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Investigating the prevalence of queuine in *Escherichia coli* RNA via incorporation of the tritium-labeled precursor, $preQ_1$

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ABSTRACT

There are over 100 modified bases that occur in RNA with the majority found in transfer RNA. It has been widely believed that the queuine modification is limited to four transfer RNA species *in vivo*. However, given the vast amount of the human genome (60–70%) that is transcribed into non-coding RNA (Mattick [10]), probing the presence of modified bases in these RNAs is of fundamental importance. The mechanism of incorporation of queuine, via transglycosylation, makes this uniquely poised to probe base modification in RNA. Results of incubations of *Escherichia coli* cell cultures with [^{3}H] $preQ_1$ (a queuine precursor in eubacteria) clearly demonstrate $preQ_1$ incorporation into a number of RNA species of various sizes larger than transfer RNA. Specifically, significant levels of $preQ_1$ incorporation into ribosomal RNA are observed. The modification of other large RNAs was also observed. These results confirm that non-coding RNAs contain modified bases and lead to the supposition that these modifications are necessary to control non-coding RNA structure and function as has been shown for transfer RNA.

1. Introduction

Queuine is one of approximately 100 modified nucleotides found in RNA. [1]. Unlike the majority of modified nucleotides that result from modifications of the genetically encoded nucleotides, queuine is incorporated into RNA by transglycosylation (Fig. 1) [2]. Presently, this modification is known to occur in the wobble position of the anticodon loops of $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asp}$, and $tRNA^{Asn}$ [3]. Investigations have determined that its presence has an effect on codon recognition and has been implicated in promoting translational fidelity. In addition, queuine has been demonstrated to have a role in the bacterial virulence of Shigella [4] and the malignancy of certain cancers [5,6]. It is known that the enzyme responsible for the incorporation of queuine into RNA, tRNA guanine transglycosylase (TGT), recognizes a UGU sequence in an RNA hairpin loop (e.g., tRNA anticodon arm) as the site for modification. Previous work in our laboratory has demonstrated that modification of other RNAs (e.g., hairpin minihelix [7,8] and 800 base mRNA [9]) with UGU sequences in a loop can occur *in vitro*. While the vast majority of modified RNA bases are only known to occur in tRNA, the possibility that modified bases in general may occur throughout other RNAs has largely been unexplored. This question is all the more important as it is now becoming more widely appreciated that 60–70% of the human genome codes for "non-coding RNA", e.g., RNA transcripts that do not code for protein [10–12]. The functions of these non-coding RNAs are beginning to be elucidated; however, the presence of base modifications in non-coding RNA, let alone the function(s) of the modifications, has only recently been shown [13].

The incorporation of queuine into RNA via transglycosylation provides a unique point of entry to study the prevalence of this base modification as it lends itself to the utilization of designed queuine analogues. Eukaryae incorporate queuine obtained through their diet whereas eubacteria biosynthesize and incorporate the queuine precursor $preQ_1$ before completing the modification on the RNA to yield queuine (Fig. 1). As a result of these differences, the enzyme (TGT) responsible for the incorporation of the modification into RNA differs between eukaryl and eubacterial organisms [14]. Tritium-labeled $preQ_1$ ([^{3}H] $preQ_1$) was synthesized previously along with tritium-labeled queuine and both were used in a study that explored differences between human and *Escherichia coli* TGTs, which concluded that divergent evolution from a common progenitor led to the eubacterial and eukaryal enzymes [14,15].

We report *in vitro* and *in vivo* experiments utilizing three *E. coli* strains: a Δ*queC* knockout that is unable to synthesize $preQ_1$, a Δ*tgt* knockout that is unable to incorporate $preQ_1$ and a wild-type *E. coli* strain. Deletion of *queC* in the Δ*queC* strain prevents the biosynthesis of endogenous $preQ_1$ and any potential TGT-substrate intermediates, thereby eliminating any competition for incorporation of our labeled compound. The Δ*tgt* strain served as a negative control to insure that any incorporation observed was TGT-dependent. Our studies clearly demonstrate TGT-dependent incorpora-

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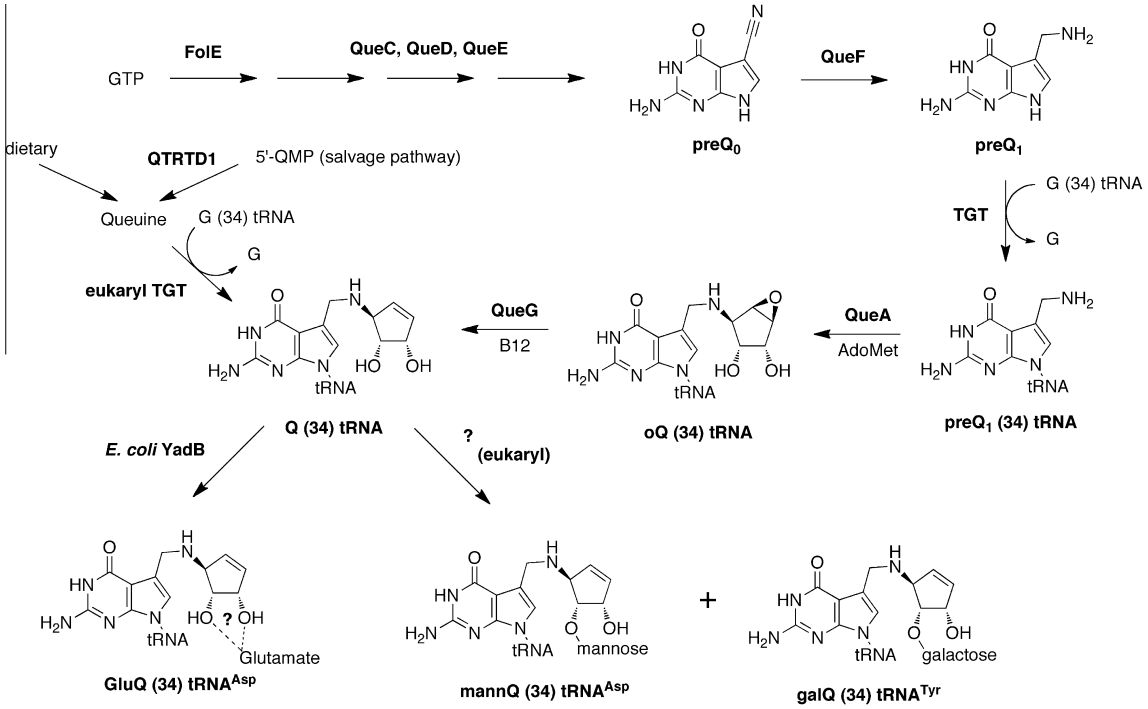

Fig. 1. Queuine biosynthetic pathways for eubacteria and eukarya. G(34) tRNA = tRNA with guanine in the 34 position, Q = queuine and oQ = epoxy-queuine [16].

tion of radiolabel into ribosomal and non-ribosomal/non-transfer RNA species.

2. Materials and methods

2.1. Reagents

Unless otherwise specified, all reagents were ordered from Sigma–Aldrich. Yeast extract and agar were ordered from Fisher Scientific; tryptone from Acros Organics; Seaplaque low-melting agarose was from Lonza. The MasterPure™ RNA Purification Kit and Gelase™ Agarose Gel-Digesting Preparation were procured from Epicentre Biotechnologies. Bio-Safe II™ was ordered from Research Products International Corporation. Fluorescent silica chromatography plates were acquired from Analtech. The [^{3}H] preQ$_1$ was obtained from a convergent synthesis with radio-labeling and isolation of final compound completed by Moravek Biochemicals [14].

2.2. Isolating total RNA

E. coli cell strains TG2 and Δ*tgt* were grown overnight at 37 °C on LB plates (5.0 g tryptone, 2.4 g yeast extract, 7.5 g agar, 5 mL 1 M NaOH) while the Δ*queC* strain was grown overnight at 37 °C on L-Kan plates (50 μg/mL kanamycin). Individual colonies were then isolated and incubated in 3 mL 2xTY broth (8.0 g tryptone, 5.0 g yeast extract, 2.5 NaCl) overnight at 37 °C with shaking. Subsequently, the total RNA was isolated following the vendor's (Epicentre MasterPure™ RNA Purification Kit) protocol.

2.3. 5′-Phosphate RNA exonuclease treatment of total RNA

Total RNA was isolated as described above. The total RNA was resuspended in 16.5 μL RNase-free water. The exonuclease digestion was conducted following the vendor's (Epicentre mRNA-ONLY™ Prokaryotic mRNA Isolation Kit) protocol.

2.4. Isolation of ribosomal RNA

E. coli Δ*queC* was grown overnight at 37 °C on L-Kan plates (50 μg/mL kanamycin). Individual colonies were then isolated and incubated in 3 mL 2X TY broth overnight at 37 °C with shaking. One milliliter of overnight culture was added to 100 mL 2X TY broth and incubated at 37 °C for three hours. The cells were pelleted at 5,000 × g for 15 min at 4 °C. The pellet was resuspended in 4 mL of buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM EDTA). The cell lysis was achieved by four rounds of a freeze thaw cycle with addition of 1 μL of PMSF (100 mM in isopropyl alcohol) and 1 μL of lysonase bioprocessing reagent, from Novagen, after the last round and incubation for 20 min. Debris were pelleted via centrifugation at 13,000 rpm in a table-top micro-centrifuge for 10 min. The supernatant was transferred to a clean tube, treated with 5 μL of DNase I (1 unit/μL) and held at room temperature for 10 min. Debris were pelleted via centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to an ultra high speed centrifuge tube and 1 mL of 2 M NH_4Cl was added. The remaining volume of the tube was filled with microbiology grade mineral oil. The ribosome pellet was obtained by centrifugation at 40,000 rpm with a Beckmann Ti70 rotor (100,000 g) for 4 h at 4 °C. The supernatant was discarded and the pellet rinsed twice with 0.5 mL of buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NH_4Cl). Subsequently, 0.5 mL of the buffer was added to the tube and the pelleted ribosomes were resuspended overnight at 4 °C.

2.5. Proteinase K digestion of ribosomal proteins

Ribosomes collected from the above-described ribosome isolation were treated with 1 μL of proteinase K (50 μg/μL). The sample was incubated at 65 °C for 15 min in a heating block. The sample was vortexed for 10 s every 5 min during the incubation. The protein was precipitated with 175 μL MPC protein precipitation solution from Epicentre Biotechnologies. The debris was pelleted via centrifugation at 13,000 rpm for 10 min. The supernatant was

decanted and the rRNA precipitated by addition of 500 μL of isopropanol. The tube was inverted 30 times and then centrifuged at 13,000 rpm for 10 min to pellet the rRNA. The rRNA pellet was washed with 70% ethanol twice and then resuspended in 5 μL of water.

2.6. In vitro incubations with TGT and [^{3}H] preQ$_1$

Total RNA isolated from *E. coli* Δ*tgt* and *E. coli* Δ*queC* and ribosomal RNA prepared from *E. coli* Δ*queC* were incubated *in vitro* with TGT and [^{3}H] preQ$_1$. The reaction conditions were as follows: 5.65μL enzyme TGT (5 μM), 10 μL total or ribosomal RNA, 20 μL 5X bicine reaction buffer, 14 μL [^{3}H] preQ$_1$ (100 μM) and 50.35 μL deionized water. A no-enzyme control reaction was also conducted where the enzyme was deleted and 56.0 μL deionized water was used. The reactions were incubated for two hours at 37 °C. Water was removed from the solutions via vacuum centrifugation for two hours and the resulting pellets were resuspended in 10 μL of deionized water and then analyzed via agarose gel separation as described below.

2.7. In vivo incubation with [^{3}H] preQ$_1$, total RNA

E. coli Δ*tgt*, Δ*queC* and TG2 cell lines were cultured with [^{3}H] preQ$_1$ added to the media. These incubations were conducted under the same conditions as described above for total RNA isolation and detection. Three milliliter cultures in 2xTY with 30 μL of [^{3}H] preQ$_1$ (650.6 μM; 1 Ci/mmol) were incubated for 12 h at 37 °C. Cell pellets from two, 1 mL samples from each 3 mL culture were obtained via centrifugation at 13,000 rpm in a table-top centrifuge. The pellets were then subjected to total RNA isolation as described above.

2.8. In vivo incubation with [^{3}H] preQ$_1$, ribosomal RNA

E. coli Δ*queC* was incubated in 3 mL culture of 2xTY 12 h at 37 °C. One milliliter of this overnight culture was used to inoculate 100 mLs of 2xTY with 30 μL of [^{3}H] preQ$_1$ (650.6 μM; 1 Ci/mmol). The culture was allowed to grow at 37 °C with shaking until an OD_{600} of 0.6 was achieved, approximately 3 h. The cells were pelleted via centrifugation at 5,000 × g for 15 min. The pellet was stored at −20 °C prior to ribosome isolation as described above.

2.9. Low-melting agarose gel electrophoresis and excision of bands

The reaction samples were separated by electrophoresis using 2% SeaPlaque low melting agarose gel at 50 V for 120 min. Three RNA species were used as controls: tRNA^{tyr} (~80 nt), VirF mRNA (~800 nt), and tRNA^{tyr} anticodon arm minihelix (ECYMH, ~25 nt). Subsequently, the gel was positioned on a silica gel chromatography plate and 250 nm UV light was used to visualize the bands via shadowing of the fluorescence of the silica plate to avoid interference that RNA stains may have presented to liquid scintillation counting. Sample bands were excised (8 per lane) and the agarose was digested with Gelase™ following vendors' protocol. The radioactivity in the resulting samples was determined by collecting the ethanol precipitated RNA on Whatman GF/C filters with subsequent liquid scintillation counting in 4 mL of BioSafe II cocktail.

2.10. Urea polyacrylamide gel electrophoresis and characterization of bands

Isolated total RNA or rRNA from previously described methods was diluted to approximately 1000 ng/μL. A serial dilution was performed to create two additional samples; one with half the concentration and the final with a quarter of the initial concentration. The reaction samples were separated by electrophoresis using 6% polyacrylamide, 8 M urea gel in TBE at 200 V for 45 min. tRNA^{tyr} (~80 nt) was used as a control. When electrophoresis was complete, the RNA bands were transferred to a Zeta-Probe membrane by semi-dry transfer at 10 V for 40 min. The membrane was then dried overnight. Once dry, the blot was placed in an exposure cassette with a TR phosphor storage screen for seven days to develop. The image was visualized using a Typhoon scanner in phosphor storage mode. After visualization, the blot of the PAGE experiment was cut into eight equal bands per lane. The bands were subjected to liquid scintillation counting in 4 mL of BioSafe II cocktail.

3. Results

3.1. RNA preparation and characterization

Fig. 2 shows an example of a total RNA preparation gel (stained with ethidium bromide for illustrative purposes) with the excision grid depicted. Fig. 3 shows an example of a ribosomal RNA gel.

3.2. In vitro incubation with TGT and [^{3}H] preQ$_1$

Total RNA isolated from *E. coli* Δ*tgt* and *E. coli* Δ*queC* was used for *in vitro* studies. Both RNA preparations were substrates for TGT *in vitro* (see Supplementary Fig. 1).

As a control, incubations of total RNA with [^{3}H] preQ$_1$ without TGT were conducted and exhibited far lower counts, which may be due to a small amount of [^{3}H] preQ$_1$ non-covalently associating and co-precipitating with the RNA. As another control, total RNA was spiked with tRNA^{tyr} prior to the experiment. The results of this study (Supplementary Fig. 1) show dramatically increased radioactivity in lane 7, which likely bled over into lanes 5 & 6 due to the high concentration of the spiked tRNA^{tyr} resulting in overloading of the gel lane. (Note that in no other RNA preparation was the tRNA^{tyr} band overloaded, therefore we conclude that there is no

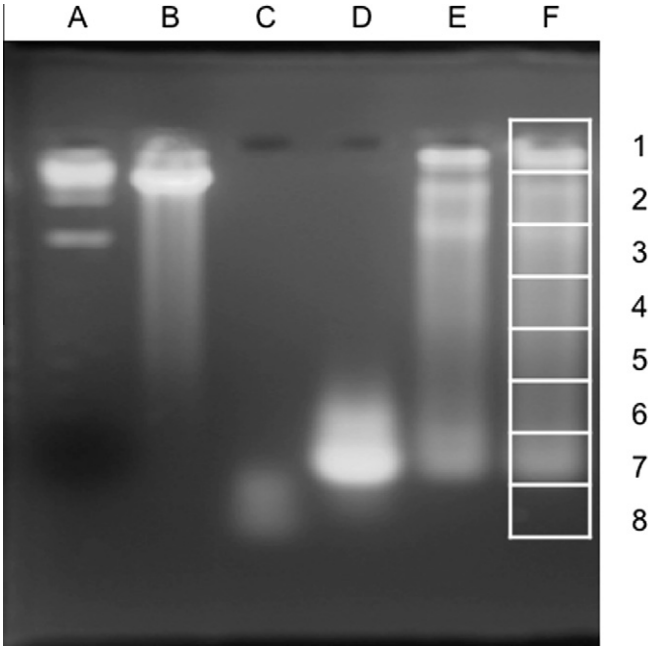

Fig. 2. Example agarose gel of total RNA preparation. Lane (A) 1 kb ladder, (B) virF mRNA, (C) minihelix/hairpin RNA, (D) tyrosine tRNA, (E) total RNA, and (F) total RNA with grid showing gel bands for radioactivity determinations.

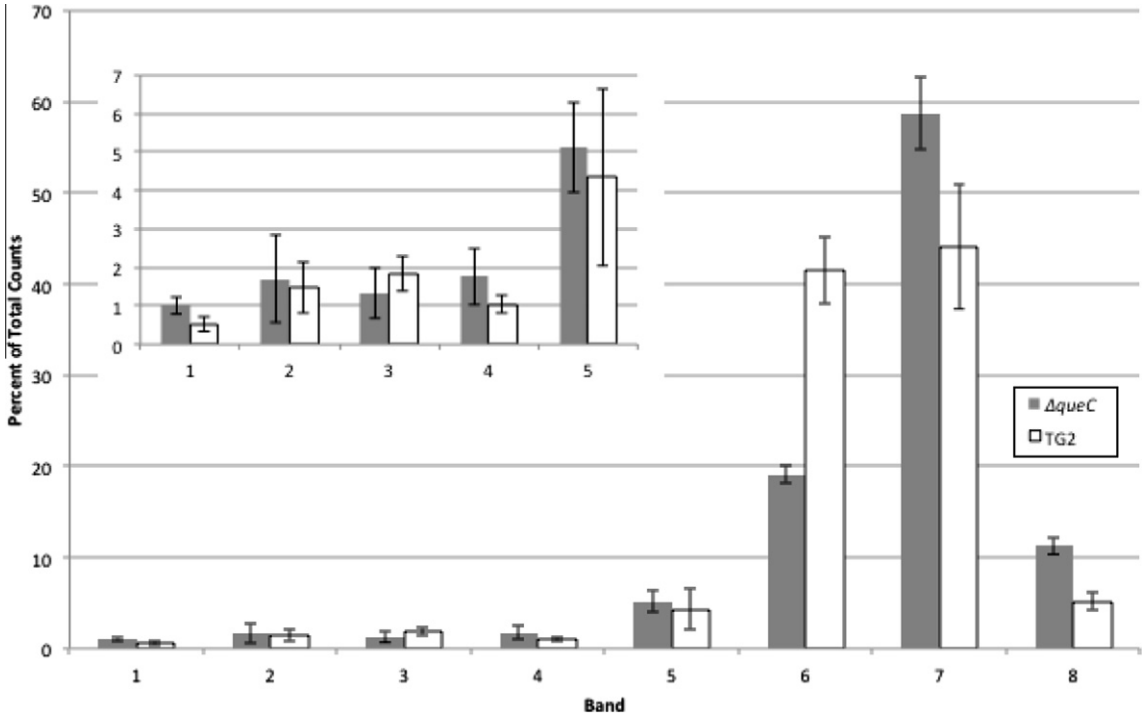

Fig. 3. Radioactivity in gel bands of total RNA isolated from *E. coli* TG2 and *E. coli* Δ*queC* incubated *in vivo* with [^{3}H] preQ$_1$. Data is the average (n = 4) percent of total counts for each gel band. Inset: data from bands 1–5 rescaled. Lanes containing total RNA isolated from *E. coli* Δ*queC* cells (filled bars) averaged 54,800 DPM per experiment with values for individual bands ranging from 260 to 41,350 DPM. Lanes containing total RNA isolated from *E. coli* TG2 (open bars) cells averaged 43,900 DPM per experiment with values for individual bands ranging from 180 to 27,000 DPM.

bleeding of the tRNA^{tyr} band into any other band than perhaps 6 in the subsequent total RNA analyses.)

Ribosomal RNA was also prepared from *E. coli* Δ*queC*. This was similarly incubated *in vitro* with TGT and [^{3}H] preQ$_1$. Supplementary Fig. 2 shows that ribosomal RNA is also a substrate for TGT with low counts in bands 7 & 8. Treatment of the ribosome preparation with proteinase K shifted the counts to lower migrating bands, consistent with dissociation of the 70S ribosome into the constituent RNAs.

3.3. In vivo incubation with TGT and [^{3}H] preQ$_1$

Results of total RNA isolated from control *in vivo* incubations of [^{3}H] preQ$_1$ with *E. coli* Δ*tgt* were very similar to standard background radioactivity (Supplementary Fig. 3). Therefore, it is clear that any radioactivity detected above 150 DPM results from TGT-catalyzed enzymatic modification of RNA (i.e., "TGT-dependent"). Studies were then conducted under the same *in vivo* conditions with *E. coli* Δ*queC* and TGT2 cell lines. The results shown in Fig. 3 reveal that the greatest amount of radioactivity incorporation is detected in the tRNA band (number 7). Ribosomes were then isolated from cultures of *E. coli* Δ*queC* incubated *in vivo* with [^{3}H] preQ$_1$. Half of the ribosome preparation was treated with proteinase K to digest the ribosomal proteins and dissociate the ribosomal RNAs prior to gel electrophoresis. As seen in Fig. 4, the untreated ribosomes exhibit significant radioactivity in all bands with the lowest levels in bands 7 & 8. As for the *in vitro* experiments, treatment of the ribosome preparation with proteinase K shifted the counts to lower migrating bands, consistent with dissociation of the 70S ribosome into the constituent RNAs.

To probe if RNA species other than ribosomal RNA is modified by TGT, total RNA isolated from *in vivo* incubation of *E. coli* Δ*queC* with [^{3}H] preQ$_1$ was treated with a 5'-phosphate-dependent RNA exonuclease, which selectively digests rRNA. The results (Supplementary Fig. 5) compare the exonuclease digestion data to total RNA results from Δ*queC* total RNA collected previously. The detected radioactivity decreases for bands 1–5; however, the counts remain significantly above background.

3.4. Urea polyacrylamide gel electrophoresis and characterization of bands

To verify the agarose gel separations, the total RNA and rRNA experiments were repeated using denaturing PAGE conditions. The visualizations of the total RNA and rRNA blots are seen in Supplementary Fig. 6. Only one band is clearly visible for the total RNA isolation, and its size indicates that it is tRNA. In the visualization of rRNA, two bands are visible near one another. It is intriguing to speculate that these might be the 5S and 16S rRNA, although it is possible that they are tRNA and 5S rRNA. To verify these results, the blotting membrane was cut into bands and subjected to liquid scintillation counting to determine where radiolabeled preQ$_1$ was present on the blot. The results of these experiments are shown in Supplementary Fig. 7. The result of the scintillation experiments match what is seen in the visualization.

4. Discussion

We investigated the prevalence of the queuine modification using tritium-labeled preQ$_1$ ([^{3}H] preQ$_1$) and low-melting agarose gel electrophoresis to resolve the known modified transfer RNA species from RNAs of other sizes. The ability to load relatively large volumes of samples likely helped to make it possible to detect low abundance RNA species. As shown in Fig. 2 by the overlaid grid, all lanes with labeled RNA were divided into 8 equal bands with band 7 representing transfer RNA as determined by control tyrosine tRNA^{tyr} in an adjacent lane. The bands were excised from the gel, the agarose was digested, the RNA was precipitated/collected and

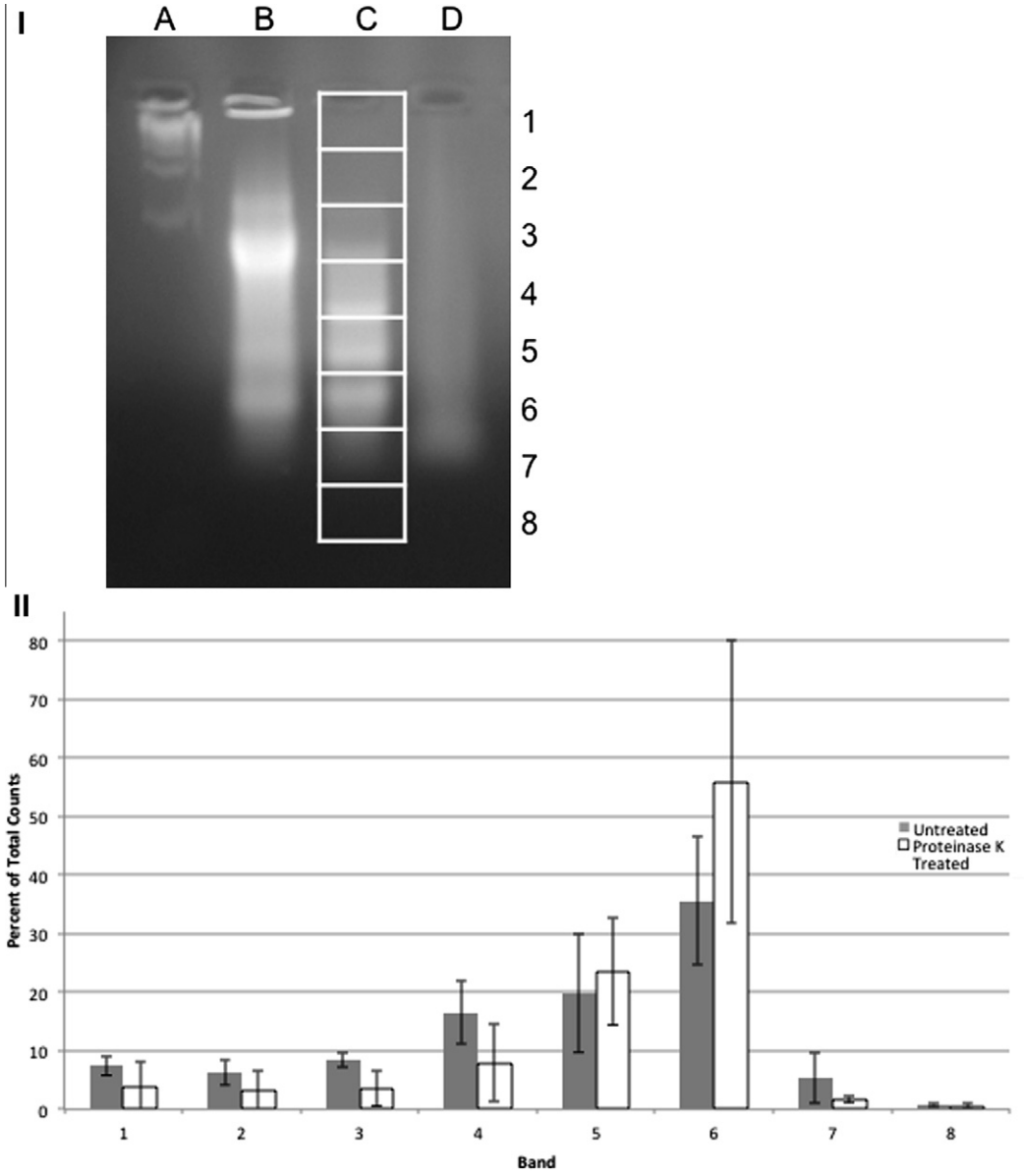

Fig. 4. Example gel and radioactivity in gel bands of ribosomal RNA (untreated and proteinase K treated) isolated from *E. coli* TG2 and *E. coli* Δ*queC* incubated *in vivo* with [^{3}H] preQ$_1$. (I) 2% low-melting agarose gel stained with EtBr to visualize bands; (A) 100 bp ladder, (B) 70 s ribosome, (C) proteinase K digested 70 s ribosome, (D) tyrosine tRNA standard. Bands were excised based on this visualization as represented by the grid on the gel and quantified by liquid scintillation counting. (II) Data is the average (n = 4) percent of total counts for each gel band for both isolated ribosome (filled bars) as well as proteinase K digest of ribosome (open bars). The average total counts per experiment in a lane for rRNA was 27,500 DPM with bands ranging from 70 to 39,000 DPM.

radioactivity determined by liquid scintillation counting. This method was found to give sufficient resolution for our purposes by initial studies with *in vitro* labeled control tRNA^{tyr}. It was observed that the radioactivity was limited in these control experiments to band 7, with a small amount of "spill over" in band 6 and in cases of extremely high loading of tRNA^{tyr}, band 5 as well.

Initial *in vitro* studies demonstrated that modification of sites other than the known transfer RNA species are indeed possible, consistent with the conclusions from previous *in vitro* studies with the enzyme. Interestingly, while there is a significant amount of radioactivity in the transfer RNA band (Supplementary Fig. 1, band 7), the majority of the radioactivity is found in bands corresponding to RNAs that migrate into the gel to a much lower extent than transfer RNAs (e.g., bands 1–5). The reason for this is unclear. It is intriguing to speculate that the total RNA preparation may not capture transfer RNA well; however, the results of the *in vivo* studies discussed below clearly show high levels of radioactivity incorporation in the transfer RNA band (e.g., band 7 in Fig. 3).

The results of *in vitro* incubations of isolated ribosomes from *E. coli* Δ*queC* show that the fully formed ribosome is also a substrate for TGT (Supplementary Fig. 2). The low radioactivity in lane 7 suggests that the radioactivity detected is not due to any transfer RNA that may have co-purified in the ribosome preparation. The proteinase K treated sample exhibited a shift in radioactivity from lanes 1 & 2 (likely the intact or partially dissociated 70S particle) mostly to lanes 4–6 (likely the dissociated ribosomal RNAs). Note that the *E. coli* 5S rRNA is 105 bases long, similar in size to transfer RNAs (~75 bases), possibly accounting for the counts in lanes 6 & 7 of the proteinase K treated sample.

The results from the *in vivo* experiments on total RNA, as shown in Fig. 3, are on the one hand more consistent with expectations and yet also surprising. Firstly, they show that preQ$_1$ can be taken

up and utilized by *E. coli* as a nutrient from the media. The results also show that transfer RNA is modified to the greatest extent. This is not surprising as transfer RNAs are the species known to be modified and exist in a greater abundance than other individual RNA species. The surprising finding is that modification occurs in RNAs from essentially all other bands (e.g., size ranges) at levels significantly above background. The fact the modification appears to be much more widespread than the four known transfer RNA species encouraged us to attempt to identify the other types of RNA that are targeted for modification, rRNA in particular.

The isolation of 70S ribosomes followed by their subsequent separation (treatment with proteinase K to free the constituent rRNA) and detection verified that rRNA is indeed a substrate for queuine modification. The larger culture volumes, required for ribosome isolation, required a lower concentration of the [^{3}H] preQ$_1$ due to the scarcity and cost of the material. However, the length of the overnight incubation and the concentration of [^{3}H] preQ$_1$ at slightly above its K_M for TGT were evidently sufficient to result in efficient incorporation. The data in Fig. 4 show that the total counts for the bands are well above background and the distribution of radioactivity suggests that rRNA species represent the majority of the modification seen in the upper bands of the gel for total RNA experiments, due to the very high abundance of rRNA. To further verify the rRNA as substrate for queuine modification, the use of a 5'-phosphate RNA exonuclease that exclusively digests rRNA was employed. The data in Supplementary Fig. 5 clearly show that the counts in the slower migrating RNA species are reduced, consistent with the digestion of the rRNA to smaller fragments that might be included in bands 6–8. The fact that counts remain in slower migrating (i.e., larger RNAs) bands (e.g., 1–4) could indicate that other RNA species such as mRNA are also sites for modification.

Denaturing PAGE analyses of total RNA and rRNA preparations were performed to validate the agarose gel results. For the total RNA, only the tRNA band is seen; however, for the rRNA two bands are observed (Supplementary Figs. 6 & 7). The observed bands in both cases do exhibit high levels of radioactivity. It is possible that the lower loading volume of the PAGE, efficiency of transfer in the blotting and efficiency of both imaging and counting tritium from pieces of the blotting membrane may all contribute to the lower sensitivity of the PAGE method, and hence lack of detection of low abundance species of RNA. In spite of this, the PAGE separation of the rRNA sample does clearly show two distinct bands, consistent with the agarose gel results for the rRNA preparations.

These results strongly suggest that RNA species (clearly rRNA) other than the known tRNAs are modified by TGT *in vivo*. It is unclear if there is a large number of RNAs of differing sizes that are modified *in vivo* or if there is a smaller number of larger RNAs that are modified *in vivo* and we are seeing them as well as their degradation products throughout bands 1–5. Further studies to isolate and identify the modified RNAs are needed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.055.

References

[1] P.F. Crain, J.A. McCloskey, The RNA modification database, Nucleic Acids Res. 24 (1996) 98–99.
[2] G.A. Garcia, J.D. Kittendorf, Transglycosylation: a mechanism for RNA modification And editing?, Bioorg Chem. 33 (2005) 229–251.
[3] F. Harada, S. Nishimura, Possible Anticodon Sequences of tRNA^{His}, tRNA^{Asn}, and tRNA^{Asp} from *Escherichia coli* B. Universal presence of nucleoside Q in the first position of the anticodons of these transfer ribonucleic acids, Biochemistry 11 (1972) 301–308.
[4] J.M. Durand, N. Okada, T. Tobe, M. Watarai, I. Fukuda, T. Suzuki, N. Nakata, K. Komatsu, M. Yoshikawa, C. Sasakawa, *VacC*, a virulence-associated chromosomal locus of *Shigella flexneri*, is Homologous to *tgt*, a gene encoding tRNA–Guanine transglycosylase (TGT) of *Escherichia coli* K-12, J. Bacteriol. 176 (1994) 4627–4634.
[5] G. Dirheimer, W. Baranowski, G. Keith, Variations in tRNA modifications, particularly of their queuine content in higher eukaryotes. Its relation to malignancy grading, Biochimie 77 (1995) 99–103.
[6] B. Emmerich, E. Zubrod, H. Weber, P.A. Maubach, H. Kersten, W. Kersten, Relationship of queuine-lacking Transfer RNAs to the grade of malignancy in human leukemias and lymphomas, Cancer Res. 45 (1985) 4308.
[7] A.W. Curnow, G.A. Garcia, tRNA–Guanine transglycosylase from *Escherichia coli* – minimal tRNA structure and sequence requirements for recognition, J. Biol. Chem. 270 (1995) 17264–17267.
[8] S.T. Nonekowski, G.A. Garcia, tRNA recognition by the *E. coli* TGT: the role of U33 in U-G-U sequence recognition, RNA 7 (2001) 1432–1441.
[9] J.K. Hurt, S. Olgen, G.A. Garcia, Site-specific modification of Shigella flexneri virF mRNA by tRNA–Guanine transglycosylase *in vitro*, Nucleic Acids Res. 35 (2007) 4905–4913.
[10] J.S. Mattick, The Functional genomics of non-coding RNA, Science 309 (2004) 1527–1528.
[11] J.S. Mattick, The hidden genetic program of complex organisms, Scientific Am. (2004) 60–67. Oct. 2004.
[12] J.S. Mattick, I.V. Makunin, Non-coding RNA, Hum. Mol. Genet. 15 (2006) R17–R29.
[13] J.E. Squires, H.R. Patel, M. Nousch, T. Sibbritt, D.T. Humphreys, B.J. Parker, C.M. Suter, T. Preiss, Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA, Nucleic Acids Res. 40 (2012) 5023–5033.
[14] Y.C. Chen, A.F. Brooks, D.M. Goodenough-Lashua, J.D. Kittendorf, H.D. Showwalter, G.A. Garcia, Evolution of eukaryal tRNA–Guanine transglycosylase: insight gained from the heterocyclic substrate recognition by the wild-type and mutant human and *Escherichia coli* tRNA–Guanine transglycosylases, Nucleic Acids Res. 39 (2011) 2834–2844.
[15] A.F. Brooks, G.A. Garcia, H.D. Showalter, A Short, Concise Synthesis of Queuine, Tetrahedron Lett. 51 (2010) 4163–4165.
[16] Z.D. Miles, R.M. McCarty, G. Molnar, V. Bandarian, Discovery of epoxyqueuosine (oQ) reductase reveals parallels between halorespiration and tRNA modification, Proc. Nat. Acad. Sci. USA 108 (2011) 7368–7372.