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A synthetic tRNA for EF-Tu mediated selenocysteine incorporation *in vivo* and *in vitro*

Corwin Miller [a,*,1], Markus J. Bröcker [a,1], Laure Prat [a], Kevan Ip [a], Napon Chirathivat [a], Alexander Feiock [a], Miklós Veszprémi [a], Dieter Söll [a,b,*]

[a] Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520, USA
[b] Department of Chemistry, Yale University, New Haven, CT 06520, USA

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ABSTRACT

Incorporation of selenocysteine (Sec) in bacteria requires a UGA codon that is reassigned to Sec by the Sec-specific elongation factor SelB and a conserved mRNA motif (SECIS element). These requirements severely restrict the engineering of selenoproteins. Earlier, a synthetic $tRNA_{Sec}$ was reported that allowed canonical Sec incorporation by EF-Tu; however, serine misincorporation limited its scope. We report a superior $tRNA_{Sec}$ variant ($tRNA_{UTuX}$) that facilitates EF-Tu dependent stoichiometric Sec insertion in response to UAG both *in vivo* in *Escherichia coli* and *in vitro* in a cellfree protein synthesis system. We also demonstrate recoding of several sense codons in a SelB supplemented cell-free system. These advances in Sec incorporation will aid rational design and directed evolution of selenoproteins.

1. Introduction

Organisms pay a high fitness cost for the benefit of endowing proteins with the unique properties of the 21st amino acid, selenocysteine (Sec) [1,2], and have evolved complex biosynthetic and translational mechanisms to incorporate Sec [3,4]. At the interface of Sec synthesis and insertion lies $tRNA^{Sec}$. Initially acylated by seryl-tRNA synthetase (SerRS) to form Ser-$tRNA^{Sec}$, the bacterial enzyme SelA catalyzes the conversion of Ser to Sec in a single step on the tRNA [3]. Once synthesized, selenocysteinyl-tRNA (Sec-$tRNA^{Sec}$) is bound by the specialized Sec-specific elongation factor SelB, which subsequently binds to a highly conserved mRNA motif denoted as Selenocysteine Insertion Sequence (SECIS), facilitating insertion of Sec at a UGA codon [3]. In bacteria, the SECIS sequence is located directly after the suppressed UGA and is thus part of the coding sequence of bacterial genes, making engineering of newly designed selenoproteins very difficult [5–7]. Recently, we reported construction of a synthetic tRNA ($tRNA^{UTu}$) that enabled SECIS-independent and EF-Tu-dependent insertion of Sec in *Escherichia coli* [8]. This $tRNA^{UTu}$ combines the aminoacyl acceptor helix of $tRNA^{Sec}$ with the backbone of $tRNA^{Ser}$, and serves as a substrate for the essential proteins SerRS, SelA, and EF-Tu. By virtue of its interaction with EF-Tu, Sec-$tRNA^{UTu}$ circumvents the need for the Sec-specific elongation factor SelB, and more importantly does not require the SECIS mRNA motif. Sec-$tRNA^{UTu}$ therefore participates in canonical translation, allowing versatile sequence-independent production of designed selenoproteins programmed by UAG.

While SelB recognizes only Sec-$tRNA^{Sec}$ [9,10], EF-Tu serves all other aminoacyl-tRNAs (aa-tRNAs). Therefore, if the SelA-dependent conversion of Ser-$tRNA^{UTu}$ to Sec-$tRNA^{UTu}$ is not complete, Ser will be incorporated instead of the desired Sec residue. This was an impediment in the earlier work in which ~30% misincorporation of Ser was observed [8]. We reasoned that by designing an improved $tRNA^{UTu}$ with better substrate properties for SelA, misincorporation could be prevented. Here we report such a tRNA ($tRNA^{UTuX}$) that allows complete Sec incorporation *in vivo* and *in vitro* in response to UAG.

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* Corresponding authors at: Department of Molecular Biophysics and Biochemistry and Department of Chemistry, Yale University, New Haven, CT 06520, USA (D. Söll).

E-mail addresses: corwin.miller@yale.edu (C. Miller), dieter.soll@yale.edu (D. Söll).

[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. In vitro Sec-tRNA formation

To characterize *in vitro* formation of Sec-tRNA, tRNA species were radiolabeled using [α-^{32}P]ATP and the *E. coli* CCA editing enzyme [11]. Ser-tRNA formation by SerRS, selenophosphate production by SelD, and Ser to Sec conversion by SelA was carried out under anoxic conditions as previously described [8]. Conversion rates were determined by autoradiography and by quantitation of aminoacyl-AMP after thin layer chromatography of nuclease P1 digests of aminoacyl-tRNA^{UTu} [12]. For use in cell free protein synthesis experiments, Sec-tRNA was phenol–chloroform extracted, ethanol precipitated, and resuspended in RNase free H_2O to desired concentration.

2.2. In vivo tRNA^{UTu} utilization assay

E. coli Δ*selA* Δ*selB* Δ*fdhF* strain MH5 was co-transformed with plasmids pACYC-[*E. coli selA*$^+$, *M. jannaschii pstk*] and pGFIB-[tRNA$^{UTu}_{am}$], or pGFIB-[tRNA$^{UTuX}_{am}$] variants as well as pRSF-[*E. coli serS-fdhF*$_{140am}$] and grown on LB medium supplemented with the corresponding antibiotics ampicillin, chloramphenicol, or kanamycin. As a control *E. coli* MH5 was co-transformed with the plasmids pACYC-[*E. coli selA*$^+$, *M. jannaschii pstk*], pRSF-[*E. coli serS-fdhF*$_{140op}$] and pET15b-[*E. coli selB*] to reconstitute the wild-type (WT) Sec formation apparatus using the genomically encoded tRNA^{Sec}. *E. coli* MH5 carrying plasmids pACYC-[*E. coli selA*$^+$ *M. jannaschii pstk*], pRSF-[*E. coli serS-fdhF*$_{140am}$] and pET15b-[*E. coli selB*] served as a second control. Overnight cultures of these clones were plated on LB agar plates supplemented with 10 μM IPTG, 1 μM Na_2MoO_4, 1 μM Na_2SeO_3 and 50 mM sodium formate, as previously

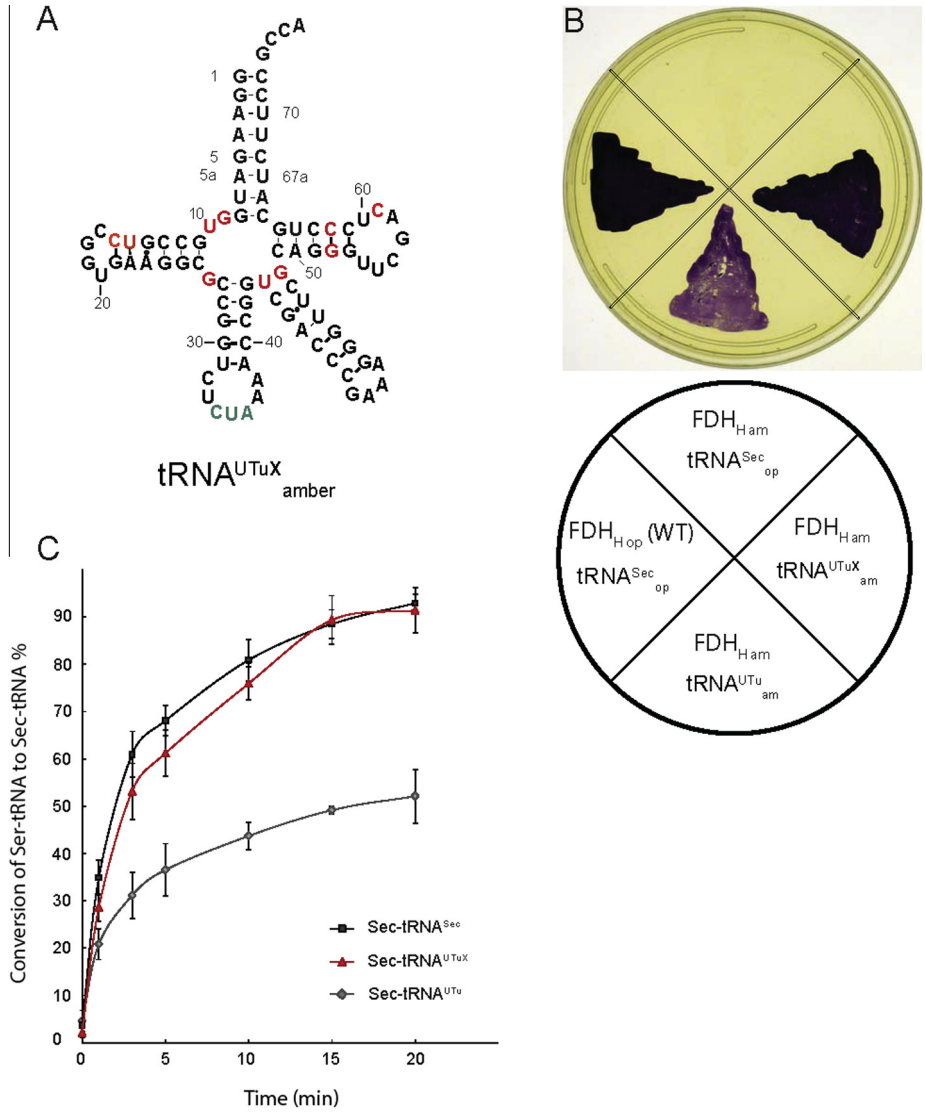

Fig. 1. (A) Secondary structure of tRNA^{UTuX}. Nucleotides that were changed from the original tRNA^{UTu} are highlighted in red and the amber anticodon is depicted in green. (B) tRNA^{UTuX} mediates functional Sec insertion in FDH$_H$. An *E. coli* Δ*selA* Δ*selB* Δ*fdhF* triple deletion strain was separately complemented with *E. coli* SelA, *M. jannaschii* PSTK alongside tRNA$^{Sec}_{op}$, *E. coli* SelB, and WT FDH$_{H140op}$; tRNA$^{UTu}_{am}$ and FDH$_{H140am}$; tRNA$^{UTuX}_{am}$ and FDH$_{H140am}$; and a negative control with tRNA$^{Sec}_{op}$, *E. coli* SelB, and FDH$_{H140am}$. FDH$_H$ activity was assessed by appearance of the purple-colored reduced BV. (C) *In vitro* conversion of Ser-tRNA^{Sec}, Ser-tRNA^{UTu}, and Ser-tRNA^{UTuX} by SelA. Reactions were pre-incubated with 5 μM SelD, 1 mM Na_2SeO_3 and 5 mM ATP at pH 7.2 under anaerobic conditions at 37 °C for 30 min and then supplemented with 1 μM SelA and 10 μM of [α^{32}-P] radiolabeled Ser-tRNA species for up to 20 min. Aliquots of 1.5 μL were taken at the indicated time points, digested with nuclease P1, and spotted onto cellulose thin layer chromatography plates. After developing, plates were analyzed by autoradiography. While approximately 50% of Ser-tRNA^{UTu} was converted, both tRNA^{Sec} and tRNA^{UTuX} support nearly full conversion to Sec-tRNA over a course of 20 min.