Expression of bovine mitochondrial tRNA$^{\text{Ser}}_{\text{GCU}}$ derivatives in *Escherichia coli*

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ABSTRACT

By replacing a stretch of five A–U base pairs in the acceptor stem with G–C pairs, mitochondrial tRNA$^{\text{Ser}}_{\text{GCU}}$ lacking a D arm could be expressed in *Escherichia coli* cells in considerable amounts. The expressed RNA with no modified nucleoside was serylated in *vivo* with the mitochondrial enzyme. The tRNA$^{\text{Ser}}_{\text{GCU}}$ derivatives carrying identity elements for alanine tRNA and the related anticodons were expressed. However, this expression event did not affect cell growth, probably because the expression started from the late log phase, which suggests that these mitochondrial tRNA derivatives are not involved in *E.coli* gene expression systems. Although there are some restrictions in the secondary structure of tRNAs that can be expressed by this method, it could prove useful for preparing large amounts of heterologous tRNAs *in vivo*.

INTRODUCTION

A number of animal mitochondrial (mt) tRNAs exhibit unique structural features: a lack of invariant or semi-invariant sequences such as GG in the D loop or TVCG in the T loop; the presence of many more A–U pairs than standard tRNAs in the stem regions (1,2), and in certain cases, the absence (or truncation) of a D arm or T arm, which appear to be important for maintaining the L-shaped tertiary structure in canonical tRNAs (3–5). Elucidation of the structure–function relationships existing in these non-canonical tRNAs should be valuable, therefore, in improving our understanding of the structural basis of tRNA functions.

However, this work is seriously hampered because only the scanty amounts of mt tRNAs can be prepared from animal mitochondria and it is often difficult to procure suitable organs in sufficient numbers. We therefore decided to attempt the over-production of mt tRNAs in *Escherichia coli* cells as a means of obtaining larger quantities. The wild-type tRNA$^{\text{Ser}}_{\text{GCU}}$ has been shown not to be expressed in *E.coli* (Taupin et al., 15th International tRNA Workshop, Cap d’Agde, May 30–June 4, 1993), apparently because of the conformational instability of the tRNA. We therefore conducted expression experiments with tRNA derivatives possessing a more stable secondary structure. As we previously demonstrated that replacing five A–U pairs in the acceptor stem of mt tRNA$^{\text{Ser}}_{\text{GCU}}$ by G–C pairs did not affect its aminocoylation by homologous seryl-tRNA synthetase (SerRS) (6), we decided to employ this pair replacement strategy as a means of stabilizing the tRNA.

The tRNA derivatives thus constructed were found to be expressed *in vivo* due to the G–C richness in the acceptor stem, which probably causes both correct 3′-processing and stabilization of the tRNA molecules. The strategy employed could prove useful for preparing large amounts of heterologous tRNAs *in vivo*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmid vector pUC19 with the mt tRNA$^{\text{Ser}}_{\text{GCU}}$ gene insert (pT7AU5) was prepared as reported previously (6,7). The plasmid of the derivative (pT7GC5) (for derivative abbreviations, see the legend to Fig. 1) was constructed in the same manner as pT7AU5. The terminator of T7 RNA polymerase was obtained from pET15b (Novagen) and inserted into the BamHI–HindIII sites of each plasmid using T4 DNA ligase (Takara Shuzo). The resulting two plasmids were named pT7AU5T and pT7GC5T, respectively. The plasmids for the other tRNA derivatives described were all constructed and transformed in the same manner as pT7GC5T.

Expression, purification and characterization of tRNA$^{\text{Ser}}_{\text{GCU}}$ and its derivatives

Production of the wild-type and derivative tRNAs was carried out by growing a 100 ml culture of an expressing clone at 28°C in LB medium supplemented with 100 mg/ml ampicillin. When the culture reached 0.5 OD600, expression of the cloned tRNA gene was induced by adding IPTG to a final concentration of 1 mM followed by incubation at 37°C. After 3 h incubation, *E.coli* cells were harvested by centrifugation and resuspended in 10 ml of 4 M guanidium thiocyanate, 25 mM sodium cyanate (pH 7.0), 0.5% sodium lauril sarcocinate and 0.1 M β-mercaptoethanol. The RNA fraction was extracted by phenol treatment followed by ethanol precipitation according to the usual method. tRNA$^{\text{Ser}}_{\text{GCU}}$ was isolated by the selective hybridization method using a solid-phase DNA probe (8). The oligodeoxyribonucleotides (22mer) used for the probes (purchased from Biologica) were 5′-AGCAGTTCTGCTACTTTTTC-3′ and 5′-AGCAGTTCTTGCATACCCGCC-3′, which were complementary to the region extending from the 5′-acceptor stem to the 5′-anticodon stem of the native tRNA$^{\text{Ser}}_{\text{GCU}}$ and tRNA$^{\text{Ser}}_{\text{GC5}}$ genes, respectively.

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Figure 1. Secondary structures of the bovine mt tRNA^Ser\(\text{GCU}\) transcript and its derivatives tRNA^Ser\(\text{GCU}(GC1)\), tRNA^Ser\(\text{GCU}(GC5)\), tRNA^Ser\(\text{GCU}(GC5A)\) and tRNA^Ser\(\text{GCU}(GC5AA)\). Arrows indicate the substitutions made in this study. The region from which the DNA probe (16mer) used in Northern blot analysis was synthesized is identified by a bold line.

The nucleotide sequence of the expressed tRNA was determined by Donis-Keller’s method (9). Nucleoside analysis was carried out using a photodiode-array detector (SPD-M10A, Shimadzu) as described previously (10).

The amino acid acceptor activity of the expressed tRNAs with the \textit{E.coli} enzyme (11) or mt SerRS (6) was assayed as described previously, using a [\(^{14}\text{C}\)]amino acid mixture (1.9 GBq/mg Atom), \textit{l}[-\(^{14}\text{C}\)]alanine (5.6 GBq/mmol) or \[^{14}\text{C}\]serine (5.8 GBq/mmol), all of which were purchased from Amersham.

**Quantitative analysis of mt tRNAs expressed in \textit{E.coli} cells**

RNA fractions extracted from BL21(DE3) cells harboring plasmid pT7AU5T or pT7GC5T at various times after induction were analyzed by electrophoresis on 8% polyacrylamide gel with 7.5 M urea and 20% formamide. After the gel was stained by toluidine blue, each band strength on the gel was monitored by 660 nm light using a densitometer (Beckmann DU, 640).

**Assay of tRNA processing using \textit{in vitro} transcribed tRNA precursors**

All the transcripts used in this study were prepared as described previously (7). tRNA was separated by electrophoresis on 8% polyacrylamide–7 M urea–30% formamide gel in TBE (0.09 M Tris-borate and 2 mM EDTA, pH 7.5) buffer.

pT7AU5 and pT7GC5 linearized by \textit{HindIII} were used as templates for the \textit{in vitro} transcription reactions with T7 RNA polymerase. The transcribed tRNA precursors were purified by electrophoresis on 10% polyacrylamide gel containing 7 M urea and 20% formamide. Each of the precursors contained 35 extra nucleotides at the 3’-end. The condition of the processing assay was as described by Cudnly \textit{et al.} (12). The reaction mixture was run on 8% polyacrylamide gel containing 7.5 M urea and 30% formamide. The S100 fraction for the 3’-processing assay was prepared from RNase I-deficient \textit{E.coli} cells (strain A19) as described previously (13).

**RESULTS**

Expression of mt tRNA genes in constructed plasmids and cell growth

The sequences of bovine mt tRNA\(^{\text{Ser\(\text{GCU}\)}}\) and its derivatives tRNA\(^{\text{Ser\(\text{GCU}(GC1)\)}}\), tRNA\(^{\text{Ser\(\text{GCU}(GC5)\)}}\), tRNA\(^{\text{Ser\(\text{GCU}(GC5A)\)}}\), and tRNA\(^{\text{Ser\(\text{GCU}(GC5AA)\)}}\) [hereafter abbreviated to tRNA\(^{\text{Ser\(\text{GCU}\)}}\), tRNA\(^{\text{Ser\(\text{GCU}(GC1)\)}}\), tRNA\(^{\text{Ser\(\text{GCU}(GC5)\)}}\), respectively] described in this study are shown in Figure 1. Synthetic genes for these mt tRNAs with the IPTG inducible T7 promoter at their 5’-end were constructed (6), and the terminator for the T7 RNA polymerase obtained from pET15b was attached to each at the 3’-end for expression of the tRNA genes \textit{in vivo}. The plasmids were named pT7AU5T for the wild-type tRNA\(^{\text{Ser\(\text{GCU}\)}}\), and pT7GC1T to pT7GC5T for the respective derivatives tRNA\(^{\text{Ser\(\text{GCU}(GC1)\)}}\), tRNA\(^{\text{Ser\(\text{GCU}(GC5)\)}}\).

With plasmids pT7AU5T and pT7GC5T, 4.0 mg tRNA mixture was recovered from 1.2 g \textit{E.coli} cells harboring each plasmid. Although there was no expression of the wild-type mt tRNA\(^{\text{Ser\(\text{GCU}\)}}\) (Fig. 2A), a large amount of tRNA\(^{\text{Ser\(\text{GCU}\)}}\) with seven G–C pairs in the acceptor stem was detected. The position of the product was confirmed by hybridization with the probe described in Figure 1. The results indicated that the product was the actual tRNA size (Fig. 2B). About 0.4 mg of tRNA\(^{\text{Ser\(\text{GCU}\)}}\) expressed in the cells was isolated by the selective hybridization method (8) and its nucleotide sequence was confirmed by
reached 0.5 when the density of the cultures on the growth of BL21(DE3) cells, the relevant genes were induced by addition of IPTG. To determine whether the expressed tRNA possessed amino acid acceptor activity, the aminoacylation of tRNAs with bovine mt SerRS was investigated in vitro. To investigate the effects of plasmids pT7AU5T and pT7GC5T on the growth of BL21(DE3) cells, the relevant genes were induced by addition of IPTG when the cell density (A600) reached 0.5. The mt tRNAs and 5S RNA were quantitatively analyzed with those of SS RNA at various cultivation times after induction (lower). The tRNA gene was induced by adding IPTG to the cells when the cell density (A600) reached 0.5. The mt tRNAs and SS RNA were quantitatively analyzed with those of SS RNA at various cultivation times after induction. The conditions for electrophoresis were the same as those in Figure 2. The gel was stained with toluidine blue.

Donis-Keller’s method (9). HPLC analysis showed no modified nucleoside in the tRNA derivative (data not shown).

To investigate the effects of plasmids pT7AU5T and pT7GC5T on the growth of BL21(DE3) cells, the relevant genes were induced by addition of IPTG when the density of the cultures reached 0.5 A600. Unlike in other tRNA expression systems, induced cells carrying either plasmid continued to grow at the same rate as cells carrying no plasmid (Fig. 3A, curves 1–3).

To determine how long the expressed tRNASer(GC5) could remain in the cells, cells were lysed every 2 h following the induction and tRNA was extracted from the lysate (Fig. 3B). tRNASer(GC5) was found to be expressed 2 h after induction and reached a maximum at 4 h. Thereafter, the expression gradually decreased. This behavior is illustrated quantitatively by curve a in Figure 3A. No wild-type tRNA was produced in E.coli at any time (data not shown).

In vitro aminoacylation of tRNAs with bovine mt SerRS and E.coli S100

To determine whether the expressed tRNA possessed amino acid acceptor activity, the KM values for serylation were measured for tRNASer(GC5), as well as for the wild-type tRNASer as a reference. As shown in Table 1, the tRNASer(GC5) expressed in vivo could be serylated with mt SerRS in a similar way to tRNASer(GC5) that was transcribed in vitro. The KM values for both of these tRNAs were several times higher than that of the wild-type tRNASer (6,14). In contrast, none of these tRNAs was aminoacylated with E.coli cell extracts (S100) (15). It was thus evident that the mt tRNASer derivative was processed correctly, but not serylated in E.coli cells.

Table 1. KM values for aminoacylation reactions of various mt tRNASer(GC5) derivatives with bovine mt SerRS and E.coli S100

<table>
<thead>
<tr>
<th>tRNA derivative</th>
<th>KM (µM) for serylation with mt SerRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine mt tRNASer(GC5) isolated from mitochondria</td>
<td>0.179</td>
</tr>
<tr>
<td>Bovine mt tRNASer(GC5) transcribed in vitro</td>
<td>0.189</td>
</tr>
<tr>
<td>tRNASer(GC5) transcribed in vitro</td>
<td>1.04</td>
</tr>
<tr>
<td>tRNASer(GC5AA) transcribed in vitro</td>
<td>1.07</td>
</tr>
<tr>
<td>tRNA derivative</td>
<td>KM (µM) for serylation with E.coli S100</td>
</tr>
<tr>
<td>tRNASer(GC5A)</td>
<td>6.1</td>
</tr>
<tr>
<td>tRNASer(GC5AA)</td>
<td>6.2</td>
</tr>
<tr>
<td>Escherichia coli tRNAAla</td>
<td>2.1</td>
</tr>
<tr>
<td>Escherichia coli tRNAAla minihelix</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Each parameter was determined from a Lineweaver–Burk plot.

a Ueda et al. (1992) (6).


Processing of in vitro transcribed tRNA precursors in E.coli cell extracts

To elucidate the relationship between the structure and the processing capability of mt tRNAs, mt tRNASer precursors for the wild-type and tRNASer(GC5), both of which contained 35 extra nucleotides in their 3′ termini, were incubated with E.coli S100 (Fig. 4). A major product band corresponding to the mature tRNA was observed only in the case of the tRNA derivative. On the other hand, the wild-type tRNA precursor was hardly processed to the mature tRNA, and was degraded within 60 min. This observation strongly suggests that 3′-processing enzymes require a substrate tRNA possessing a G–C-rich acceptor stem.

The melting temperature of tRNASer was 57.5°C in the presence of Mg2+ (14,16), whereas that of tRNASer(GC5) was 62.5°C. Thus, G–C richness in the acceptor stem seems to increase the stability of the expressed tRNAs, which in turn influences the 3′-processing capability of mt tRNASer precursors in E.coli cells.

Expression in E.coli cells of tRNASer derivatives possessing different numbers of G–C pairs in the acceptor stem

To determine the minimum number of G–C pairs necessary for the 3′-processing of tRNASer derivatives, plasmids pT7GC1T, pT7GC2T, pT7GC3T and pT7GC4T were constructed in which the A–U pairs in the acceptor stem were replaced by one to four G–C pairs (Fig. 1) and introduced into E.coli BL21(DE3) cells in which the expression of the tRNA derivatives was examined (Fig. 5). All the tRNASer derivatives except for tRNASer(GC1) were found to be expressed in the cells. Since the amount of expressed tRNA increased exponentially with the number of G–C pairs, it is very likely that at least three G–C pairs at the top of the...
Experiments were therefore planned to determine whether cell Northern hybridization analysis (data not shown) clearly demon-
named tRNA Ser (GC5AA) (Fig. 1).

The tRNA Ser (GC5A) was changed to the alanine anticodon GGC was
Ser (GC5) gene in which an alanine identity element (G3–U70 in
derivatives in
′
acceptor stem are necessary for the 3′-processing of tRNASer
derivatives in E.coli cells.

Growth of cells carrying mt tRNA derivatives with alanine
identity

Since the identity elements of E.coli tRNAAla are known to be
only the G3–U70 base pair and A73 (17,18), we considered it
possible that the introduction of alanine identity elements into the
derivatives of mt tRNAs expressed in E.coli cells would enable the
tRNAs to be charged with alanine even in E.coli cells.

Experiments were therefore planned to determine whether cell
growth was influenced by plasmids containing the mt tRNA-
Ser(GC5) gene in which an alanine identity element (G3–U70 in
the standard numbering) was introduced and the anticodon was
replaced by one for alanine or amber suppressor tRNA.

tRNArep(GC5A) is a tRNArep(GC5) derivative in which the
base pair A3–U57 (U70 in the standard numbering) and the discriminator base G60 (G73 in the standard numbering) were
replaced by G3–U57 and A60, respectively, and the serine
anticodon GCU was replaced by the suppressor anticodon CUA.
Another tRNArep(GC5) derivative in which the anticodon of
tRNArep(GC5A) was changed to the alanine anticodon GGC was
named tRNArep(GC5AA) (Fig. 1).

No inhibition of cell growth was observed in E.coli cells
harboring either of the plasmids carrying genes for the derivatives

tRNArep(GC5A) and tRNArep(GC5AA), even 8 h after induction
(Fig. 3A), although staining with toluidine blue (Fig. 3B) and
Northern hybridization analysis (data not shown) clearly demon-

Figure 4. Time course of 3′-processing of precursors for tRNASerGCU and its
derivative in E.coli S100. 5′-labeled tRNA precursors were incubated with
E.coli S100 for 15 (lanes 2 and 3), 60 (lanes 3 and 8) and 120 min (lanes 4 and
9) and the resultant products were electrophoresed on 8% polyacrylamide gel
with 7.5 M urea and 30% formamide. Lanes 1 and 6, radioactive precursors of
tRNArepGCU and tRNArep(GC5) (97 nt long) prepared by T7 RNA polymerase
from plasmids pT7AU5T and pT7GC5T, respectively; lanes 5 and 10,
radioactive tRNArepGCU and tRNArep(GC5) prepared by T7 RNA polymerase
from plasmids pT7AU5T and pT7GC5T, respectively.

The expressed tRNAdep(GC5A) and tRNAdep(GC5AA) could
both be charged with alanine by E.coli S100 in vitro (Table 1, lower),
and it was confirmed by acid polyacrylamide gel electrophoresis (19)
that both of the expressed tRNAs were, in fact, alanylated in
E.coli cells (data not shown).

DISCUSSION

There have been several reports on the expression in E.coli cells
of homologous (20–24) and heterologous tRNAs (25), as well as
one on a minihelix derived from E.coli tRNAgly (26). However,
these tRNAs had a normal structure or formed part of the E.coli
tRNA. In the present work, we found that derivatives of bovine
mitochondrial tRNAdepGCU lacking a D arm could be produced in
E.coli cells by introducing a stretch of G–C base pairs into the
acceptor stem of the tRNA gene loaded onto the expression vector
plasmid, and that the expressed tRNAs were chargeable with mt
seryl-tRNA synthetase.

Four conditions need to be met for a heterologous tRNA gene
to be expressed in E.coli BL21(DE3) cells: (i) a plasmid containing
the tRNA gene should be efficiently transcribed in vivo by T7 RNA
polymerase, (ii) the expressed precursor should be recognized by
processing enzymes in order to be converted to the mature tRNA,
and (iv) the tRNA should be stable enough to remain in the cells.

Both the plasmid carrying the wild-type tRNA gene (tRNA Ser
mitochondrial) and (iv) the tRNA should be stable enough to remain in the cells.

The expressed tRNAdep(GC5) and tRNAdep(GC5AA) were
separately transcribed and translated in vivo (Table 1, lower),
and it was confirmed by acid polyacrylamide gel electrophoresis (19)
that both of the expressed tRNAs were, in fact, alanylated in
E.coli cells (data not shown).

stated that these tRNAs were actually produced in the E.coli cells.

The cell growth and amounts of the tRNAs expressed were
approximately the same as those for tRNAdep(GC5), which
possessed no alanine identity element.

Figure 3A shows the relationship between cell growth and
expression of tRNAdep(GC5) and tRNAdep(GC5AA) as monitored by the ratio of tRNA versus 5S RNA in cells harboring pT7GC5T
and pT7GC5AAAT, respectively. Both tRNAs started to be
expressed >2 h after induction, when the cell growth of both
strains had almost reached the stationary phase, and the expression
reached maximum in the mid-stationary phase. This observation
clarifies why heterologous mt tRNAs expressed in E.coli cells
have no appreciable influence on cell growth.

The expressed tRNAdep(GC5A) and tRNAdep(GC5AA) could
both be charged with alanine by E.coli S100 in vitro (Table 1, lower),
and it was confirmed by acid polyacrylamide gel electrophoresis (19)
that both of the expressed tRNAs were, in fact, alanylated in
E.coli cells (data not shown).
demonstrated that even mt tRNA precursors having unusual secondary structures could be processed normally. The in vivo 3’ processing assay also showed that while depletion of the D arm did not influence the processing, some G–C pairs in the acceptor stem were indispensable. It was also found that the more G–C pairs there are in the acceptor stem, the more efficiently the tRNA is expressed (Fig. 5), which is probably related to the stability of the tRNA.

The expressed tRNA<sup>ser</sup>(G GS5) was found to be chargeable in vitro with mt SerRS, but not with <i>E. coli</i> S100, which is consistent with the findings of our previous work (15). However, once the identity determinant for <i>E. coli</i> expressed in <i>E. coli</i> cells are folded into the active form in their tertiary structures, as is the case with canonical tRNAs.

It is intriguing that cell growth was never influenced by the expression of the mt tRNAs, irrespective of their charging activity with <i>E. coli</i> S100. This behavior differs from that of expressed mimihelcines derived from <i>E. coli</i> tRNA<sup>Gly</sup>, which compete with tRNA<sup>Gly</sup> for glycine charging and cause inhibition of cell growth (26). The reason may lie in the fact that both mt tRNA<sup>ser</sup>(G GS5) and tRNA<sup>ser</sup>(G GS5AA) start to be expressed in <i>E. coli</i> cells only from the very late log phase (almost the stationary phase) (Fig. 3A), which suggests that there may be some unknown mechanism that delays the expression of heterologous tRNA genes until the cells have nearly reached the stationary phase so that the expressed tRNAs do not hinder the <i>E. coli</i> gene expression systems, including the translation system. Further studies are needed to reveal this postulated mechanism.

It has already been determined that although seryl-tRNA<sup>ser</sup>(GCU) of bovine mitochondria is able to bind to bacterial EF-Tu to form a ternary complex with GTP (28), it cannot be transferred to the ribosomal A site (29; Y. Okogawa et al., unpublished data). Therefore, it is evident that even when alanyl-tRNA<sup>ser</sup>(GCS5) or alanyl-tRNA<sup>ser</sup>(G GS5AA) is present in <i>E. coli</i> cells (which has been shown to be), the tRNA would be non-functional in the <i>E. coli</i> translation system.

The system for the heterologous expression of tRNAs in <i>E. coli</i> cells described here may be applicable to various fields of study. As the expressed tRNAs are not modified, they may be good substrates for tRNA-modification enzymes. Furthermore, since even non-canonical tRNAs lacking a D arm could be expressed in vivo, the system may be a useful means of providing various kinds of tRNA substrates for tRNA-processing enzymes, especially 3’-processing enzymes, which have so far been little studied in comparison with 5’-processing enzymes (30,31). It could also provide a powerful method for preparing larger amounts of RNA samples, especially for use in structural analysis such as by X-ray crystallography or NMR spectroscopy. Though the system has some limitations in terms of the RNA structures that can be obtained, a sufficient amount of RNA (~0.5 mg tRNA per 1 g cells) can be recovered. It should be also noted that since the system works in minimal medium, it could be a useful method for preparing many kinds of labeled RNA molecules.

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