces cerevisiae*, demonstrating the biological relevance of the G₋₁-independent aminoacylation activity. In contrast, in *Caenorhabditis elegans*, which is another Thg1-independent eukaryote, the G₋₁ residue is maintained, but here its acquisition is noncanonical. In this case, the G₋₁ is encoded and apparently retained after 5' end processing, which has so far only been observed in Bacteria and organelles. Collectively, these observations unearth a widespread and previously unappreciated diversity in eukaryotic tRNA^{His} identity mechanisms.

Keywords: G₋₁ residue; histidyl-tRNA synthetase; tRNA identity; tRNA^{His}; tRNA^{His} guanylyltransferase

INTRODUCTION

Recognition and aminoacylation of a specific tRNA by its cognate aminoacyl-tRNA synthetase (aaRS) is quantitatively important for error-free protein synthesis. tRNAs and aaRS utilize various identity elements that facilitate discrimination between substrate and nonsubstrate tRNA at a biochemical level, and are often necessary and sufficient for aminoacylation in vitro and in vivo (McClain 1993; Giege et al. 1998; Ardell 2010). Identity elements are commonly present in the primary sequence of a tRNA, but some are acquired post-transcriptionally in the form of extra nucleotides or modifications of existing nucleotides.

A critical identity element for histidyl-tRNA (tRNA^{His}) is a single additional guanosine residue at the -1 position (G₋₁) that is present in all three domains of life. Biochemical analyses of histidyl-tRNA synthetase (HisRS) from *Escherichia coli* and *Saccharomyces cerevisiae* revealed that G₋₁, and specifically the 5'-monophosphate on this additional residue,

contributes substantially toward discrimination of tRNA^{His} from nonsubstrate tRNAs, although the discriminator nucleotide at position 73 and nucleotides of the GUG anticodon can further aid recognition (Himeno et al. 1989; Nameki et al. 1995; Rudinger et al. 1997; Rosen and Musier-Forsyth 2004; Rosen et al. 2006).

Two distinct biochemical pathways have been identified that lead to acquisition of G₋₁. In Bacteria and many Archaea, the G₋₁ residue is encoded in the tRNA^{His} gene sequence (Jühling et al. 2009). The presence of genomically encoded G₋₁ opposite the bacterial C₇₃ leads to formation of an atypical 8-bp acceptor stem in pre-tRNA^{His}, causing alternative cleavage of the 5'-leader by Ribonuclease P (RNase P) and retention of the encoded G₋₁ in mature tRNA^{His} (Orellana et al. 1986). Alternatively, in Eukarya and other Archaea, the G₋₁ residue is not genomically encoded and instead is added after tRNA 5' end processing by a unique 3'-5' nucleotide addition reaction catalyzed by the tRNA^{His} guanylyltransferase (Thg1) (Cooley et al. 1982; Gu et al. 2003).

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The occurrence of the G_{-1} residue is highly conserved in Bacteria, Archaea, and Eukarya, with two notable exceptions: (1) the α -proteobacterium *Caulobacter crescentus*, where the isolated tRNA^{His} lacks a G_{-1} and in turn the corresponding HisRS enzymes uses alternative identity elements for tRNA^{His} aminoacylation (Ardell and Andersson 2006; Wang et al. 2007; Yuan et al. 2011), and (2) the protist *Acanthamoeba castellanii*, where it was recently shown that the HisRS (*AcaHisRS*) readily aminoacylates a G_{-1} -lacking tRNA in vitro (Rao et al. 2013). Despite the latter example representing the only such occurrence in a eukaryote described to date, neither the mechanistic basis for altered substrate specificity, nor the ability of the noncanonical *A. castellanii* synthetase to function independently of G_{-1} in vivo has been demonstrated.

Here we show that the alternative tRNA^{His} identity mechanism exhibited by *A. castellanii* is not a unique instance of noncanonical recognition among Eukarya. In *Trypanosoma brucei*, like *A. castellanii*, mature tRNA^{His} lacks the G_{-1} element, and the corresponding HisRS from *T. brucei* also catalyzes aminoacylation of tRNA^{His} independent of G_{-1} . Through biochemical analysis, we demonstrate that the noncanonical tRNA^{His} recognition mechanisms exhibited by *T. brucei* and *A. castellanii* HisRS result from a substantial dependence on the tRNA^{His} anticodon for substrate recognition and do not require the 5'-monophosphate that is critical for identity in canonical G_{-1} -dependent HisRS enzymes. Moreover, we identify a third completely distinct eukaryotic pathway for tRNA^{His} identity in *Caenorhabditis elegans*, wherein the tRNA^{His} retains G_{-1} , but in this case the identity element is acquired by a non-Thg1-dependent pathway that predictably results from noncanonical 5' end processing mechanisms. Unlike the α -proteobacterial case, which seems to have arisen from a common ancestral trait, the scattered distribution of organisms that possess noncanonical tRNA^{His}/HisRS suggests a polyphyletic origin for these activities, implying that these mechanisms may have arisen multiple independent times throughout evolution and that reliance on G_{-1} among eukaryotes is not as ubiquitous as previously accepted. Taking advantage of these newly described non- G_{-1} -dependent HisRS enzymes, we also describe the construction of an *S. cerevisiae* genetic system that exhibits robust growth in the absence of the G_{-1} -addition enzyme *THG1*, thus permitting future investigation into alternate functions of Thg1-catalyzed 3'-5' polymerase activities in biology.

RESULTS

Mature tRNA^{His} in *T. brucei* lacks the G_{-1} nucleotide

Despite the widespread distribution of Thg1 family members throughout eukaryotes, a number of species do not encode an identifiable *THG1* gene (Fig. 1; Jackman et al. 2012). Previous studies focused on *A. castellanii*, which lacks a bona fide Thg1 capable of adding G_{-1} to tRNA^{His}, but encodes two related

family members, which are Thg1-like proteins (TLPs) that apparently function in other processes beyond tRNA^{His} metabolism (Abad et al. 2011; Rao et al. 2011). However, a number of other eukaryotes do not encode any identifiable gene with sequence similarity to either Thg1 or TLP, and thus seem to lack any enzyme capable of catalyzing 3'-5' addition (highlighted in red in Fig. 1). We first chose to investigate the disease-causing parasite *Trypanosoma brucei* as a representative organism. To evaluate the G_{-1} status of *T. brucei* tRNA^{His}, we used a previously described approach to sequence the 5' end of tRNA^{His} by circularization of isolated tRNA from *T. brucei* and amplification of the junction between the 5'- and 3' ends of tRNA^{His} (Rao et al. 2013). Nine clones were obtained for otherwise fully mature tRNAs (containing the G_{+1} nucleotide ligated directly to A_{76} of the intact 3'-CCA end) (Fig. 2A, clones 1–9). The other seven clones contained additional nucleotides at the junction between G_{+1} and A_{76} that appear to correspond to aberrant processing intermediates (Fig. 2A, clones 10–16). The five clones with additional A, C, or CCA sequences (Clones 10–14) likely correspond to additional nucleotides added to the 3' end by the CCA-adding enzyme. However, the possibility that the single additional C nucleotide observed in clone 13 is derived from incomplete processing at the 5' end cannot be excluded, since the tRNA^{His} gene contains an encoded C_{-1} immediately adjacent to G_{+1} . Interestingly, two clones (15 and 16) contained an additional U residue between the G_{+1} nucleotide

FIGURE 1. Thg1 is not ubiquitous among eukaryotes. A phylogenetic tree to demonstrate Thg1 diversity in eukaryotes from the indicated supergroups was generated using PhyloT and visualized using the Interactive Tree of Life (iTOL) tool. Organism names indicated in color represent organisms that lack an identifiable Thg1 gene; species shown in blue lack any identifiable Thg1 protein, but encode Thg1-like proteins (TLPs) that catalyze RNA repair reactions; species shown in red lack any gene with identifiable similarity to Thg1 (either Thg1 or TLPs).

FIGURE 2. Recognition of tRNA^{His} in *T. brucei* does not require the G₋₁ identity element. (A) tRNA 5'- and 3'-end sequences of 16 independent clones obtained by circularization and RT-PCR using primers targeting the *T. brucei* tRNA^{His} shown on the diagram. Additional nucleotides observed between A₇₆ and G₋₁ in some clones (presumably derived from alternative processing events) are shown in gray italics. (B) In vitro aminoacylation assays with purified HisRS enzymes from *S. cerevisiae* (Sce), *A. castellanii* (Aca), and *T. brucei* (Tbr), enzymes were used in serial dilutions from ~125 to 5 ng/μL. Reactions contained *S. cerevisiae* tRNA^{His} substrates either lacking (Δ) or containing (G₋₁) the G₋₁ residue as indicated. Lanes indicated by – are no enzyme controls. Positions of radiolabeled 5'-³²P-A₇₆ derived from unreacted substrate (p*A) and 5'-³²P-A₇₆ with the ester-linked amino acid (p*A-His), derived from products, are as indicated.

and the mature 3'-CCA end, despite the fact that U is not derived from any annotated 5'-leader sequence or would not predictably result from aberrant activity of the CCA-adding enzyme. The lack of G₋₁ in any obtained clone (from either mature or alternatively processed tRNA) clearly indicates that *T. brucei*, like *A. castellanii*, lacks the highly conserved G₋₁ identity element on tRNA^{His}.

Noncanonical eukaryotic HisRS enzymes do not depend on G₋₁ for aminoacylation either in vitro or in vivo

We tested whether purified *T. brucei* (TbrHisRS) requires G₋₁ to recognize and aminoacylate its tRNA^{His} substrates in vitro by performing aminoacylation assays with tRNA^{His} transcripts containing (G₋₁) or lacking (Δ) the G₋₁ nucleotide (Wolfson et al. 1998). Purified TbrHisRS displayed a clear lack of dependence on the presence of G₋₁ for tRNA^{His} aminoacylation (Fig. 2B). The ability of TbrHisRS to aminoacylate tRNA^{His} independent of G₋₁ in vitro is consistent with the lack of G₋₁ observed on isolated tRNA^{His} from *T. brucei*, and therefore another noncanonical HisRS/tRNA^{His} pair, in addition to the previously described pair in *A. castellanii*, is identified in Eukarya.

Although the observed in vitro activities of TbrHisRS and AcaHisRS are consistent with the lack of G₋₁ on isolated tRNA^{His} from each organism, these altered tRNA^{His} recognition properties had not been demonstrated in a biological system with fully modified tRNA. In *S. cerevisiae* Thg1 is essential for normal growth, due to the strong dependence of SceHisRS in the presence of the G₋₁ residue for tRNA^{His} recognition. The requirement for Thg1 for viability can only be bypassed by overexpression of both SceHisRS and its tRNA^{His} substrate, although these strains still exhibit a growth defect compared with THG1-containing yeast strains (Preston and Phizicky 2010). This suggests an epistasis between Thg1 function and tRNA^{His} aminoacylation, and that the dependence on Thg1 could be bypassed by expression of a G₋₁-independent HisRS in *S. cerevisiae*. To test this, we transformed plasmids for expression of AcaHisRS or TbrHisRS into the previously described *S. cerevisiae* thg1Δ strain in which viability is maintained by the presence of wild-type THG1 on a URA3 plasmid (Gu et al. 2003; Abad et al. 2010). Growth on media containing 5-fluoroorotic acid (5-FOA) causes selective loss of the THG1-expressing URA3 plasmid and in the absence of another source of THG1 (such as in the vector control strain), cells selected on 5-FOA are not viable (Fig. 3A).

As hypothesized, *S. cerevisiae* thg1Δ strains expressing AcaHisRS grow similarly on 5-FOA-containing media to the SceThg1-expressing control strain, thus indicating that AcaHisRS genetically complements SceThg1 function in vivo (Fig. 3A). To ensure that the observed growth of AcaHisRS-complemented yeast is not due to the presence of residual G₋₁-containing tRNA^{His}, we isolated total RNA from the AcaHisRS-complemented strain and analyzed the 5' end of tRNA^{His} by primer extension, which revealed a lack of any detectable G₋₁ on the tRNA (Fig. 3C). Thus, AcaHisRS aminoacylates tRNA^{His} in the absence of G₋₁, both in vitro and in vivo in yeast. Interestingly, TbrHisRS failed to complement yeast growth in the presence of 5-FOA, suggesting that these cells still require Thg1 for viability (Fig. 3A). The observed phenotype was not due to lack of TbrHisRS expression; indeed AcaHisRS and TbrHisRS were expressed to similar levels in the complemented strains as judged by Western blots targeting a Flag epitope added to each protein (Fig. 3D).

To determine whether there was a biochemical explanation in terms of catalytic efficiency for the difference in ability of AcaHisRS and TbrHisRS to complement the thg1Δ phenotype, we compared steady-state kinetic parameters for aminoacylation of SceRNA^{His}. Steady-state assays were performed at saturating concentrations of histidine and ATP with ³²P-labeled A₇₆ to visualize the extent of aminoacylation. Since previous kinetic characterization of SceHisRS utilized a different (charcoal filter) assay with radiolabeled amino acid, the steady-state kinetic parameters for SceHisRS were also determined under the same buffer conditions reported previously for G₋₁-containing tRNA^{His} (Rosen et al. 2006). The K_{M,tRNA} for SceHisRS observed

FIGURE 3. Genetic complementation of *thg1Δ* in *S. cerevisiae*. (A) The *thg1Δ* yeast strain JY20 (see Materials and Methods for relevant genotype) was transformed with either empty *CEN LEU2* plasmid (Vector) or the same plasmid containing the indicated genes (*S. cerevisiae* THG1 or HisRS enzymes from *S. cerevisiae* [SceHisRS], *A. castellanii* [AcaHisRS], *T. brucei* [TbrHisRS], or *C. elegans* [CelHisRS]) under control of a galactose-inducible promoter. Independent transformants (A and B) from each strain were tested by replica plating on the indicated media; pictures represent growth after 4 d at 30°C. (B) The *thg1Δ* strain JY20 was transformed with the same *CEN LEU2* plasmids (empty vector or *TbrHisRS*) used in A and with a 2μ *HIS3* plasmid containing tRNA^{His} genes (+200-bp flanking sequence) from either yeast (*Sce*) or *T. brucei* (*Tbr*), as indicated. Independent transformants (A and B) were tested by replica plating on the indicated media; pictures represent growth after 5 d at 30°C. (C) Analysis of 5' end status of tRNA^{His} by primer extension of total RNA isolated from Thg1-complemented or AcaHisRS-complemented cells. Reactions used labeled primer (indicated by starred line on tRNA diagram) corresponding to the D-loop and stem of *S. cerevisiae* tRNA^{His}. Position of the unextended primer is shown in the lane with no added RNA (-); positions of the primer extension stop corresponding to tRNA with G₋₁ and without G₋₁ (5' end at G₊₁) are indicated with arrows. (D) Western blot to test for expression of the indicated enzymes in JY20 (*thg1Δ*) yeast transformed with *CEN LEU2* plasmids expressing either *S. cerevisiae* Thg1 (*Thg1*) or various HisRS enzymes from *A. castellanii* (*Aca*), *T. brucei* (*Tbr*), and *C. elegans* (*Cel*). Antibodies targeted N-terminal Flag epitopes incorporated into each construct and were used to probe blots from crude lysates prepared from each strain. Expected sizes for the indicated enzymes are as follows: Thg1 (29 kDa); SceHisRS (59 kDa); AcaHisRS (55 kDa); TbrHisRS (54 kDa); CelHisRS (60 kDa). The reason for the second (lower) apparent molecular weight band observed in strains expressing CelHisRS is not known. Positions of molecular weight standards are as shown on the left of the image, determined with commercial dye-labeled standards.

with this assay was $<0.3 \mu\text{M}$ (reflecting the lowest concentration of tRNA that could be tested in the assay, see Supplemental Fig. S1) compared with $0.14 \mu\text{M}$ measured earlier. The measured k_{cat} is ~ 60 -fold lower, as expected since 5'-tri-

phosphorylated tRNA^{His} transcripts were used here and *Sce*-HisRS displays a preference for 5'-monophosphorylated tRNA^{His} (Table 1; Rosen et al. 2006). However, since neither *Aca*HisRS nor *Tbr*HisRS exhibit a similar phosphate preference (see below), these assays utilized the 5'-triphosphorylated form of each tRNA that is readily produced by in vitro transcription. The measured catalytic efficiency (as reflected by k_{cat}/K_M) of aminoacylation of *Scet*tRNA^{His} by *Aca*-HisRS and *Tbr*HisRS was very similar to that observed with *Sce*HisRS and its optimal (G₋₁-containing) substrate, regardless of the presence of the G₋₁ residue, and *Aca*HisRS even displays an approximately fivefold preference for the ΔG_{-1} tRNA^{His} (Table 1; Supplemental Fig. S1).

Despite similar overall efficiencies of aminoacylation (as judged by k_{cat}/K_M), *Tbr*HisRS exhibited a substantially (~ 20 – 50 -fold) higher K_M than either *Sce*HisRS or *Aca*HisRS for *S. cerevisiae* tRNA^{His} (Table 1; Supplemental Fig. S1). We reasoned that the inability of *Tbr*HisRS to complement the yeast *thg1Δ* phenotype could be attributed to a preference for features of its native *T. brucei* tRNA^{His}. Indeed, we observed that k_{cat}/K_M of *Tbr*HisRS for *Tbr*tRNA^{His} was ~ 84 -fold higher as compared with that observed with *Scet*tRNA^{His}, and importantly, that the $K_{M,\text{tRNA}}$ was substantially decreased (Table 1; Supplemental Fig. S1). Thus, we hypothesized that overexpression of this preferred tRNA in yeast might confer the ability to complement the yeast *thg1Δ* phenotype. We transformed the same yeast strain used for the previous complementation tests with a 2μ (multiple copy) vector that encodes either *Scet*tRNA^{His} or *Tbr*tRNA^{His}, and again tested growth on media containing 5-FOA. Interestingly, *Tbr*HisRS supported growth of the yeast *thg1Δ* strain in transformants that overexpressed either *Scet*tRNA^{His} or *Tbr*tRNA^{His} (Fig. 3B). Thus, consistent with the elevated $K_{M,\text{tRNA}}$ values (Table 1), *Tbr*HisRS may require higher levels of tRNA^{His} for efficient aminoacylation, but again expression of the noncanonical HisRS can complement the loss of *THG1*.

C. elegans contains a genomically encoded G₋₁ on tRNA^{His}

Although numerous examples of eukaryotes that lack Thg1 are found among lower eukaryotic genomes (Jackman et al. 2012), among well-sequenced metazoa there is only a single organism identified so far that lacks an identifiable Thg1 enzyme in its genome, which is *Caenorhabditis elegans* (Fig. 1). Given the previous observations with *A. castellanii* and *T. brucei*, we tested whether the lack of a Thg1 enzyme in *C. elegans* could be accommodated by a similar mechanism that does not require the presence of the G₋₁ identity element on tRNA^{His}. However, *C. elegans* HisRS (*Cel*HisRS) did not support growth in the absence of *Sce*Thg1 (Fig. 3A), and moreover, purified *Cel*HisRS exhibits a detectable dependence on G₋₁, although unlike *Sce*HisRS, *Cel*HisRS exhibits a small amount of activity even with the ΔG_{-1} substrate (Fig. 4A).

TABLE 1. Steady-state kinetic parameters for aminoacylation by HisRS enzymes

HisRS	tRNA ^{His} a	k_{cat} (min ⁻¹)	$K_{M,tRNA}$ (μM)	$k_{cat}/K_{M,tRNA}$ (M ⁻¹ s ⁻¹)	Loss of efficiency (G ₋₁ /Δ) ^b
<i>Sce</i>	<i>Sce</i> Δ	ND ^c	ND ^c	ND ^c	470 ^c
<i>Sce</i>	<i>Sce</i> G ₋₁	0.21 ± 0.03	<0.3 ^d	~2 × 10 ⁴ d	–
<i>Aca</i>	<i>Sce</i> Δ	0.88 ± 0.10	<0.3 ^d	~1 × 10 ⁵ d	0.2
<i>Aca</i>	<i>Sce</i> G ₋₁	0.94 ± 0.15	0.7 ± 0.4	2.2 ± 0.9 × 10 ⁴	–
<i>Tbr</i>	<i>Sce</i> Δ	6.0 ± 0.9	19 ± 5	0.5 ± 0.1 × 10 ⁴	–
<i>Tbr</i>	<i>Tbr</i> Δ	25 ± 3	1.0 ± 0.5	4.2 ± 1.6 × 10 ⁵	–
<i>Tbr</i>	<i>Sce</i> _{U73} Δ	≥10 ^e	≥25 ^e	6.8 ± 0.4 × 10 ³ e	–

^atRNA^{His} transcripts representing sequences from *S. cerevisiae* (*Sce*) or *T. brucei* (*Tbr*) that either lack (Δ) or contain G₋₁ (G₋₁).

^bLoss of efficiency is expressed by $k_{cat}/K_{M,tRNA}(G_{-1})/k_{cat}/K_{M,tRNA}(\Delta)$.

^cKinetic parameters for activity of *Sce*HisRS with tRNA^{His} that lacks G₋₁ have not been determined (ND) under the same experimental conditions, but Loss of efficiency due to removal of G₋₁ is as reported in Rudinger et al. (1997).

^dUpper limit to $K_{M,tRNA}$ is based on the lowest concentration of tRNA testable in the assays; k_{cat}/K_M was estimated from the fit of the Michaelis–Menten equation to the data obtained at $[S] \geq K_M$ (Supplemental Fig. S2).

^eLower limit to $K_{M,tRNA}$ is based on the highest concentration of tRNA testable in the assays; k_{cat}/K_M was calculated from the slope of the linear $v_o/[E]$ versus $[S]$ plot and used to determine the corresponding lower limit to k_{cat} .

To directly address the nature of tRNA^{His} in *C. elegans*, 5' end sequencing of circularized *C. elegans* tRNA^{His} was performed, revealing that the G₋₁ nucleotide is indeed present, since 12/12 clones derived from mature tRNA contained a G₋₁ (Fig. 4B, clones 1–12). Among these 12 clones, multiple tRNA^{His} genes are represented, since sequences were obtained that contain either G₇–C₆₆ base pairs (encoded by 11 of the 17 *C. elegans* tRNA^{His} genes) or U₇–A₆₆ base pairs (encoded by the remaining six genes). Thus, the sample of sequences obtained here appears to be representative of the overall tRNA pool in *C. elegans* and not just derived from a small subset of G₋₁-containing tRNA^{His}. These data are consistent with widespread occurrence of G₋₁ on tRNA^{His} in *C. elegans*, which would be recognized by a canonical G₋₁-dependent HisRS.

Interestingly, among the 17 annotated tRNA^{His} genes in *C. elegans*, we noted that 16 contain a genomically encoded guanosine at –1 position in the leader sequence of tRNA^{His}, thus suggesting that a G₋₁ residue could be incorporated during transcription of the pre-tRNA. No prior examples of a similar genomically encoded G₋₁ have been documented in any nucleus-encoded eukaryotic tRNA^{His} gene, and the lack of the typical Thg1-dependent mechanism for acquisition of G₋₁ appears to have been compensated for by encoding the G₋₁ nucleotide in the precursor transcript, which is unprecedented in Eukarya.

Four sequences were obtained from immature tRNA^{His} species, all of which correspond to tRNA with matured 5' ends but unprocessed 3' ends (Fig. 4B). One sequence corresponds to the product of tRNA gene Y40H7A.t1 (Clone 13), which is clearly identifiable based on its unique 3'-trailer sequence (ACUAUUUU). As with the tRNAs described above, this gene also encodes a G₋₁ nucleotide that is detected

in the immature tRNA. The other three immature tRNA sequences (Clones 14–16) were all clearly attributable to another unique gene (B0513.t1), which is the only one of the 17 tRNA^{His} genes that does not encode G₋₁. The observation of clones derived from this gene confirms that the tRNA is transcribed, but the fact that no fully mature sequences (after 3' end processing) were obtained raises questions about whether this single tRNA species that apparently lacks G₋₁ is functional. From the limited number of clones obtained for this analysis, we cannot rule out the possibility that there is also a pool of mature tRNA^{His} derived from this gene (and presumably lacking G₋₁), but the abundance of other mature clones that contain the G₋₁ nucleotide suggests that these would be minor species in the cell.

FIGURE 4. *C. elegans* utilizes an encoded G₋₁ identity element for tRNA^{His} recognition. (A) In vitro aminoacylation assays with purified HisRS enzymes from *S. cerevisiae* (*Sce*) and *C. elegans* (*Cel*); enzymes were used in serial dilutions from ~125 to 5 ng/μL. Reactions contained *S. cerevisiae* tRNA^{His} substrates either lacking (Δ) or containing (G₋₁) the G₋₁ residue as indicated. Lanes indicated by – are no enzyme controls. (B) tRNA 5'- and 3'-end sequences of 16 independent clones obtained by circularization and RT-PCR using primers targeting the *C. elegans* tRNA^{His} shown on the diagram. The additional G₋₁ residue observed in 13 clones is underlined; nucleotides corresponding to identifiable 3' end trailer sequences in some clones are shown in gray italics. The N₇–N₆₆ base pair that distinguishes different tRNA^{His} genes is also highlighted for each sequence.

Distinct mechanism of tRNA^{His} recognition by AcaHisRS and TbrHisRS

Since AcaHisRS and TbrHisRS clearly lack a dependence on the canonical G₋₁ element, we investigated the biochemical basis for tRNA^{His} recognition. Since tRNA identity is often associated with the anticodon, we tested the role of the GUG anticodon using tRNA^{His} variants in which the GUG anticodon was replaced with the GAA sequence of tRNA^{Phe} (Jackman and Phizicky 2006a). Both AcaHisRS and TbrHisRS are strongly dependent on the tRNA^{His} anticodon, GUG (Fig. 5A,B). Interestingly, transplantation of the GUG anticodon into an otherwise wild-type tRNA^{Phe} supported some, albeit reduced, acquisition of aminoacylation activity with AcaHisRS, but a similar effect was not observed for TbrHisRS.

We noted that native TbrtRNA^{His} contains an unusual U₇₃ discriminator nucleotide, which is not found in other annotated tRNA^{His} species (Fig. 2A). However, alteration of the wild-type A₇₃ discriminator to U₇₃ in the context of ScetRNA^{His} resulted in only modest, if any, improvement in *k*_{cat}/*K*_M for TbrHisRS aminoacylation, and the *K*_M remained high for this ScetRNA^{His} variant (Table 1). Although an effect of the U₇₃ discriminator in conjunction with other elements of TbrtRNA^{His} cannot be ruled out, U₇₃ alone is not a sufficient identity element for recognition by TbrHisRS, and additional elements remain to be identified.

The 5'-monophosphate on G₋₁, more so than the guanine base itself, was shown to be critical for tRNA^{His} recognition

through previous kinetic analysis of canonical HisRS enzymes (Rosen and Musier-Forsyth 2004; Rosen et al. 2006). Therefore, an alternate interaction that similarly positions the 5'-monophosphate in the HisRS active site, even in the absence of G₋₁, could explain the altered G₋₁ dependence exhibited by noncanonical AcaHisRS and TbrHisRS. To test this, we compared aminoacylation activity of the various HisRS enzymes with the in vitro transcribed ScetRNA^{His} substrate, which naturally contains a 5'-triphosphate (ppp), to the activities observed with the same tRNA treated with either tobacco acid pyrophosphatase (TAP), which cleaves the α-β phosphate linkage to yield a 5'-monophosphate (p), or calf intestinal phosphatase (CIP), which removes all terminal phosphates and yields a 5'-OH end (-). Control activity assays with SceHisRS recapitulated the previous observation of optimal activity with 5'-monophosphorylated tRNA^{His}, followed by reduced activity with the ppp-tRNA and little or no detectable aminoacylation of the OH-tRNA under these conditions (Fig. 5C).

However, with both AcaHisRS (Fig. 5D) and TbrHisRS (Supplemental Fig. S2), aminoacylation assays revealed a strikingly different pattern of activity, with neither enzyme dependent on the number, or even the presence of any phosphates on the 5' end of tRNA^{His}. The observed dependence of the amount of aminoacylated product on the concentration of each enzyme included in the reactions is nearly identical, suggesting that the overall efficiency of aminoacylation is similar for the three tRNAs. These observations are in stark contrast to the role of 5'-terminal phosphorylation of

FIGURE 5. G₋₁-independent HisRS from *A. castellanii* and *T. brucei* depend on the presence of the tRNA^{His} anticodon but do not require a tRNA 5'-monophosphate. In vitro aminoacylation assays were performed with serial dilutions (~125–5 ng/μL) of AcaHisRS (A) or TbrHisRS (B) with the indicated *S. cerevisiae* tRNA variants. Lanes indicated with – are no enzyme controls; p*A and p*A-His represent substrate and product of the reactions, as described above. C demonstrates the classical 5'-monophosphate dependence observed with SceHisRS (serial dilutions from 125 to 5 ng/μL) and *S. cerevisiae* tRNA^{His} substrate containing the indicated 5'-phosphorylation status; ppp, 5'-triphosphate; p, 5'-monophosphate; –, 5'-hydroxyl. (D) In vitro aminoacylation of the same 5'-phosphate variants analyzed in C with AcaHisRS (serial dilutions from 125 to 0.125 ng/μL).

tRNA^{His} for *E. coli* and *SceHisRS* and underscore the distinct nature of the molecular mechanism used to interact with tRNA^{His} by the noncanonical *AcaHisRS* and *TbrHisRS*.

A. castellanii encodes both canonical and noncanonical HisRS enzymes

Eukaryotes can encode separate tRNA^{His} genes in nuclear and organellar genomes, raising the possibility of different mechanisms of 5'-processing to yield G₋₁ in the two compartments, as is observed in *S. cerevisiae* where G₋₁ is encoded in the mitochondrial tRNA^{His} and added by Thg1 to the nucleus-encoded tRNA (Burkard and Söll 1988; L'Abbé et al. 1990; Marechal-Drouard et al. 1996b). Although *T. brucei* does not encode any mitochondrial tRNAs (mt-tRNAs) and therefore utilizes nucleus-encoded tRNA for mitochondrial translation, *A. castellanii* also encodes a tRNA^{His} gene in its mitochondrial genome. Interestingly, despite the lack of G₋₁ in the nucleus-encoded tRNA^{His}, *A. castellanii* mt-tRNA^{His} encodes a G₋₁ opposite a C₇₃ discriminator nucleotide, and thus predictably retains the G₋₁ residue on the mt-tRNA during 5' end processing. Accordingly, the *A. castellanii*

nuclear genome encodes two distinct HisRS, only one of which has an identifiable mitochondrial targeting sequence and hence is predicted to localize to the mitochondria. Apart from the typical highly conserved catalytic residues, sequence comparison of the cytosolic and mitochondrial HisRSs from *A. castellanii* reveals relatively low similarity, indicating that they are most likely not products of gene duplication (Fig. 6).

To investigate the possibility that two distinct HisRS enzymes with different tRNA^{His} specificity are expressed in the same organism, we tested the biochemical dependence of the purified *A. castellanii* mitochondrial HisRS (*Acamt-HisRS*) on the G₋₁ identity element. For these assays, we used a transcript based on mitochondrial tRNA^{His} (either cloned from *A. castellanii* [*Acamt*-tRNA^{His}] or *S. cerevisiae* [*Scemt*-tRNA^{His}]), since *Acamt*-HisRS was not active with cytosolic *Scet*tRNA^{His} transcripts (Fig. 7A). Indeed, purified *Acamt*-HisRS exhibited a preference for G₋₁-containing tRNA^{His} with either mt-tRNA substrate (Fig. 7B,C). As reported previously, *SceHisRS* also readily aminoacylated the mitochondrial tRNA^{His} even in the absence of G₋₁ (Fig. 7C; Su et al. 2011).

FIGURE 6. Multiple sequence alignment of HisRS enzymes. The HisRS sequences from *E. coli* (*E.co*), *C. crescentus* (*C.cr*), *A. castellanii* predicted mitochondrial HisRS- (*A.ca(m)*), *T. brucei* (*T.br*), *A. castellanii* predicted cytosolic HisRS (*A.ca(c)*), *S. cerevisiae* (*S.ce*), *H. sapiens* (*H.sa*), *D. discoideum* (*D.di*), and *C. elegans* (*C.el*) were aligned using Clustal Omega (Sievers et al. 2011; Sievers and Higgins 2014). Coloring of organism labels indicates whether the HisRS is canonical G₋₁-dependent (blue) or noncanonical G₋₁-independent (red). The black line separates HisRS enzymes derived from bacteria/organelles (above the line) and eukaryotic cytosol (below the line). Conserved motifs I and II and HisA and HisB boxes are indicated by blue lines above the alignment; the motif II amino acids that interact with G₋₁ in bacterial HisRS are colored in red on the alignment. The numbers in parentheses after each organism label indicate the number of N-terminal amino acids that are not shown on this alignment. Sequence alignment image was prepared using GeneDoc.

FIGURE 7. Role of G_{-1} as $tRNA^{His}$ identity element for mitochondrial HisRS. All in vitro aminoacylation assays were performed with A_{76} ^{32}P -labeled $tRNA^{His}$ substrates which either lacked (Δ) or contained (G_{-1}) a G_{-1} . (A) Aminoacylation of yeast cytosolic $tRNA^{His}$ was tested with *A. castellanii* mitochondrial HisRS (Acamt-HisRS) using yeast HisRS (*Sce*) as control. Effect of G_{-1} on aminoacylation of mitochondrial $tRNA^{His}$ substrates by *S. cerevisiae* HisRS (*Sce*) and *A. castellanii* mitochondrial HisRS (Acamt-HisRS) was tested using mitochondrial $tRNA^{His}$ from (B) *A. castellanii* (Acamt-RNA^{His}) and (C) *S. cerevisiae* (Scemt-RNA^{His}). Assays contained 125–5 and 250–10 ng/ μ L of *Sce* and Acamt-HisRS, respectively.

DISCUSSION

The critical nature of the G_{-1} residue for recognition of $tRNA^{His}$ and the highly conserved nature of the domain-specific pathways that lead to incorporation of this identity element are widely acknowledged (Rudinger et al. 1997; Giege et al. 1998; Heinemann et al. 2012). However, a deeper investigation of the eukaryotic family tree revealed multiple diverse organisms in which these rules for specifying $tRNA^{His}$ identity are not universally followed (Fig. 1). On the basis of G_{-1} occurrence and biochemical properties of HisRS enzymes, we propose that $tRNA^{His}$ /HisRS pairs can now be broadly divided into at least three classes. These include (1) the well-characterized scenario where G_{-1} is present and there is a canonical HisRS (e.g., *E. coli* and *S. cerevisiae*), (2) the scenario where G_{-1} is absent and a non-canonical HisRS ensures $tRNA^{His}$ identity (e.g., *C. crescentus*, *A. castellanii*, and *T. brucei*), and finally, (3) the case where G_{-1} is present, but it is obtained by an atypical mechanism (e.g., *C. elegans*). The practically ubiquitous presence of an encoded G_{-1} residue throughout $tRNA^{His}$ gene sequences in Bacteria suggests that the first scenario remains the overwhelming rule in this domain. Although only a limited number of reports detail the actual G_{-1} status of $tRNA^{His}$ from bacterial species, the ~20 α -proteobacteria from the RCS clade that lack this encoded residue remain the only exceptions to this rule identified to date (Wang et al. 2007).

However, in Archaea and Eukarya, the data described here suggest the possibility of significantly more variability. A

number of archaeal species from both euryarchaeal and crenarchaeal clades contain an encoded G_{-1} and C_{73} that would predictably lead to cotranscriptional G_{-1} incorporation as in Bacteria, but a roughly equivalent number of available archaeal genomes lack this encoded G_{-1} . Thus, if present, G_{-1} must be acquired by an alternative pathway in these organisms. Interestingly, members of the Thg1 enzyme family (Thg1-like proteins, or TLPs) are found in Archaea that lack an encoded G_{-1} , and could thus function analogously to Thg1 in $tRNA^{His}$ metabolism (Abad et al. 2010). However, archaeal TLPs are also found in species that contain the encoded G_{-1} , raising questions about their function in these species, and moreover, a substantial biochemical preference for catalyzing $tRNA$ 5' end repair has now been associated with TLPs, including several from Archaea (Rao et al. 2011).

In Eukarya, these newly identified mechanisms for specifying $tRNA^{His}$ identity indicate a more diverse landscape than could have been previously appreciated from the study of a relatively limited number of model organisms. Most notably, in contrast to the single isolated lineage of G_{-1} -independent HisRS enzymes observed in the RCS clade of α -proteobacteria, noncanonical HisRS enzymes are found sporadically throughout the eukaryotic family tree, and in distantly related eukaryotes, suggesting that differences in $tRNA^{His}$ recognition may have arisen multiple times throughout evolution (Fig. 1). While a fully quantitative picture of the relative proportions of each type of $tRNA^{His}$ /HisRS pair in Eukarya awaits further investigation, examples of species that lack an identifiable Thg1 homolog are readily identified among all taxa. Therefore, it is reasonable to assume that additional eukaryotic species exhibit alternative mechanisms for $tRNA^{His}$ recognition that may be similar to, or even different from, the mechanisms identified here.

These data also constitute the first biochemical investigation of the basis for $tRNA^{His}$ recognition by a G_{-1} -independent eukaryotic HisRS. Although in the absence of G_{-1} , the $tRNA^{His}$ anticodon clearly contributes to $tRNA^{His}$ recognition, additional identity elements remain to be identified, since little to no aminoacylation was observed by either *Tbr* or *Aca*HisRS with the $tRNA^{Phe}$ chimera containing the $tRNA^{His}$ anticodon, GUG (Fig. 5). Interestingly, while *T. brucei* $tRNA^{His}$ contains an unusual U_{73} nucleotide at the discriminator position, this relatively conspicuous feature does not uniquely contribute to recognition by noncanonical *Tbr*HisRS (Table 1). Moreover, the currently accepted model for $tRNA^{His}$ binding to HisRS enzymes is based almost entirely on the need to correctly position the $tRNA$ 5'-monophosphate in the active site; indeed the identity of the actual nucleotide base found at the -1 position of $tRNA^{His}$ contributes quantitatively less to $tRNA$ recognition than does the phosphorylation status at the $tRNA$ 5' end (Hawko and Francklyn 2001; Rosen and Musier-Forsyth 2004; Rosen et al. 2006). Moreover, the fact that this critical role for the 5'-monophosphate is shared by *E. coli* and *S. cerevisiae* HisRS led to the idea that this is an evolutionarily conserved pathway,

despite local differences in G₋₁:N₇₃ recognition between enzymes from different domains of life. Again, the lack of dependence on the 5'-phosphorylation status observed here reveals that a completely different manner of tRNA recognition occurs with G₋₁-independent HisRS enzymes, and that the basis for tRNA selectivity remains to be fully determined.

Although the noncanonical HisRS enzymes investigated in this work share some biochemical similarities with the α -proteobacterial HisRS from *C. crescentus* (*CcrHisRS*), there are some important differences that distinguish these two types of HisRS (Ardell and Andersson 2006; Yuan et al. 2011). As with *AcaHisRS* and *TbrHisRS*, the nucleotides of the anticodon contribute greatly to recognition by *CcrHisRS*, and single alterations to these residues decrease k_{cat}/K_M for *CcrHisRS* by 100–1000-fold (Yuan et al. 2011). However, *CcrHisRS* also strongly prefers the presence of a G₊₁–U₇₂ wobble base pair, and conversion to a Watson–Crick +1–72 pair (as is found in eukaryotic tRNA^{His}, including in *A. castellanii* and *T. brucei*) causes a substantial decrease in k_{cat}/K_M for *CcrHisRS*. Furthermore, the presence of a G₋₁ residue is actually somewhat detrimental for the efficiency of aminoacylation by *CcrHisRS*, whereas aminoacylation by *AcaHisRS* and *TbrHisRS* occurs with similar catalytic efficiency in the absence or presence of G₋₁. Moreover, *AcaHisRS* and *TbrHisRS* enzymes differ from *CcrHisRS* in the motif (motif II) that is associated with recognition of G₋₁, and specifically do not have an analogous residue to the G118 residue of *CcrHisRS* demonstrated to play an important role in recognition of tRNA^{His} without G₋₁ (Fig. 6; Ardell and Andersson 2006; Ardell 2010). Thus, although a reduced dependence on G₋₁ superficially appears to be a common theme for substrate recognition between *AcaHisRS*, *TbrHisRS*, and *CcrHisRS*, this detailed comparison of their respective protein sequences and biochemical preferences for tRNA^{His} substrates suggests that these are distinct and likely independently evolved mechanisms. Notably, *A. castellanii* and *T. brucei* are both human pathogens for which treatment has proven to be challenging. A better understanding of the mechanistic features that distinguish these enzymes may lead to the development of novel compounds that could selectively target HisRS from these pathogens.

The identification of the non-Thg1-dependent pathway for acquisition of G₋₁ raises interesting questions about the nature of tRNA^{His} processing in *C. elegans*, and presumably other *Caenorhabditis* species, which similarly lack Thg1. To date, the only mechanism that allows retention of a genomically encoded G₋₁ residue is the bacterial pathway that depends on the presence of a C₇₃ discriminator nucleotide capable of base-pairing with the encoded G₋₁, thus creating an alternative cleavage site for the 5'-maturation enzyme RNase P (Burkard et al. 1988). However, *C. elegans* tRNA^{His} retains the universal eukaryotic A₇₃ discriminator nucleotide and thus G₋₁ cannot participate in a similar Watson–Crick interaction (Fig. 4B). If *C. elegans* G₋₁ is retained after 5' end processing, this would require an alternative pathway

in which either RNase P or another 5'-nuclease exhibits an unusual cleavage specificity that accommodates a non-Watson–Crick G₋₁:A₇₃ base pair at the cleavage site. Although *C. elegans* is the only eukaryotic system in which a Thg1-independent mechanism for G₋₁ acquisition has been observed to date, other eukaryotes (such as *Bigeloviella natans*) similarly lack a Thg1 enzyme and yet encode a G residue in the –1 position of their nuclear tRNA^{His} genes (Chan and Lowe 2009). Therefore, as with the noncanonical HisRS/tRNA^{His} pairs, this pathway is likely to account for tRNA^{His} identity in a number of eukaryotes.

Based on the widespread observation of an encoded G₋₁ (and bacteria-like C₇₃) in organellar tRNA^{His} genes, the G₋₁ residue was predicted to serve as an identity element not only in the cytosol, but also for organellar-encoded tRNA^{His}. In *A. castellanii*, we have identified the first example of both modes of tRNA^{His} recognition operating in the same organism, with two distinct HisRS enzymes that exhibit different G₋₁-dependence encoded in the nuclear genome. These observations indicate a division of labor in *A. castellanii*, wherein two independent and coexisting mechanisms for tRNA^{His} identity have evolved that are strictly compartmentalized. Inconsistencies in the occurrence of even the encoded G₋₁ on tRNA^{His} have been previously reported in plant mitochondria and chloroplast (Marechal-Drouard et al. 1996a,b; Placido et al. 2005, 2010).

The yeast genetic system in which the requirement for the tRNA^{His}-related function of Thg1 has been bypassed by expression of a G₋₁-independent *AcaHisRS* provides an exciting opportunity to study other potential functions for Thg1 enzymes in eukaryotes. The original observation of the Watson–Crick templated 3'–5' polymerase activity catalyzed by *SceThg1* was at odds with the fact that Thg1 in yeast (and other eukaryotes) was apparently only required to add a single G₋₁ nucleotide opposite A₇₃ in tRNA^{His} (Jackman and Phizicky 2006b). The reason for eukaryotic Thg1 enzymes to have even retained the ability to recognize a Watson–Crick base pair was not understood. Previous studies that bypassed the essential requirement for Thg1 by overexpression of HisRS and tRNA^{His} were complicated by the fact that there was still a strong growth defect evident in this strain, and whether this was due to incomplete aminoacylation or other defects associated with loss of Thg1 could not be completely decoupled (Preston and Phizicky 2010). However, the *AcaHisRS*-complemented *thg1Δ* strain grows similarly to the *THG1*-expressing strain under these conditions, and therefore will allow investigation of alternative function(s) of Thg1 enzymes, including those that may involve 3'–5' polymerase activity. Indeed, several unexplained cell cycle defects have been associated with depletion of Thg1 in *S. cerevisiae* and humans, and a biochemical interaction between *SceThg1* and the Orc2 component of the origin-recognition complex is not understood (Guo et al. 2004; Rice et al. 2005). It is intriguing to speculate that these effects may not be completely explained by a simple role for Thg1 solely in tRNA^{His} maturation.

MATERIALS AND METHODS

tRNA^{His} 5' end sequencing

5'-End sequencing of tRNA^{His} was performed by circularization of total RNA isolated from *T. brucei* and *C. elegans*, using T4 RNA ligase, followed by reverse transcription and PCR-amplification of the ligated junction, as described previously (Rao et al. 2013).

Cloning and purification of HisRS and tRNA^{His}

HisRS from *T. brucei* (*TbrHisRS*) was cloned by RT-PCR from total *T. brucei* RNA (generously provided by Juan Alfonzo, Ohio State University). *S. cerevisiae* HisRS (*HTS1*; *SceHisRS*) was PCR amplified from a clone generously provided by Eric Phizicky, University of Rochester. Genes for *A. castellanii* mitochondrial HisRS (*Acamt-HisRS*) and *C. elegans* HisRS (*CelHisRS*) were PCR amplified from synthetic constructs (Genewiz, PA). PCR products were all cloned using ligation-independent cloning in to the previously described AVA421 for expression as N-terminal His₆-tagged proteins (Quartley et al. 2009). All HisRS constructs were overexpressed at 18°C in *E. coli* BL21 (DE3) pLysS and purified by metal-ion affinity chromatography using the protocol described in Abad et al. (2011). Purified proteins were dialyzed against buffer containing 50% glycerol, 20 mM Tris (pH 7.5), 4 mM MgCl₂, 0.05 M NaCl, 1 μM ethylenediamine tetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT).

tRNA^{His} genes (*T. brucei* tRNA^{His} and *A. castellanii*/*S. cerevisiae* mitochondrial tRNA^{His}) were cloned by either RT-PCR from total RNA (*T. brucei* and *A. castellanii*) or PCR from genomic DNA (*S. cerevisiae*) for in vitro transcription by T7 RNA polymerase. Constructs with and without G₋₁ were generated by the use of alternate 5'-end primers during amplification. In vitro transcription reactions were performed with linearized DNA plasmids digested for run-off transcription and transcripts were purified on denaturing acrylamide gels, followed by phenol extraction and ethanol precipitation.

Yeast strains, media, and complementation

HisRS genes (as described above) were PCR-amplified and cloned into a vector for expression under control of a galactose-inducible promoter with an N-terminal Flag epitope [*CEN LEU2 P_{GAL}-Flag-HisRS*] and transformed into yeast strain JY20 (relevant genotype: *thg1Δ::kanMX his3-1 leu2Δ ura3Δ [CEN URA3 P_{THG1}-THG1]*). The plasmid for expression of *T. brucei* tRNA^{His} in *S. cerevisiae* was generated by PCR amplification of the *T. brucei* tRNA^{His} gene (with 100-bp upstream and downstream sequences) from *T. brucei* genomic DNA and restriction cloning into the yeast 2μ YEplAC vector [2μ *HIS3 TbrtRNA^{His}*]. YEplAC and yeast tRNA^{His}-expressing [2μ *HIS3 ScttRNA^{His}*] vectors were generously provided by Eric Phizicky. Growth was tested by replica plating and in liquid at 30°C in media containing either 2% dextrose or galactose and 0.1% 5-fluoroorotic acid (FOA), if indicated.

tRNA labeling and aminoacylation assays

tRNA^{His} substrates were radiolabeled with ³²P at the 5'-phosphate of A₇₆ by the activity of tRNA nucleotidyltransferase as described (Wolfson et al. 1998). Aminoacylation assays were performed and

reaction products were resolved as described previously (Rao et al. 2013). Steady-state parameters were determined using a minimum of fivefold excess of [tRNA^{His}]/[HisRS], in the presence of saturating concentrations of histidine (40 μM) and ATP (2.5 mM). Linear initial rates were determined at varied concentrations of tRNA^{His} (0.3–30 μM), plotted as a function of [tRNA^{His}] and fit to the Michaelis–Menten equation using Kaleidagraph (Synergy software). To generate 5'-monophosphorylated and 5'-hydroxyl tRNA^{His} substrates, A₇₆³²P-labeled tRNA^{His} was incubated with either tobacco acid pyrophosphatase (10 units; Epicentre) or calf intestinal alkaline phosphatase (20 units; NEB), respectively, according to the manufacturer's instructions and subsequently purified by phenol extraction and ethanol precipitation.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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