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Minimal translation of the tmRNA tag-coding region is required for ribosome release

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Abstract

The trans-translation system in bacteria promotes recycling of stalled ribosomes and targets incomplete peptides for proteolysis. In *Escherichia coli*, loss of trans-translation function has little effect on growth under normal laboratory conditions. Among the subtle phenotypes of tmRNA-deficient mutants is the inability to plate certain λ*imm*^{P22} phages. This phenotype is dependent on the ribosome recycling functions of the trans-translation system but is independent of its proteolysis-targeting activity. The experiments described here show that translation of the first (resume) codon of the tmRNA open reading frame by a tRNA is both necessary and sufficient for ribosome recycling. While a variety of sense codons can replace the naturally-occurring GCA alanine codon as the resume codon, both AAA and AAG lysine codons are non-functional resume codons. These results suggest that the main function of tmRNA in releasing stalled ribosomes is to supply a stop codon and so facilitate termination and subsequent ribosome recycling.
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The trans-translation system found in bacteria and some organelles serves several cellular functions. The first is to target for proteolysis, any incomplete polypeptides generated by translation of truncated mRNAs lacking stop codons [1]. This is achieved by recruitment of a complex containing EF-Tu·GTP, SmpB, Ala-tmRNA, and ribosomal protein S1 to a ribosome stalled on a truncated mRNA lacking stop codons [2]. Here, tmRNA acts first as an acceptor tRNA in a standard peptidyltransferase reaction. In a second step, tmRNA assumes the role of an mRNA and the ribosome switches templates from the truncated mRNA to an open reading frame (orf) found within tmRNA. Translation of this reading frame results in the addition of a peptide tag (ANDENYALAA in *Escherichia coli*) onto the incomplete polypeptide which is subsequently recognized and degraded by a variety of proteases [3].

Proteins carrying tmRNA-encoded tags are unstable and visualization of tagged proteins usually requires the use of protease-deficient strains, or the use of a tmRNA encoding an altered, proteolysis-resistant tag. A second function for tmRNA has been uncovered through the use of tmRNAs encoding such protease-resistant tags. In *E. coli*, tmRNA is dispensable for normal cell growth but is required for propagation of hybrid λ*imm*^{P22} phages, induction of Mu lysogens and insensitivity to low levels of antibiotics [4–6]. A mutant tmRNA that converts the two C-terminal alanine codons of the tag sequence to aspartic acid codons (the DD mutant) is protease-resistant and supports λ*imm*^{P22} growth and induction of a Mu Cts prophage as effectively as wild-type tmRNA, while sensitivity to antibiotics is only slightly increased [4–6]. Moreover, in *Neisseria gonorrhoeae* where trans-translation function is essential for viability, either of two different tmRNA mutants encoding protease-resistant tags support cell growth and viability at levels indistinguishable from wild-type tmRNA [7]. The molecular basis of the phage plating defects in tmRNA-deficient cells is unknown, although the

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λ*imm*^{P22} phenotype requires the P22 CI protein [4]. The phenotype nonetheless has provided a convenient means of distinguishing between proteolysis-targeting and ribosome releasing functions of the trans-translation system. Together, these results suggest that tmRNA has a second function that is independent of its role in targeting proteins for proteolysis. Such a function is usually interpreted in terms of tmRNA's ability to release stalled ribosomes and promote their recycling. Both protease-targeting and ribosome release functions of tmRNA require an aminoacylated tmRNA [4,8]. Northern blotting analyses have shown that the truncated mRNA onto which tmRNA is initially recruited is invariably degraded during the process of trans-translation [9] and tmRNA appears to play an active role in this process [10]. Thus, a third function of tmRNA is to facilitate decay of the truncated non-stop mRNA.

tmRNA encoding protease resistant tags have been used in a large number of mutagenesis studies [11–13]. While some of these mutant tmRNAs have also been shown to support the ribosome release function, the existence of any differential sequence requirements for tagging *vs*. ribosome release functions and the range of tag sequences that can support ribosome release is currently unclear. The tmRNA encoded tag is ten amino acids long in *E. coli* but can be up to thirty five residues long in certain Mycoplasma species [14]. The recent discovery of tmRNA-like molecules in the mitochondria of several jakobids, apparently lacking any tag-encoding mRNA region has led to the suggestion that these highly reduced tmRNAs might still retain the ribosome releasing function of conventional tmRNAs, but lack tagging activity [15]. If these tmRNAs are indeed functional in ribosome release, this would suggest that translation of tmRNA might not be required for ribosome release functions.

This study addresses the tag sequence requirements for the ribosome release function of tmRNA in *E. coli*. Two tmRNA mutants in which the resume codon is replaced with termination triplets do not support ribosome release. However, mutants containing minimal, single-codon orfs are fully functional. It is concluded that translation of a sense codon at the resume position is essential for ribosome release and that the main function of the tag orf in ribosome release may be to provide a stalled ribosome with the means of accessing a stop codon, by supplying a termination triplet in *trans*.

Experimental procedures

Materials

Strains, plasmids, and phages. A strain carrying a Δ*ssrA::Kan* deletion of the tmRNA coding region was provided by Dr. Alan Herr, University of Utah. P1 transduction was used to move this deletion mutation into MC85 (*F⁻ thi-Δ(lac-pro) trpE91*) [16] to give MC288. A derivative of MC85 that maintains plasmids at low copy number was constructed by transducing the *pcnB8* mutation from strain MRi93 [17] into MC85 to give MC120. The Δ*ssrA::Kan* deletion was then introduced into MC120 to give strain MC289. The suppressor-free strain, CSH108 (F' *lacIZ, proAB, araΔ(gpt-lac)5 gyrA argE$_{am}$ rpoB*) as well as the tRNA suppressor-containing strains CSH112 (*F⁻, araΔ(gpt-lac)5 gyrA argE$_{am}$ rpoB metB supB*) and CSH113 (*F⁻, araΔ(gpt-lac)5 gyrA argE$_{am}$ rpoB metB supC*) were obtained from Cold Spring Harbor Laboratories [18]. The *ΔssrA::Kan* mutation was then transduced into each strain resistance to give MC302, MC304, and MC305, respectively.

All tmRNA mutants were constructed on plasmids and introduced *via* transformation into strains carrying a deletion of the chromosomal *ssrA*$^{+}$ gene, encoding wild-type tmRNA. Mutants were typically expressed at low gene dosage in the *pcnB8* strain MC289, to avoid complications arising from over-expression of mutant tmRNAs.

The λ*imm*^{P22} hybrid phages, *dis* and *hy25* were obtained from the laboratory of Dr. David Friedman, University of Michigan [4]. All tmRNA mutants were tested for their ability to plate λ*imm*^{P22} *hy25* and selected mutants were also examined for their effects on λ*imm*^{P22} *dis* propagation. No differences in phage yield were observed between the two hybrid phages.

Methods

Mutagenesis. Site directed mutagenesis of tmRNA was carried out by the megaprimer PCR method [19] using MC85 chromosomal DNA as a template, a single mutagenic primer and upstream and downstream flanking primers. The primers upstream and downstream of the native *ssrA* promoter and terminator carried recognition sites for *Eco*RI and *Hin*dIII, respectively. The mutant 600 bp PCR fragments were digested with *Eco*RI and *Hin*dIII and ligated to double-digested plasmid pTZ19u (USB Corporation, Cleveland OH). A plasmid expressing wild-type tmRNA was constructed by amplifying the *ssrA* coding and control regions from MC85 with just the upstream and downstream primers. The structure of the mutant plasmids was verified by nucleotide sequencing. Standard DNA manipulation, cloning and transformation procedures were carried out as described [20].

Growth of phage. The efficiency of plating (EOP) of λ*imm*^{P22} phages on tmRNA mutants was calculated by comparing plaque formation on lawns of the mutant of interest with that on an isogenic *ssrA*$^{+}$ strain, as described [4]. All phage titers were verified in duplicate experiments.

Results and discussion

tmRNA tag mutants

During the process of trans-translation, the ribosome switches mRNA templates from a truncated mRNA, to the mRNA domain of tmRNA. Translation on tmRNA resumes at a conserved GCA codon, the so-called resume codon. Previous mutagenesis experiments showed that a stop codon at this position was non-functional in a tagging assay [11]. We have reconstructed this resume codon mutant (UAA-1, Fig. 1) and tested its ability to support growth of λ*imm*^{P22} phage. The results presented in Table 1 show that the UAA-1 mutant is completely ineffective in this tmRNA function and does not differ in this respect from a complete deletion of the tmRNA coding region. A further tag mutant (UAA-2) was constructed which left the resume codon intact but changed the second codon of the tag orf to a stop codon. This was achieved by inserting a U residue immediately after the GCA resume codon, generating a single codon (GCA) orf, followed by an in-frame UAA triplet. The UAA-2 tmRNA mutant supports λ*imm*^{P22} phage growth as well as a wild-type strain

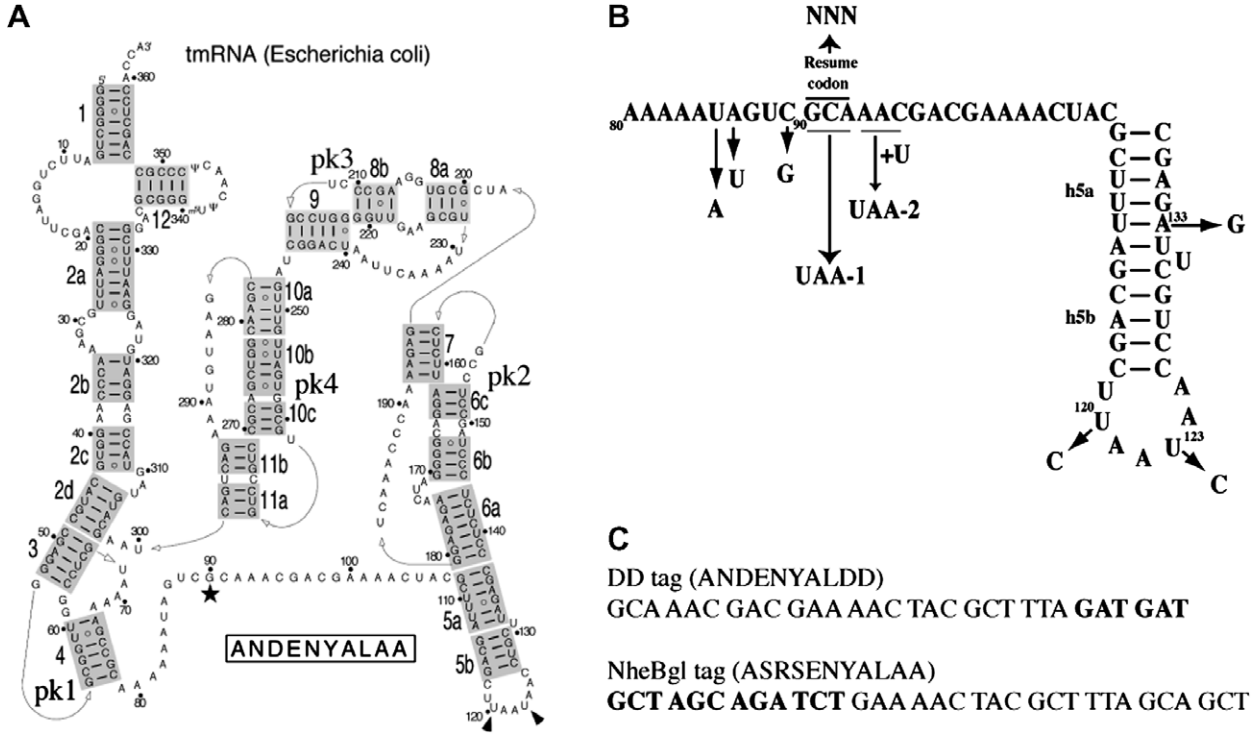

Fig. 1. Structure of tmRNA and the location of mutants analyzed in this study. (A) The secondary structure of *E. coli* tmRNA [28]. An asterisk (*) marks the resume codon and the sequence of the ten-residue tag sequence is indicated underneath (boxed). (B) The mutants constructed in the mRNA-like domain. (C) The nucleotide and amino acid sequence of the DD and *NheBgl* tag mutants. Altered codons are indicated in bold.

Table 1
Effects of tmRNA mutations on growth of λimm^{P22} *hy25* phage

Mutant	Feature	EOP
Wild-type	Wild-type tmRNA	1.0
DD	Altered tag sequence	0.8
U85A	Alteration of base upstream of resume codon	0.7
A86U	Alteration of base upstream of resume codon	2.6×10^{-5}
C89G	Alteration of base upstream of resume codon	0.8
UAA-1	Resume codon changed to UAA stop codon	1.3×10^{-5}
UAA-2	Codon following resume codon changed to stop codon	0.9
NheBgl	Codons 1–3 of tag altered	0.2
U120C/U123C	Tandem UAA UAA codons at end of tag changed to CAA CAA, extends tag-coding orf	0.7
U120C/U123C/A133G	Tandem UAA UAA codons at end of tag changed to CAA CAA combined with A133G, extends tag-coding orf	0.8
U357C	Acceptor stem mutant, prevents charging	1.7×10^{-5}
*Δ*ssrA::Kan	Complete deletion of tmRNA coding region	4.6×10^{-5}

Wild-type and mutant tmRNAs were expressed from plasmid-borne *ssrA* genes at low gene dosage, in the *ΔssrA::Kan pcnB*$^-$ strain, MC289. The efficiencies of plating (EOP) of the indicated mutants were obtained by comparing plaque formation on lawns of mutant strains to that of an *ssrA*$^+$ strain. Numbers represent the averages of at least duplicate experiments.

(Table 1). A similar, tagging-proficient mutant carrying both an altered resume codon and an adjacent stop codon (UCA UAA) was constructed by Trimble et al. [21]. However, in this double mutant, the UCA-decoding serine tRNA reading the resume codon can re-pair with a UCU codon in the −2 reading frame with high efficiency. In the UAA-2 mutant constructed here, the potential for re-phasing of the tmRNA reading frame is absent, as there are no adjacent or overlapping GCN codons. These results suggest that a minimal open reading frame, consisting of just a single codon is necessary and sufficient for the ribosome release functions of tmRNA.

Further support for the conclusion that translation of the resume codon by a tRNA is necessary for trans-translation comes from the observation that the function of a tmRNA mutant with a UAA resume codon is restored in strain MC304 which carries the UAA-decoding suppressor tRNA, *supB*. In the suppressor-free strain MC302, expressing the UAA resume codon mutant tmRNA, λimm^{P22} phage plates at an efficiency of 2.3×10^{-5}. However, in

the isogenic *supB* strain MC304, the same UAA resume codon mutant now plates λ*imm*^{P22} phage with an efficiency of 0.2. Surprisingly, however, the UAA suppressor *supC*, which like *supB* decodes both UAA and UAG stop codons, fails to suppress the UAA resume codon mutant. Potentially, reading of a UAA stop codon by the *supC* tRNA, or competition between the suppressor tRNA and release factors may differ between mRNA and tmRNA resume codon templates, resulting in poor decoding of UAA by *supC*.

Two mutants that were previously shown to prevent tagging of a reporter protein also prevent ribosome release; an acceptor stem mutant (U357C) which prevents tmRNA aminoacylation [4,8] as well as the A86U mutation upstream of the resume codon (Fig. 1) [22] are both also non-functional in ribosome release. Conversely, the U85A and C89G mutations upstream of the resume codon [22] do not affect ribosome release functions (Table 1).

Resume codon mutants

The finding that a sense codon at the resume position is essential for the ribosome release function of tmRNA raised the question of whether all sense codons, or just a restricted set were functional resume codons. Previous experiments had demonstrated that at least 12 different sense codons were functional as resume codons in tmRNA-directed tagging [11]. Using an oligonucleotide containing all four possible bases at the resume codon positions, a library of tmRNA fragments was constructed in plasmid pTZ19u and introduced into strain MC289. The transformants were screened for their ability to plate λ*imm*^{P22} phage and selected tmRNA mutants were sequenced. These experiments revealed that mutants with AAU, AUU, ACU, GGU, GGG, GAG, GUU, GUG, UGG, UAU, UAC, UGU, CCU, CAA, and CUG triplets at the resume codon showed only minor differences from wild-type tmRNA in their ability to support phage growth (Fig. 2). Three non functional mutants were also recovered. Sequencing of the tmRNA coding regions in these mutant plasmids showed that the resume codon was altered to AAA or AAG lysine codons or a UAG stop codon (Fig. 2). All three mutants were still non-functional when expressed at high gene dosage in a *pcnB*$^{+}$ strain (data not shown). These experiments confirm the requirement for a sense codon at the resume position and show that many sense codons are functional resume codons. However, both lysine codons are non-functional. Alignment of the tag peptides of bacterial tmRNAs shows that while an alanine codon is overwhelmingly the most common triplet found at the resume position, glycine codons are also present in several tmRNAs [14]. Other codons are much less common. Significantly, no examples of lysine codons at the resume position have yet been reported. In *E. coli*, a single species of tRNA^{Lys} decodes both AAG and AAA lysine codons. This tRNA carries several post-transcriptional modifications and an unusual anticodon structure that may contribute to its ability to shift reading frames at high frequency in response to adjacent stimulator elements [23]. Potentially, the unique features of this tRNA that contribute to its propensity to shift reading frames are also are responsible for its reduced function at the resume codon.

tmRNA mutants with extended tag orfs

The finding that tmRNAs expressing minimal orfs can still support the ribosome releasing function prompted an investigation of the functionality of tmRNAs with extended tags. Such extended tags were constructed by removing successive, in-frame stop codons and replacing them with sense codons. The mRNA-like region of wild-type tmRNA contains two tandem UAA stop codons

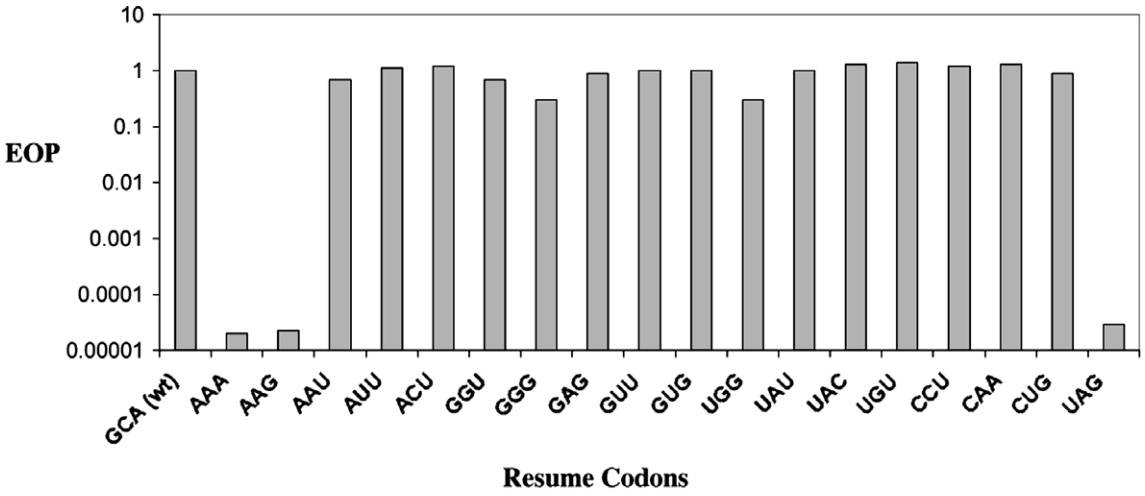

Fig. 2. Effects of the identity of the resume codon on growth of λ*imm*^{P22} *hy25* phage. Phages were grown on strains expressing tmRNAs with wild-type or mutant resume codons. Numbers represent the averages of at least duplicate assays. The plating efficiency of phage (EOP) on mutant strains is expressed relative to growth on a wild-type *ssrA* strain, which is assigned a value of 1.0.

which define the 3′ end of the tag orf (Fig. 1). Alteration of these stop codons to CAA glutamine codons extends the open reading frame by four codons to the UAG stop codon at nucleotides 132–134. Strains expressing this mutant tmRNA (U120C/U123C; Fig. 1) support λ*imm*^{P22} phage growth at wild-type efficiencies (Table 1). When the U120C/U123C mutant contains an additional A133G mutation that changes the UAG stop codon to a UGG sense codon, translation of the tag orf is extended by a further 30 codons and is now predicted to terminate at the UGA codon at nt 222–224 (Fig. 1). Again, strains expressing this mutant (U120C/U123C, A133G) plated λ*imm*^{P22} phage at wild-type efficiencies. Wower et al. [24] have described the construction of similar, extended-tag mutants and have reported that these mutations reduce tagging of a reporter protein. The difference between the results of Wower et al. and the data presented here most likely derives from differences in the assay systems used by both groups. Growth of λ*imm*^{P22} phage may require a relatively low threshold level of tmRNA activity, while the *in vivo* tagging assay of Wower et al. [24] is a quantitative assay, sensitive to even modest decreases in tmRNA function, that may not be detected in the λ*imm*^{P22} plating assay. Substitutions at the N- and C-terminal ends of the tag sequence are also well tolerated: Alteration of the terminal two alanine residues of the tmRNA tag to aspartate (the DD mutant) yields a tmRNA that can tag polypeptides with a protease resistant tag and also support ribosome release (Ref. [4] and Table 1). A tag mutant was constructed (*NheBgl*) which resulted in the introduction of adjacent *Bgl*II and *Nhe*I restriction sites into the coding sequence and changed the first four codons of the tmRNA tag from ANDE to ASRSE (Fig. 1). Nonetheless, the NheBgl mutant plated λ*imm*^{P22} phage at wild-type efficiency (Table 1). Together, these experiments show that tmRNAs carrying a variety of changes at the N- and C-terminal regions of the tag sequence are functional in ribosome release.

tmRNA tag functions required for ribosome release

The main conclusion from this work is that ribosome release activity of the trans-translation system requires at least some translation of the tmRNA orf. The experiments presented here, together with previous work thus show that two tmRNA functions, tagging and ribosome release, both require translation of the mRNA-like domain of tmRNA. Moreover, both tmRNA functions are also similarly affected by mutations at position C86 upstream of the tag orf, or by mutations in the tRNA-like domain that prevent charging [4,8,22]. The distinction between tagging and ribosome release functions may lie largely in the nature of the tmRNA-encoded tag: Targeting of peptides for proteolysis requires the addition of a specific tag, while ribosome release has much less stringent requirements on the length and identity of the tag sequence. A minimal orf, consisting of a single codon is sufficient for the ribosome release function of tmRNA. However, tmRNAs that carry stop codons at the resume position cannot support either ribosome release (Table 1) or tagging functions [11].

In wild-type cells, upon recruitment of the trans-translation system by ribosomes stalled on mRNAs lacking stop codons, translation of the tmRNA orf not only targets polypeptides for proteolysis but also provides a termination signal at the end of the tmRNA orf (Fig. 1). After translation and termination on the tmRNA orf, post-termination complexes, consisting of a 70S ribosome with a deacylated tRNA bound to the mRNA-like domain of tmRNA, can then recruit the ribosome recycling factor (RRF), EF-G·GTP and IF-3. Together, these factors facilitate release of both the deacylated tRNA and the tmRNA (in its role as the mRNA template) and promote dissociation of the ribosomal subunits [25]. Thus, by providing a stalled ribosome with the means of terminating, tmRNA also allows these ribosomes to proceed to the subsequent ribosome recycling steps. The recycling factor, RRF has a stringent requirement for a deacylated tRNA in the P site [25]. In tmRNA mutants that contain a termination triplet at the resume position, the corresponding post-termination complex carries deacylated tmRNA in the P site. Although tmRNA carries a tRNA-like domain, its structure is clearly distinct from that of a canonical tRNA [26] and critically, the tRNA-like domain does not engage in codon-anticodon interaction. Consequently, deacylated tmRNA is likely a poor substrate for RRF and thus, terminated ribosomes carrying tmRNAs with nonsense codons at the resume position may be poorly recycled.

The relative importance of ribosome releasing *vs*. proteolysis-targeting functions of tmRNA differs between organisms: While only the ribosome releasing activity of tmRNA is required for complementation of the phenotypes associated with loss of trans-translation function in *E. coli* and *Neisseria*, the proteolysis-targeting function is required for complementation of tmRNA-defective phenotypes in *Caulobacter crescentus* [27]. The work presented here indicates that both functions require a functional orf.

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