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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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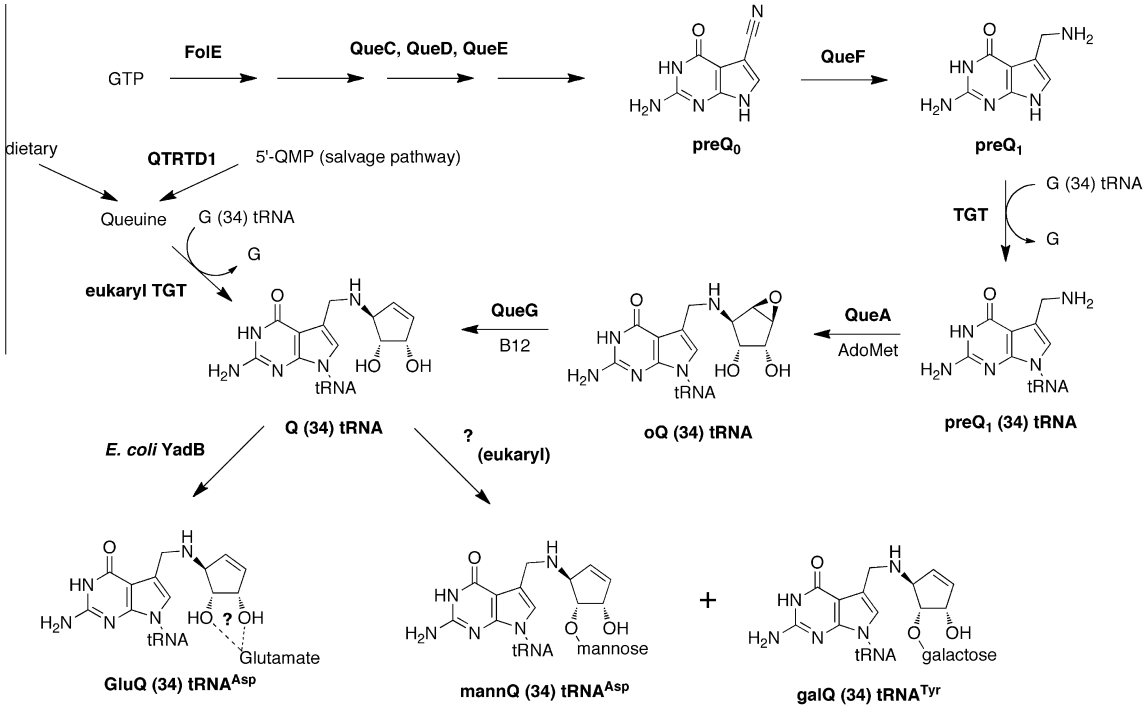

Fig. 1. Queuine biosynthetic pathways for eubacteria and eukarya. G(34) tRNA = tRNA with guanine in the 34 position, Q = queuine and oQ = epoxy-queuine [16].

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