5'-Terminal nucleotide variations in human cytoplasmic tRNA$^{\text{HisGUG}}$ and its 5'-halves

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ABSTRACT
Transfer RNAs (tRNAs) are fundamental adapter components of translational machinery. tRNAs can further serve as a source of tRNA-derived noncoding RNAs that play important roles in various biological processes beyond translation. Among all species of tRNAs, tRNA$^{\text{HisGUG}}$ has been known to uniquely contain an additional guanosine residue at the −1 position (G$_{-1}$) of its 5'-end. To analyze this −1 nucleotide in detail, we developed a TaqMan qRT-PCR method that can distinctively quantify human mature cytoplasmic tRNA$^{\text{HisGUG}}$ containing G$_{-1}$, U$_{-1}$, A$_{-1}$, or C$_{-1}$ or lacking the −1 nucleotide (starting from G$_{1}$). Application of this method to the mature tRNA fraction of BT-474 breast cancer cells revealed the presence of tRNA$^{\text{HisGUG}}$ containing U$_{-1}$ as well as the one containing G$_{-1}$. Moreover, tRNA lacking the −1 nucleotide was also detected, thus indicating the heterogeneous expression of 5'-tRNA$^{\text{HisGUG}}$ variants. A sequence library of sex hormone-induced 5'-tRNA halves (5'-SHOT-RNAs), identified via cP-RNA-seq of a BT-474 small RNA fraction, also demonstrated the expression of 5'-tRNA$^{\text{HisGUG}}$ halves containing G$_{-1}$, U$_{-1}$, or G$_{1}$ as 5'-terminal nucleotides. Although the detected 5'-nucleotide species were identical, the relative abundances differed widely between mature tRNA and 5'-half from the same BT-474 cells. The majority of mature tRNAs contained the −1 nucleotide, whereas the majority of 5'-halves lacked this nucleotide, which was biochemically confirmed using a primer extension assay. These results reveal the novel identities of tRNA$^{\text{HisGUG}}$ molecules and provide insights into tRNA$^{\text{HisGUG}}$ maturation and the regulation of tRNA half production.

Keywords: tRNA; tRNA$^{\text{HisGUG}}$; tRNA half; SHOT-RNA; −1 nucleotide

INTRODUCTION
Transfer RNAs (tRNAs) are noncoding RNAs (ncRNAs) with lengths of 60- to 90-nucleotide (nt) that play central roles as adapter molecules in the translational machinery. Although tRNA molecules are stable and abundant, the expression profiles of individual tRNAs vary dynamically among different cells and tissues (Dittmar et al. 2006; Pavon-Eternod et al. 2009; Zhou et al. 2009; Mahlab et al. 2012) and this variation has been implicated in the translational regulation of mRNA expression (Gingold et al. 2014), animal development (Marshall et al. 2012; Rideout et al. 2012; Schmitt et al. 2014), and disease (Daly et al. 2005; Pavon-Eternod et al. 2010; Zhou et al. 2012; Clarke et al. 2016). Accumulating evidence regarding tRNA-derived ncRNAs has further increased the complexity of tRNA biology. In many organisms, tRNAs are not always end products but are processed further into smaller ncRNAs, many of which are known to be functional molecules with roles in various biological processes beyond translation (Garcia-Silva et al. 2012; Gebetsberger and Polacek 2013; Anderson and Ivanov 2014; Saikia and Hatzoglou 2015; Shigematsu and Kirino 2015; Telonis et al. 2015; Diebel et al. 2016). These tRNA-derived ncRNAs are in general classified into two groups: tRNA halves that range either from the 5'-end to the anticodon loop (5'-half) or from the anticodon loop to the 3'-end (3'-half) of a mature tRNA, and shorter tRNA-derived fragments (tRFs) that originate from various regions of mature tRNAs or their precursor transcripts (pre-tRNAs).

To date, two distinct classes of tRNA halves have been identified: tRNA-derived stress-induced RNAs (tiRNAs) (Thompson et al. 2008; Fu et al. 2009; Hsieh et al. 2009; Yamasaki et al. 2009; Saikia et al. 2012) and sex hormone-dependent tRNA-derived RNAs (SHOT-RNAs) (Honda et al. 2015). Although both tiRNAs and SHOT-RNAs are produced from mature tRNAs via angiogenin (ANG)-mediated cleavage of the anticodon loop (Fu et al. 2009; Yamasaki et al. 2009; Honda et al. 2015), the molecular factors that trigger their production are different. The expression of tiRNAs...
is triggered by a variety of stress stimuli, including oxidative stress, heat/cold shock, and UV irradiation (Shigematsu et al. 2014; Saikia and Hatzoglou 2015). The accumulation of tRNAs has been implicated in stress granule formation (Emara et al. 2010; Lyons et al. 2016), translational regulation (Yamasaki et al. 2009; Ivanov et al. 2011), and the pathogenesis of neurodevelopmental disorders (Blanco et al. 2014). In contrast, the expression of SHOT-RNAs is promoted by signaling pathways associated with sex hormones (e.g., estrogen and androgen) and their receptors (e.g., estrogen receptor [ER] and androgen receptor [AR]). SHOT-RNAs are specifically expressed in ER- or AR-positive breast and prostate cancers and have functional significance in cell proliferation (Honda et al. 2015).

Because ANG leaves a 2′,3′-cyclic phosphate (cP) on its 5′-cleavage products (Shapiro et al. 1986), ANG-generated 5′-tRNA halves contain a cP at the 3′-end (Honda et al. 2015). These cP-containing RNAs cannot be captured accurately by standard RNA-seq methods because they are not ligated to a 3′-adapter during library preparation procedure. We circumvented the issue by developing a cP-RNA-seq method that can exclusively sequence cP-containing RNAs (Honda et al. 2015, 2016) and used this method to determine the expression repertoire of 5′-SHOT-RNAs (5′-tRNA halves) in human BT-474 ER-positive breast cancer cells; accordingly, we identified eight cytoplasmic (cyto) tRNA species as the major sources of SHOT-RNAs (Honda et al. 2015). Although 5′-SHOT-RNAs from cyto tRNA_LysGUG and tRNA_HisGUG were particularly enriched, the molecular mechanism by which specific tRNAs are selectively cleaved for SHOT-RNA production remains elusive.

Among all species of tRNAs, tRNA_{HisGUG} is unique in that it contains an additional guanosine residue at the −1 position (G_{−1}) of its 5′-end (Sprinzl et al. 1998). This G_{−1} residue is conserved across phyla and has been observed in bacteria (Singer and Smith 1972; Orellana et al. 1986), yeast (Keith and Pixa 1984), fruit fly (Alteweg and Kubli 1980), and mammals (Boisnard and Petrissant 1981; Rosa et al. 1983). In Escherichia coli, this G_{−1} residue is genome-encoded, and anomalous RNase P cleavage of pre-tRNA{HisGUG} between positions −1 and −2 yields G_{−1}-containing tRNA_{HisGUG} (Orellana et al. 1986; Burkdard et al. 1988). In yeast, G_{−1} is not derived from the genomic sequence; instead, tRNA_{HisGUG} guanylyltransferase (Thg1) post-transcriptionally adds this residue to the 5′-end (Gu et al. 2003). The conservation of G_{−1} residue addition via different mechanisms in different organisms implies the functional significance of the G_{−1} residue. Indeed, the G_{−1} residue is a critical determinant for the aminoacylation of tRNA_{HisGUG} by the cognate histidyl-tRNA synthetase (HisRS) in both E. coli (Himenoo et al. 1989) and yeast (Rudinger et al. 1994; Rosen et al. 2006). In yeast, the loss of this G_{−1} residue consequent to the depletion of Thg1 or its polymerase activity causes a severe reduction in the tRNA_{HisGUG} aminoacylation levels, resulting in growth impairment (Gu et al. 2005; Jackman and Phizicky 2008; Preston and Phizicky 2010). The G_{−1} residue is also implicated in post-transcriptional nucleotide modification because yeast lacking this residue has been shown to acquire additional 5-methylcytidine (m^5C) modifications (Gu et al. 2005; Preston et al. 2013), although a biological role for the interplay between the absence of G_{−1} and the presence of m^5C is unknown. In contrast to the presence and significance of G_{−1} in these studies, some organisms such as α-proteobacteria (Wang et al. 2007; Jackman et al. 2012), Acanthamoeba (Rao et al. 2013), and Trypanosoma (Rao and Jackman 2015) lack G_{−1}. HisRS does not require G_{−1} for aminoacylation in these species.

Despite the advent of next-generation sequencing (NGS) technologies and their widespread use in RNA-seq for transcriptome analyses, the −1 nucleotide of tRNA_{HisGUG} has not been investigated in RNA-seq-based studies. This could be partly attributable to the expectation that tRNA_{HisGUG} contains post-transcriptional modifications that would interfere with reverse transcription (Kellner et al. 2010), such as a 1-methyl-guanosine at nucleotide position 37 (m^1G_{37}) (Boisnard and Petrissant 1981) (nucleotide position [np] is based on the tRNA numbering system [Sprinzl et al. 1998]). Indeed, human and Bombyx 3′-haves of cyto tRNA_{HisGUG}, possessing G_{37} (likely modified to m^1G_{37}), could not be amplified by RT-PCR despite being successfully detected by Northern blot (Honda et al. 2015). The presence of the RT-interfering modification would lead to underrepresentation and bias in the tRNA_{HisGUG} sequence information generated from RNA-seq data. While analyzing a cP-RNA-seq library of 5′-SHOT-RNAs from BT-474 cells (Honda et al. 2015), we reasoned that this library would be useful for observing the −1 nucleotide on human cyto tRNA_{HisGUG} for the following reasons. First, 5′-SHOT-RNA_{HisGUG} (5′-half of cyto tRNA_{HisGUG}) does not contain RT-inhibitory modifications, and therefore sequence analyses should not be biased by modifications. Second, 5′-SHOT-RNA_{HisGUG} is produced from mature aminoacylated cyto tRNA_{HisGUG}, and therefore information about the −1 nucleotide in mature tRNA might be retained in this 5′-half. Third, 5′-SHOT-RNA_{HisGUG} was the second most abundant species in the 5′-SHOT-RNA library from BT-474 cells, and thereby sufficient sequence reads of the 5′-half are available for an estimation of the −1 nucleotide state.

In this study, we investigated the 5′-terminal nucleotides of 5′-SHOT-RNA_{HisGUG} expressed in human BT-474 cells and observed an unexpected level of variation that was not limited to G_{−1}. Furthermore, we developed a TaqMan qRT-PCR-based method that could distinctively quantify each tRNA variant containing a different 5′-terminal nucleotide and thus clarified a 5′-terminal nucleotide variation of the mature cyto tRNA_{HisGUG} expressed in the same BT-474 cells. This identification and comparison of the 5′-terminal nucleotides and their variations among mature cyto tRNA_{HisGUG} and 5′-half molecules have yielded insights
Experimental validation of the predominant expression of 5′-SHOT-RNA\textsuperscript{HisGUG} lacking the −1 nucleotide

Because our cP-RNA-seq scheme includes several chemical and enzymatic RNA treatments (Honda et al. 2015, 2016), unexpected variations of the 5′-termini of 5′-SHOT-RNA\textsuperscript{HisGUG} might have resulted from undesired procedural RNA damage. To exclude this possibility and confirm that our cP-RNA-seq results reflect the cellular state of RNA expression, we conducted a primer extension assay for 5′-SHOT-RNA\textsuperscript{HisGUG}. In this assay, a radiolabeled DNA primer complementary to np 6–25 of cyto tRNA\textsuperscript{HisGUG} (Fig. 2A) was specifically hybridized to 5′-SHOT-RNA\textsuperscript{HisGUG} present in gel-purified small RNA fractions (20–50 nt) from BT-474 cells; subsequently, reverse transcription was carried out from the primer. When using synthetic tRNA\textsuperscript{HisGUG} initiating from G\textsubscript{1} as a template, the 5′-nt primer extension was detected as a 25-nt band (Fig. 2B). In contrast, the use of synthetic tRNA\textsuperscript{HisGUG} containing G\textsubscript{1} yielded an additional extension of 1 nt and a 26-nt band that was clearly distinct from the above-mentioned 25-nt band. An equal mix of these two synthetic tRNAs yielded two bands of equal abundance, indicating the ability of this assay to estimate the presence or absence of the −1 nucleotide. By performing reactions using dideoxynucleotides, we confirmed that the reverse transcription was correctly run on tRNA\textsuperscript{HisGUG} in both synthetic RNA and cellular RNA samples (Supplemental Fig. S2). Analyses of BT-474 small RNA fractions revealed the marked and more abundant presence of the 25-nt band in comparison with the 26-nt band (Fig. 2B). Quantification of the band intensities suggested that >70% of the 5′-SHOT-RNA\textsuperscript{HisGUG} lacks the −1 nucleotide (Supplemental Fig. S3), which is consistent with the cP-RNA-seq-based analyses shown in Figure 1. These results indicate that the majority of the 5′-SHOT-RNA\textsuperscript{HisGUG} molecules expressed in BT-474 cells lack the −1 nucleotide and initiate from G\textsubscript{1}. In the primer extension assay, we did not observe a clear 24-nt band corresponding to 5′-SHOT-RNA\textsuperscript{HisGUG} initiating from C\textsubscript{2}; therefore, the presence of such RNA in cP-RNA-seq data might result from undesired procedural RNA damage.
Development of a TaqMan qRT-PCR-based method for the discriminative quantification of 5′-terminal variants of mature cyto tRNA^{HisGUG}

SHOT-RNAs originate from mature aminoacylated tRNAs (Honda et al. 2015); accordingly, we reasoned that the 5′-terminal variations of 5′-SHOT-RNA^{HisGUG} would mirror those of mature cyto tRNA^{HisGUG}, although these variations did not match the canonical, reported variations in mature tRNA. Because RNA-seq data are not appropriate for analyses of mature tRNA^{HisGUG} sequences, we developed a TaqMan qRT-PCR-based method that can discriminatively quantify each 5′-terminal variant of mature cyto tRNA^{HisGUG} containing G_{1}, U_{1}, A_{1}, C_{1}, or G_{1} as the 5′-terminal nucleotide. We focused on a single major isodecoder encoded by nine of 16 genes in the human genome (Supplemental Fig. S1).

In the developed method, mature tRNA fractions (70–90 nt) were first gel-purified from total RNA, after which an acceptor-stem disrupter (AS-disrupter), a DNA oligo complementary to np 55–76 (3′-end) of the cyto tRNA^{HisGUG}, was hybridized to the purified fractions (Fig. 3A,B; Supplemental Fig. S4). Subsequently, a DNA/RNA chimeric 5′-adapter was ligated to the 5′-ends of mature tRNA^{HisGUG}, and the ligation product was amplified and quantified by TaqMan qRT-PCR to eventually generate an 86-nt (tRNA starting from G_{1}) or 87-bp cDNA (tRNA containing −1 nucleotide). The AS-disrupter was utilized to disrupt mature tRNA^{HisGUG} structure; this disruption was expected to enhance the accessibility of the adapter, primer, and enzymes to the tRNA and thereby increase the reaction efficiencies following hybridization. In addition, dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG) 8000, both of which enhance RNA ligation efficiency, were added to the adapter ligation reaction. Indeed, the combined inclusion of the AS-disrupter hybridization step and addition of PEG/DMSO to the ligation reaction increased the detection efficiency of synthetic tRNA^{HisGUG} with G_{1} by more than 95-fold and prevented the synthesis of nonspecific cDNA bands (Fig. 3C). The TaqMan probe was designed to target the boundary of the adapter and the 5′-end of mature tRNA^{HisGUG}, thus allowing an exclusive analysis of the tRNA 5′-end in the ligation product. Indeed, we were unable to detect an amplification signal in the absence of T4 RNA ligase (Fig. 3C). Because the TaqMan probe has a single-nucleotide resolution (Ranade et al. 2001; Honda and Kirino 2015), our design scheme was expected to discriminatively quantify each 5′-terminal variant of tRNA^{HisGUG} without cross-reactivity with other variant species. We confirmed the exclusive specificity of our TaqMan probes to quantify perfectly matched target sequences without cross-reactivity from other variants (Supplemental Table S1). To examine the quantification ability, our method was applied to different amounts of synthetic mature tRNA^{HisGUG} (0.1–100 fmol). To mimic tRNA quantification using a total tRNA fraction, an E. coli tRNA fraction was mixed with synthetic RNA as a carrier; we confirmed that the E. coli tRNA fraction did not yield an amplification signal in our system. For all five synthetic 5′-terminal variants, the quantifications demonstrated clear linearity between the log of tRNA input and the Ct value (Supplemental Fig. S5), indicating that this method has a dynamic range of at least three orders of magnitude, and discriminatively quantifies the 5′-terminal variants. We further validated our method by quantifying a mixture of different synthetic tRNA variants. Since 5′-SHOT-RNAs^{HisGUG} starting from G_{1}, U_{1}, A_{1}, C_{1}, or G_{1} as the 5′-terminal nucleotide were mainly detected (Fig. 1D), corresponding mature synthetic RNAs^{HisGUG} were mixed at several different ratios and subjected to the method with an E. coli tRNA carrier. The amount of each detected tRNA was calculated based on the standard curves (Supplemental Fig. S5). As shown in Figure 3D, the resultant relative abundances of detected tRNAs well reflected those of the tRNAs added to the reactions, allowing us to conclude that our method can estimate the relative abundance of 5′-terminal variants of mature tRNA^{HisGUG}.

The majority of the mature tRNA^{HisGUG} molecules contain the −1 nucleotide

Given the high specificity and quantification ability of our TaqMan qRT-PCR method, we utilized this method to determine the relative abundances of the 5′-terminal variants of endogenous mature cyto tRNA^{HisGUG} expressed in BT-474 cells. Using our synthetic tRNA results as standards, we determined the relative abundances of the five potential 5′-terminal variants of tRNA^{HisGUG}. In contrast to the 5′-terminal variations of 5′-SHOT-RNA^{HisGUG} which were dominated by G_{1} (Fig. 1C), ~60% of the mature tRNA^{HisGUG} contained G_{1} as a 5′-terminal nucleotide (Fig. 4A). A significant proportion of tRNAs contained U_{−1} (~20%), and a similar
proportion lacked the −1 nucleotide. We could not detect amplification signals from tRNAs containing A−1 and C−1, likely because those tRNA species were not expressed at sufficient levels to allow detection by our system. The predominance of the −1 nucleotide-containing tRNAHisGUG was validated by a primer extension assay using the mature tRNA fractions from BT-474 cells. In contrast to the 5′-SHOT-RNAHisGUG analyses, in which the 25-nt band was more abundant than the 26-nt band (Fig. 2B), mature tRNA analyses revealed a predominance of the 26-nt band (Fig. 4B). Because of the low resolution of this method for mature tRNA, no clear band around 25 nt appeared, and thereby it was difficult to estimate the relative abundance of the −1 nucleotide lacking tRNA. However, this result at least validated our TaqMan qRT-PCR result that the −1 nucleotide-containing tRNAHisGUG is the major mature species in BT-474 cells.

Potential mechanisms by which distinct 5′-terminal variations are formed in mature cyto tRNAHisGUG molecules and 5′-halves

This study identified the 5′-terminal variations of human mature cyto tRNAHisGUG in BT-474 cells. Although more than half of the tRNAs contained G−1, substantial amounts of previously unreported mature tRNA species either containing U−1 or lacking the −1 nucleotide were also identified. In earlier studies, mammalian cyto tRNAHisGUG from HeLa cells and sheep liver were analyzed using chromatography, and neither U−1-containing nor −1 nucleotide-lacking tRNAs were detected (Boisnard and Petrissant 1981; Rosa et al. 1983). This difference might be attributable to differences in the detection method sensitivities. During the course of this study, an advanced tRNA sequencing method has been reported in which methylations, including m1G, were removed from tRNAs via engineered AlkB demethylase prior to reverse transcription, thereby reducing sequencing bias from these methylations (Zheng et al. 2015). We investigated the −1 nucleotide variations of tRNAHisGUG in the reported less-biased tRNA sequencing data set from HEK293T cells (Supplemental Table S2). As a result, we observed a major population of mature tRNAHisGUG containing G−1 (47%), and substantial populations of mature tRNAs containing U−1 (18%) or lacking the −1 nucleotide (30%); these results were similar to the 5′-variations that we observed in mature tRNA from BT-474 cells. These results suggest the universality of the presence of mature tRNAHisGUG containing U−1 or lacking the −1 nucleotide, as well as molecules containing G−1, among human cultured...
tRNAs containing each 5′-terminal nucleotide; SHOT-RNA lacking the 5′-terminal nucleotide; SHOT-RNAs exhibiting 5′-terminal variations of tRNAHisGUG. The generative mechanism –1 nucleotide-lacking tRNAs, resulting in a considerable accumulation of –1 nucleotide-lacking SHOT-RNAs. The generative mechanism –1 nucleotide-lacking SHOT-RNAHisGUG molecules mostly lack the –1 nucleotide. This inconsistency might be attributable to ANG cleavage activity to generate SHOT-RNAs. ANG might selectively cleave –1 nucleotide-lacking tRNAs, resulting in a considerable accumulation of –1 nucleotide-lacking SHOT-RNA molecules. However, ANG is a small protein, and thus selective cleavage should be facilitated by cofactors. Alternatively, ANG might cleave tRNA irrespective of the 5′-terminal nucleotide; SHOT-RNA lacking the –1 nucleotide might then become more stable within the cells than SHOT-RNA containing G−1, or an unknown ribonuclease might trim the –1 nucleotide from SHOT-RNAs. The generative mechanism and biological significance of these 5′-terminal variations in mature tRNAHisGUG molecules 5′-halves remain to be elucidated.

FIGURE 4. Variations in 5′-terminal nucleotide from mature tRNAHisGUG expressed in BT-474 cells. (A) Of note, 70- to 90-nt mature tRNA fractions of BT-474 cells were subjected to TaqMan qRT-PCR quantification of each 5′-terminal variant of mature tRNAHisGUG. Expression levels were estimated using standard curves from synthetic RNAs (Supplemental Fig. S5), and the relative abundances of mature tRNAHisGUG molecules 5′-variations in mature tRNA and the functional significance of these 5′-terminal nucleotide-lacking SHOT-RNAHisGUG exhib-

MATERIALS AND METHODS

Bioinformatics analyses of 5′-SHOT-RNAHisGUG

Human cyto tRNAHisGUG sequences were identified using the tRNAscan-SE program (Lowe and Eddy 1997) and are shown in Supplemental Figure S1. The 5′-SHOT-RNA library was previously obtained by cP-RNA-seq of gel-purified 30- to 50-nt RNAs from BT-474 cells (Honda et al. 2015) and can be found in the Gene Expression Omnibus Database (GEO accession no. SRX1060214). Reads previously mapped to mature cyto tRNAHisGUG sequences (Honda et al. 2015) were extracted and used for this study.

Cell culture

BT-474 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) containing 10% (v/v) FBS.

In vitro synthesis of tRNAHisGUG

Templates for the in vitro synthesis of human cyto tRNAHisGUG (with or without N−1 nucleotide) were prepared by annealing two ssDNAs (5′-GCTTAATACGACTCACTATAGGCCGTGATCGTATAGTTAAGTACCTCCTGGTTGGTGGC-3′ and 5′-mUmGGTGGCCGTACCGGATTCGAACCGAGGTTGCTGCGGCCACAACGC-3′) in a solution containing 10 mM Tris–HCl (pH 8.0) and 20 mM MgCl₂. After blunting the formed duplex using sequenase (Affymetrix), the resultant dsDNAs were used as templates for transcription with T7 RNA polymerase (New England Biolabs). Synthesized RNAs were gel-purified using denaturing PAGE.

5′-End identification of tRNAHisGUG by TaqMan qRT-PCR

The sequences of the adapter, AS-disrupter, primers, and TaqMan probes for TaqMan qRT-PCR are shown in Supplemental Table S4. The 70- to 90-nt RNA fraction, which contained mature tRNAs, was initially gel-purified from total RNA using denaturing PAGE. To ligate the 5′-adapter, to the 5′-end of cyto tRNAHisGUG, 500 ng of the RNA fraction were incubated with 100 pmol of AS-disrupter in a 4-µL reaction mixture at 90°C for 2 min and subsequently incubated at 37°C. RNA was then added immediately to a ligation reaction mixture (total volume: 10 µL) containing 200 pmol of 5′-adapter, T4 RNA ligase 1 (New England Biolabs), 10% (v/v) DMSO, and 5% PEG8000 and incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. Next, 1 µL of the ligation mixture was subjected to cDNA synthesis with 1 µM of RT primer and SuperScript III (Invitrogen), the resultant dsDNAs were used as templates for transcription with T7 RNA polymerase (New England Biolabs). Synthesized RNAs were gel-purified using denaturing PAGE.

Primer extension assay

To detect 5′-SHOT-RNAHisGUG, 30- to 50-nt RNAs were first gel-purified from BT-474 total RNA. Subsequently, 50 ng of gel-purified
RNA or 0.1 pmol of synthetic tRNA\textsuperscript{HisGUG} were incubated with SuperScript III, the corresponding reaction buffer (Invitrogen), and 0.1 pmol of 5'-\textsuperscript{32}P-labeled DNA primer (5'-GTACTAACCAC TATACGATC-3') at 55°C for 30 min. The reaction mixtures were developed using denaturing PAGE containing 7 M urea and 20% formamide. To analyze mature tRNA\textsuperscript{HisGUG}, 70- to 90-nt mature tRNAs were gel-purified from the total RNAs extracted from BT-474 cells. Gel-purified RNAs (1 µg) were then subjected to a primer extension assay as described above.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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