Contributions of Pseudoknots and Protein SmpB to the Structure and Function of tmRNA in \textit{trans}-Translation*

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Bacteria contain transfer-messenger RNA (tmRNA), a molecule that during \textit{trans}-translation tags incompletely translated proteins with a small peptide to signal the proteolytic destruction of defective polypeptides. TmRNA is composed of tRNA- and mRNA-like domains connected by several pseudoknots. Using truncated ribosomal protein L27 as a reporter for tagging \textit{in vitro} and \textit{in vivo}, we have developed exceptionally sensitive assays to study the role of \textit{Escherichia coli} tmRNA in \textit{trans}-translation. Site-directed mutagenesis experiments showed that pseudoknot 2 and the abutting helix 5 were particularly important for the binding of ribosomal protein S1 to tmRNA. Pseudoknot 4 not only facilitated tmRNA maturation but also promoted tagging. In addition, the three pseudoknots (pk2 to pk4) were shown to play a significant role in the proper folding of the tRNA-like domain. Protein SmpB enhanced tmRNA processing, suggesting a new role for SmpB in \textit{trans}-translation. Taken together, these results provide unanticipated insights into the functions of the pseudoknots and protein SmpB during tmRNA folding, maturation, and protein synthesis.

An interruption of the elongation step of protein synthesis results in the production of truncated proteins and leaves ribosomes stalled at the 3’ end of mRNA templates lacking a stop codon(s). To remove the defective polypeptides and recycle the ribosomes, bacteria have developed \textit{trans}-translation, a quality control mechanism that tags the C termini of defective polypeptides with a short peptide recognized by housekeeping proteases. This peptide tag is encoded by a short open reading frame in the mRNA-like domain (MLD). The TLD and the MLD are connected by a pseudoknot-rich domain consisting of four pseudoknots (pk1 to pk4) in most tmRNAs.

The three-dimensional model of \textit{E. coli} tmRNA suggests a structure in which the TLD is connected to the circularly arranged MLD and pseudoknots through coaxially stacked helices (6). Recently, the entry of tmRNA into a stalled \textit{E. coli} ribosome has been visualized by cryo-electron microscopy (7). At this particular step of \textit{trans}-translation, the TLD, pk1, and the MLD contact the ribosome, whereas the pk2 to pk4 segment forms an arc that remains outside the ribosome.

Three proteins facilitate binding of tmRNA to the ribosome. Elongation factor Tu forms a ternary complex with GTP and aminoacyl-tmRNA, as in regular protein synthesis (8, 9). Protein SmpB binds to the ternary complex \textit{in vitro} as well as to stalled ribosomes \textit{in vivo} (10–12). Ribosomal protein S1 contacts the MLD and the pseudoknot-rich domain both on and off the ribosome (13).

Although pseudoknots are predominant tmRNA features, little is known about their contributions to tmRNA structure and function. Previous \textit{in vitro} experiments suggested that pk1 is essential for tmRNA folding and protein tagging, whereas the three remaining pseudoknots, pk2 through pk4, are interchangeable and replaceable with stretches of single-stranded RNA (14–16). However, these data were derived exclusively using an insensitive assay in which poly(U)-programmed ribosomes produce hydrophobic polypeptides (polyphenylalanine) that are inefficiently tagged and because of their heterogeneity are difficult to analyze qualitatively.

To understand how tmRNA facilitates the tagging of truncated proteins, we investigated the contributions of the pseudoknots pk2 to pk4 and protein SmpB. The key to achieving our goals was development of two exceptionally sensitive assays for testing the functionality of tmRNA \textit{in vivo} and \textit{in vitro}. A truncated ribosomal protein, L27, produced by ribosomes programmed with mRNA lacking stop codons, was tagged with a protease-resistant histidine-rich peptide in the presence or absence of protein SmpB. A stable fusion protein was easily detected in fractionated \textit{E. coli} lysates either by staining with Coomassie Blue or by using anti-His-tag antibodies. We found that disrupting pseudoknots pk2 to pk4 had differential effects not only on tmRNA tagging activity and on binding of ribosomal protein S1 to tmRNA but also on tmRNA maturation. Taken together, these results indicated that pk2, pk3, and pk4 are important for the proper overall folding of the tmRNA. The ability of SmpB to reverse certain defects in tmRNA processing suggests that this protein plays a hitherto undiscovered regulatory role in \textit{trans}-translation.

**EXPERIMENTAL PROCEDURES**

\textbf{Bacterial Strains—} \textit{E. coli} strain XL1-B was the host for purification and maintenance of new plasmids. Expression strains IW410 and

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§ The abbreviations used are: tmRNA, transfer-messenger RNA; TLD, tRNA-like domain; MLD, mRNA-like domain; IPTG, isopropyl 1-thio-β-D-galactopyranoside; Ni-NTA, nickel nitritotrriacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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IW363 were constructed by P1 transduction of gene ssrA deletion from W3110 ssrA::kan’ into BL21(DE3) and BL21(DE3)pLysS, respectively (17).

Primers—Oligodeoxyribonucleotides used for constructions of plasmids and mutagenesis of the rpmA and ssrA genes were as follows: PR1, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR2, 5’-GATGCCGGCCACAGGTCTGCTGGCCGCTAGAAAGGATGACTG-3’; PR3, 5’-GGTGCCGGATCACGACTCCAGAATCC-3’; PR4, 5’-TAGTTCTCTTCGGAGCCCGCTCATTAGGCGGGCTGCTTCGATGCTGATAAATTT-3’; PR5, 5’-GAGTGGACTCCAGAATCC-3’; PR6, 5’-TAGTCTCTTCGCGA-3’; PR7, 5’-GCTGATTGTGCGTACATCGATAAGCTTTAATGCG-3’; PR8, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR9, 5’-GTTGGCCTCGTAAAAAGC-3’; PR10, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR11, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR12, 5’-GAGGCTTACTAGGAGAGAGGAGAGGACTACAT-3’; PR13, 5’-GAGGCTTACTAGGAGAGAGGAGAGGACTACATCGGAGGATGACTG-3’; PR14, 5’-GTTGGCCTCGTAAAAAGC-3’; PR15, 5’-GTTGGCCTCGTAAAAAGC-3’; PR16, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR17, 5’-GTTGGCCTCGTAAAAAGC-3’; PR18, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR19, 5’-GTTGGCCTCGTAAAAAGC-3’; PR20, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’; PR21, 5’-GACAGCTTATCATCGATAAGCTTTAATGCG-3’.

Plasmid pETrpmA-At-1 contained the E. coli rpmA gene in which the stop codon was replaced with the trpA terminator sequence (19). To construct this plasmid, the rpmA gene obtained from plasmid pETrpmA was amplified using PR3 and PR4 and cloned into pET-23a (see Fig. 2A) (20).

Plasmid pETrpmA-At-2 was a derivative of pETrpmA-At-1 into which the ssrA gene with its flanking regions (133 bp upstream and 157 bp downstream) was inserted. A PCR fragment containing the ssrA gene was cloned into the PvuII site in pETrpmA-At-1.

Plasmid pETrpmA-At-3 was prepared by inserting the smpB gene upstream of the ssrA gene in plasmid pETrpmA-At-2 to obtain their chromosomal configuration. Primers PR5 and PR6 were used to amplify the insert.

Mutagenesis—To replace the rpmA-trpA gene with wild-type rpmA sequence in plasmid pETrpmA-At-3, mutagenic primer PR7 was used. The resulting plasmid pETrpmA-3 was suitable for expression of protein T7-L27 in vivo.

For replacing the sequence encoding the proteolytic peptide ANDEHNNHHHHH (H6 tag) in E. coli ssrA gene in all pETrpmA-type plasmids, PCR-directed mutagenesis was carried out with PR8 and PR9. Similarly, for the replacement with ANHNNHHHHHH (H8 tag), PCR-directed mutagenesis was carried out with PR10 and PR11.

The base pairing in helix 5 of tmRNA(H6) and tmRNA(H8) was determined for replacing the sequence encoding the proteolytic peptide ANDEHNNHHHHH (H6 tag) in E. coli rpmA gene encoded by plasmid pETrpmA-2 into plasmid pETrpmA-3 carrying mutant ssrA gene encoding tmRNA(H6hp) and tmRNA(H8hp).

TmRNAs lacking pseudoknots or containing disrupted pseudoknots were introduced into plasmids pETrpmA-At-2 and pETrpmA-At-3 carrying mutant ssrA genes encoding tmRNA(H6hp) and tmRNA(H8hp).

For the in vitro tagging experiments, the ssrA gene in plasmid pTM was replaced with its derivative encoding tmRNA(H6hp) (13). All dele-
tion mutations listed above were re-cloned into plasmid pTMtRHhp using restriction enzymes SphI and PvuII. The mutations were verified by DNA sequencing.

**In Vitro RNA Synthesis and Analysis—tRNAs were transcribed in vitro**, purified, ^32P-labeled, aminoacylated, and tested for binding to protein S1 as described earlier (13, 21).

**[^35S]-Labeling of Proteins in Vivo**—Proteins were labeled with[^35S]Met as described (22).

**Protein Tagging in Vivo**—In initial in vivo tagging experiments the rpmA and ssrA genes were expressed simultaneously from the plasmids pETrpmA-At-1 and pAcArpmA in E. coli strains BL21(DE3) and IW410. Most of the in vivo tagging experiments were performed in E. coli IW363 cells transformed with either pETrpmA-At-2 or pETrpmA-At-3. Freshly transformed cells were grown at 37 °C for 2 h in 2×YT broth supplemented with the appropriate antibiotics (kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml; ampicillin, 200 μg/ml) to 0.3–0.4 A_{600}. Cultures were diluted to an A_{600} of 0.05 and incubated in the absence or presence of 1 mM IPTG for 3 h. 1 ml aliquots of each cell culture were collected for RNA and protein analysis. Cells were lysed and fractionated on 12.5% SDS-polyacrylamide gels as described (23). Proteins were detected by staining with Coomassie Blue. Stable expression of tagged L27 was assayed by replacing the ANDENYALAA proteolysis tag with a protease-resistant peptide. We chose a string of six or eight histidines (His tag) to allow the isolation and optional detection of even minute quantities of the tagged fusion protein by affinity chromatography on Ni-NTA-Sepharose and anti-His-tag antibodies, respectively.

**RESULTS**

**Monitoring tmRNA-dependent Tagging of Truncated Proteins in Vitro**—Ribosomal protein L27, encoded by the rpmA gene, can be overexpressed in E. coli without detrimental effects (24). Thus, a truncated L27 encoded by mRNA lacking a stop codon(s) was expected to be suitable for studying tmRNA-dependent tagging in cell lysates by detection of polypeptides using SDS-PAGE and staining with Coomassie Blue. Stable expression of tagged L27 was assured by replacing the ANDENYALAA proteolysis tag with a protease-resistant peptide. We chose a string of six or eight histidines (His tag) to allow the isolation and optional detection of even minute quantities of the tagged fusion protein by affinity chromatography on Ni-NTA-Sepharose and anti-His-tag antibodies, respectively.

The main components of the in vivo assay are the three plasmids, pETrpmA-At-1, -2, and -3 (Fig. 2A), which direct the expression of truncated L27 either in the presence or absence of tmRNA and protein SmpB. Plasmid pETrpmA-At-1 was constructed by deleting the His-tag-encoding segment from pET-23a (Novagen) followed by inserting a modified rpmA gene under control of the T7 promoter. The 5′ end of the rpmA gene was fused to the T7-tag, a DNA segment that encodes the N-terminal segment of the T7 major capsid protein. The stop codon of the rpmA gene was replaced with trpA, an efficient trpA terminator (19). These manipulations yielded T7-rpmA-At, a modified rpmA gene encoding T7-L27-At, a 11,226-Da polypeptide consisting of 105 amino acids. T7-L27-At, when
tagged by the tmRNA mutants with the protease-resistant ANDEHHHHHHH (H$_6$) tag or AANHHHHHHHHH (H$_8$) tag (see Fig. 1B), produced the 116-amino acid long proteins T7-L27-H$_6$ (Mr 12,444) or T7-L27-H$_8$ (Mr 12,474 Da). Because T7-L27-At and its tagged derivative can be separated by SDS-PAGE, tagging is easily monitored (Fig. 2C).

Expression of protein T7-L27-At in IW410, an _E. coli_ strain lacking the ssrA gene, was prominent (Fig. 3). In contrast, in the ssrA gene-containing BL21(DE3) strain, T7-L27-At was degraded, demonstrating that this protein is suitable for monitoring tmRNA-directed tagging.

To prevent the degradation of tagged T7-L27-At, we constructed tmRNA(H$_6$) and tmRNA(H$_8$), encoding protease-resistant H$_6$ and H$_8$ tags (Fig. 1B). The alterations were introduced into plasmid pACssrA where the ssrA gene is under control of its own promoter. As shown in Fig. 4, tmRNA(H$_6$) was expressed less efficiently than the wild-type tmRNA. A similarly reduced level of expression was observed for tmRNA(H$_8$) (not shown). We attribute this effect to the disruption of base pairing in helix 5 because higher expression levels were observed with wild-type tmRNA and tmRNA(H$_6$ hp) in which helix 5 is restored (see Fig. 1B). The mechanism for how the disruption of helix 5 reduces the expression of the tmRNA(H$_6$/H$_8$)-encoded peptides remains to be resolved.

To tag T7-L27-At in _in vivo_, _E. coli_ IW410 cells were transformed simultaneously with pETrpmA-At-1 and derivatives of pACssrA encoding either wild-type tmRNA or tmRNA(H$_6$). The synthesis of truncated L27 was induced in logarithmically growing cells by the addition of IPTG. Cells continued to grow for 3 h, and their protein content was analyzed by SDS-PAGE. As shown in Fig. 5, similar sets of proteins were apparent in uninduced and induced cells, indicating that the truncated L27 was tagged by plasmid-borne wild-type tmRNA and then proteolytically degraded. In contrast, a prominent band corresponding to T7-L27-At was visible in lysates of the IPTG-induced cells expressing either tmRNA(H$_6$) or tmRNA(H$_8$). Using Coomassie Blue staining, tagged proteins could not be detected.

To demonstrate that tmRNA(H$_6$) and tmRNA(H$_8$) were able to tag _in vivo_, it was necessary to use $^{35}$S-labeled T7-L27-At, affinity chromatography, and anti-His-tag antibodies. IW410 cells were transformed simultaneously with plasmids pETrpmA-At-1 and pACssrA and treated with IPTG in a medium containing $^{35}$S-labeled His-tagged T7-L27-At proteins were captured on Ni-NTA-Sepharose and analyzed by SDS-PAGE. Protein T7-L27-At was tagged very poorly by tmRNA(H$_6$) and tmRNA(H$_8$) (see Fig. 6). Using Coomassie Blue staining, tagged proteins could not be detected.

To account for some of the observed poor tagging of protein T7-L27-At in vivo, we inserted the ssrA gene into plasmid pETrpmA-At-1 to form pETrpmA-At-2. Because the rpmA and ssrA genes were part of the same molecule, their products were expected to be expressed at a constant ratio. To increase the reproducibility of these experiments, we inserted the ssrA gene into plasmid pETrpmA-At-2, which encoded either tmRNA(H$_6$) or tmRNA(H$_8$), which was then stained with Coomassie Blue.
A

B

FIG. 7. Maturation of wild-type tmRNA, tmRNA(H₆), and tmRNA(H₈) expressed from plasmid pETrpmA-At-2. Northern blot analysis of 1 µg of total RNA extracted from un-induced and IPTG-induced E. coli TW363 cells separated on a 1.5% formamide-agarose gel and blotted to a Zeta-probe membrane. ³²P-Labeled oligonucleotide TM4 was hybridized to both precursor tmRNA (p-tmRNA) and mature tmRNA. △, no tmRNA; H₆, H₈, and wt denote tmRNA(H₆), tmRNA(H₈), and wild-type tmRNA, respectively.

FIG. 8. In vitro tagging of truncated protein L27 by tmRNA(H₆) and tmRNA(H₈). Cell lysates from IPTG-induced E. coli TW363 cells harboring plasmid pETrpmA-At-2, which encodes either tmRNA(H₆) or tmRNA(H₈) and protein T7-L27-At, were fractionated on a 12.5% SDS-polyacrylamide gel. A, Coomassie-stained gel. B, Western blotting with anti-His-tag antibody. His-tagged proteins T7-L27-At are denoted as T7-L27-H8/H6.

FIG. 9. In vitro tagging of truncated protein L27 by tmRNA(H₆). Tagging reactions were assembled by addition of circular plasmid pETrpmA-At-1 and purified tmRNA(H₆) to the T7 S30 transcription/translation mixture (Promega). Some reactions were also supplemented with purified His-tagged protein SmpB and partially purified E. coli alanine-tRNA synthetase (25). After 60 min of incubation at 37 °C, His-tagged proteins were captured on Ni-NTA-Sepharose and then fractionated on a 12.5% SDS-polyacrylamide gel. Tagging was visualized by Western blotting with anti-His-tag antibodies. tmRNA(H₆), tagged T7-L27-At, His-tagged SmpB, and alanine-tRNA synthetase are denoted as tmRNA*; T7-L27-H8; SmpB-H6; and AlaRS, respectively.

tmRNA (not shown). Precursors of tmRNA(H₆) and tmRNA(H₈) were trimmed to their mature form as efficiently as the precursors of wild-type tmRNA (compare Figs. 7 and 15).

Tagging of protein T7-L27-At by tmRNA(H₆) and tmRNA(H₈) was tested in TW363, a derivative of E. coli strain BL21(DE3)pLysS lacking the ssrA gene. As shown in Fig. 8A, inserting the ssrA and T7-rpmd-A genes into a single plasmid and restoring helix 5 in tmRNA(H₆) and tmRNA(H₈) improved the tagging efficiency of protein T7-L27-At to a level that allowed the detection of the fusion protein by Coomassie Blue staining. Effective His-tagging of protein T7-L27-At was confirmed by its capture on Ni-NTA-Sepharose and the use of anti-His-tag antibodies (Fig. 8B). Both tmRNA(H₆) and tmRNA(H₈) tagged protein T7-L27-At efficiently. Together, these findings demonstrated that, at least in E. coli, the disruption of helix 5 inhibits maturation of precursor tmRNAs and tmRNA-dependent tagging. Therefore, helix 5 constitutes a functionally important element of tmRNA structure and function.

Monitoring tmRNA-dependent Tagging of Truncated Proteins in Vitro—As shown above, certain alterations in tmRNA affected tmRNA-dependent protein tagging by preventing the processing of tmRNA precursors. To differentiate between defects in protein tagging and tmRNA maturation, we developed an in vitro assay that employed plasmids pT7tmRNAH₈hp and pETrpmA-At-1. pT7tmRNAH₈hp linearized with restriction enzyme BstNI provided a template suitable for in vitro synthesis of tmRNA(H₈) by T7 RNA polymerase. pETrpmA-At-1, when added to an in vitro transcription/translation system, was used for the synthesis of stop-free mRNA encoding truncated protein L27. Although the in vitro tagging of truncated protein L27 by tmRNA(H₈) was less efficient than the in vivo reaction, formation of the fusion protein could be easily monitored by fractionation of the reaction products on SDS-polyacrylamide gels followed by Western blotting with anti-His-tag antibodies.

During the optimization of the in vitro protein tagging assay, we found that in addition to plasmids pETrpmA-At-1 and tmRNA(H₈), the transcription/translation reactions had to be supplemented with protein SmpB (Fig. 9). The most efficient tagging was observed when tmRNA(H₈) and protein SmpB were provided in equimolar quantities. This finding was consistent with the earlier demonstration that protein SmpB is an essential component of the trans-translation apparatus in vitro (25, 26).

Functions of Pseudoknots in tmRNA-dependent Protein Tagging—TLD and MLD are mimics of tRNA and mRNA, respectively, and function in trans-translation as expected (1, 2, 27, 28). Pseudoknots, on the other hand, form in many of the larger RNAs where they are involved in a wide variety of biological processes (29–31).

To determine the roles of the pseudoknots, we first tested the tagging potential of tmRNAs that lacked two or more pseudoknots. Three derivatives of tmRNA(H₆) missing pk2 and pk3 (tmRNAΔpk2/pk3), pk3 and pk4 (tmRNAΔpk3/pk4), or pk2-pk4 (tmRNAΔpk2-pk4) were unable to tag protein T7-L27-At in vivo (not shown). The tmRNAΔpk3/pk4 construct was able to mediate tagging of protein T7-L27-At in vitro (Fig. 10) but with greatly reduced efficiency. This result suggested that the loss of pk2 is more detrimental for tmRNA-dependent tagging than the depletion of pk3 or pk4.

Previously, ribosomal protein S1 was shown to be associated with the MLR and pseudoknots pk2 to pk4 (17). Therefore, we investigated the ability of purified E. coli S1 to bind to mutant tmRNAs that lacked various pseudoknots. tmRNAs were ³²P-labeled at their 3' ends with yeast ³⁵S ATP/CTP RNA nucleotidyltransferase in the presence of [α-³²P]ATP and aminoacylated with alanine as described (13). Typically 80–95% of tmRNAΔpk2/pk3, tmRNAΔpk3/pk4, and tmRNAΔpk2-pk4 could be charged (not shown), indicating that the TLDs of the mutant tmRNAs were properly folded (32).

Gel mobility shift assays demonstrated that the pseudoknot deletion mutants retained their ability to bind protein S1 (Fig. 11). However, the affinity of S1 to tmRNA was particularly low when pk2 was deleted (compare panels b and d with panel c in Fig. 11). Protein S1 bound to tmRNA derivatives lacking pk2 with an apparent binding constant, $K_a$, of $5 \times 10^6$–$1 \times 10^7$ m$^{-1}$.
Fig. 10. In vitro tagging of truncated protein L27 by tmRNA(H8hp) mutants lacking pk2-pk3, pk3-pk4, and pk2-pk4. Each tagging reaction contained equimolar amounts of tmRNA and His-tagged protein SmpB. Tagging was visualized by Western blotting with anti-His-tag antibodies. Δpk2-pk3, Δpk3-pk4, and Δpk2-pk4 denote mutant tmRNA lacking pk2-pk3, pk3-pk4, and pk2-pk4, respectively. Lane M shows molecular mass markers. For more details, see the legend for Fig. 9. C, control.

Fig. 11. Gel-shift analysis of binding between [32P]-labeled truncated tmRNA(H8hp) derivatives and ribosomal protein S1 from E. coli. Titration of [32P]-labeled tmRNA(H8hp) (panel a), tmRNAΔpk2-pk3 (panel b), tmRNAΔpk3-pk4 (panel c), and tmRNAΔpk2-pk4 (panel d) with protein S1. Aliquots of binding mixtures were analyzed by electrophoresis on a 5% polyacrylamide gel and autoradiography. Concentrations of protein S1: lane 1, no protein; lane 2, 1 × 10−5 μM; lane 3, 5 × 10−5 μM; lane 4, 1 × 10−5 μM; lane 5, 5 × 10−7 μM.

Fig. 12. In vivo tagging of truncated protein L27 by tmRNA(H8hp) and its derivatives with single, double, and triple pseudoknot substitutions. C denotes a control reaction without tmRNA. For more details, see the legends for Figs. 9 and 10.

Fig. 13. In vitro tagging of truncated protein L27 by tmRNA(H8hp) and SmpB. Tagging was visualized by Western blotting with anti-His-tag antibodies. Two independent clones were tested for each mutant to highlight reproducibility of the assay. tmRNA+ denotes tmRNA(H8hp).

Because protein SmpB enhanced protein tagging in vitro (see Fig. 13), we speculated that co-expression of tmRNA mutants and SmpB may also improve protein tagging in vivo. In pETrpmA-At-3 the smpB gene was inserted upstream of the sraA gene in pETrpmA-At-2 to mimic the chromosomal organization of these two genes (Fig. 2A). TmRNApk2L, TmRNApk3L, and TmRNApk4L, when co-expressed with protein SmpB, tagged so efficiently that protein T7-L27-H8 could be easily detected by staining with Coomassie Blue (Fig. 14A). As much as 50% of protein T7-L27-At was tagged by tmRNA(H8hp). The levels of tagging by tmRNApk2L and tmRNApk3L were similar. However, the expression of protein T7-L27-At in cells producing tmRNApk3L was consistently lower than in cells expressing tmRNA(H8hp), tmRNApk2L, and tmRNApk4L. The tagging by tmRNApk4L, tmRNApk2/3L, and tmRNApk3/4L was significantly impaired and was detected only with anti-His-tag antibodies (Fig. 14B). The tmRNApk2–4L could not tag at all. Together, the observations indicated that pk4 is very important for protein tagging.

The tagging patterns seen in Figs. 13 and 14 suggested that certain defects displayed by tmRNAs with disrupted pseudoknots might be related to tmRNA maturation. To test this possibility we isolated total RNA from IW363 cells before and after induction of protein T7-L27-At synthesis with IPTG. The RNAs were examined by Northern blot analysis with an oligonucleotide probe complementary to nucleotides 54–67 of E. coli tmRNA. As seen in Fig. 15, E. coli coped very well with processing of tmRNA precursors before IPTG induction. More than 90% of precursor tmRNA(H8hp), tmRNApk2L, tmRNApk3L, and tmRNApk2/3L were trimmed to their mature forms. Maturation of precursor tmRNApk4L, tmRNApk3/4L, and tmRNApk2–4L was less effective, but still, more than 70% of the molecules were processed. Upon IPTG induction, the processing of precursor tmRNApk4L, tmRNApk3/4L, and tmRNApk2–4L was affected the most. More than 50% of these tmRNA derivatives remained unprocessed. Because pk4 was disrupted in these tmRNA mutants, the maturation pattern shown in Fig. 15 indicated that pk4 is
more important for tmRNA processing than pk2 and pk3.

Because protein SmpB improved tmRNA-dependent tagging in vivo, we compared the maturation of mutant tmRNAs with a single pseudoknot disrupted in the presence and absence of SmpB. Total RNA was isolated from the IPTG-induced IW363 cells harboring plasmids pET rpmA-At-2 or pET rpmA-At-3 and examined by Northern blot analysis. This analysis demonstrated that the majority of the precursor tmRNApk2L and tmRNApk3L and 90% of tmRNApk4L remained unprocessed when SmpB was absent (Fig. 16). In contrast, in the presence of protein SmpB, more than 85% of precursor tmRNA(H₈hp), tmRNApk2L, and tmRNApk3L was processed to their mature forms. Most of the tmRNApk4L mutant remained unprocessed, confirming that pk4 plays a prominent role in tmRNA maturation.

**FIG. 14.** In vivo tagging of truncated protein L27 by tmRNA(H₈hp) and its derivatives with single, double, and triple pseudoknot substitutions in the presence of plasmid-encoded protein SmpB. Logarithmically growing E. coli IW363 cells transformed with plasmid pET rpmA-At-3 were induced with 1 mM IPTG. Total RNA was isolated from the IPTG-induced E. coli IW363 cells separated on a 5% denaturing polyacrylamide gel and blotted to a Zeta-probe membrane. 32P-Labeled oligonucleotide TM4 was hybridized to both precursor tmRNA (p-tmRNA) and mature tmRNA. The lower panel shows the graphical representation of PhosphorImager-derived data from the Northern blot. Open bars, p-tmRNA; filled bars, tmRNA.

**FIG. 15.** Maturation of mutant tmRNA co-expressed with protein SmpB. Northern blot analysis of 1 μg of total RNA extracted from un-induced and IPTG-induced E. coli IW363 cells harboring plasmid pET rpmA-At-3 separated on a 5% denaturing polyacrylamide gel and blotted to a Zeta-probe membrane. 32P-Labeled oligonucleotide TM4 was hybridized to both precursor tmRNA (p-tmRNA) and mature tmRNA. The lower panel shows the graphical representation of PhosphorImager-derived data from the Northern blot. Gray bars, no IPTG induction/precursor tmRNA; open bars, no IPTG induction/mature tmRNA; hatched bars, IPTG induction/p-tmRNA; filled bars, IPTG induction/mature tmRNA. tmRNA* denotes tmRNA(H₈hp).

**FIG. 16.** Maturation of tmRNA mutants expressed from plasmids pET rpmA-At-2 (no smpB gene) and pET rpmA-At-3 (plus smpB gene). Northern blot analysis of 1 μg of total RNA extracted from IPTG-induced E. coli IW363 cells separated on a 5% denaturing polyacrylamide gel and blotted to a Zeta-probe membrane. 32P-Labeled oligonucleotide TM4 was hybridized to both precursor tmRNA (p-tmRNA) and mature tmRNA. The lower panel shows the graphical representation of PhosphorImager-derived data from the Northern blot. Open bars, p-tmRNA; filled bars, tmRNA.

**DISCUSSION**

For protein tagging, the 457-nucleotide precursor of E. coli tmRNA must be trimmed to its mature form, aminoacylated, delivered to the ribosome, and then perform its dual tRNA- and mRNA-like functions (2, 17, 28, 33). We have studied these processes in vivo and in vitro by using suitably modified tmRNA derivatives with focus on the relative contribution of pseudoknots pk2 to pk4, helix 5, and proteins SmpB.

Using novel sensitive assays for monitoring tmRNA-dependent tagging of truncated protein L27, we demonstrated that deletions and disruptions of pseudoknots pk2-pk4 and helix 5 impair protein tagging in vitro and in vivo. The level of tagging was different for each modification. For example, tmRNApk2/pk3 and tmRNApk2-pk4, mutants lacking pk2, did not tag in vitro. TmRNApk3L tagged well but inhibited the expression of the truncated protein L27 in vivo. TmRNApk4L, tmRNApk3/4L, and tmRNApk2–4L, all of which contained a disrupted pk4, showed no tagging activity in vivo. However, tmRNApk4L was as active as tmRNApk2L and tmRNApk3L in tagging in vitro.

Differences between the in vitro and in vivo tagging profiles suggested that different functions of tmRNA may be impaired (compare Figs. 13 and 14). Indeed, further testing using gel mobility shift assays demonstrated that the deletion of pk2 in particular weakened the interactions between tmRNA and ribosomal protein S1, suggesting that pk2 may be an important S1 binding site in tmRNA. This result is consistent with footprinting studies, which demonstrated a decreased reactivity of pk2 toward single-strand-specific probes when tmRNA is bound to protein S1 in vitro (34). Furthermore, cryo-electron microscopy revealed that pk2 remains available for interac-
tions with protein S1 when tmRNA enters the ribosome (7).

Our work demonstrated that mutant tmRNAs with alterations in helix 5 and the pk2-pk4 region adversely affect maturation of tmRNA precursors. Because formation of the double-stranded acceptor stem is a prerequisite for trimming the termini of the precursor tmRNA (17, 33), this finding implied that disruption of pseudoknots impairs the folding of the 3′ and 5′ termini of tmRNA into a tRNA-like structure. However, pk2 to pk4 were not essential for tmRNA maturation. As shown in Fig. 15 (upper panel, last lane), even upon disruption of base pairing in the pk2-pk4 segment E. coli was able to process ~50% of the tmRNAPk2–4L precursors to mature, tagging-incompetent RNA molecules.

Because we observed similar cellular levels of vigorously tagging mature tmRNA(H8hp) and tagging-impaired mature tmRNA mutants, at least one pseudoknot, preferably pk4, is required for tmRNA-directed protein tagging. In contrast, it was suggested previously that pk2, pk3, and pk4 are inter
dependent and can be replaced with single-stranded RNAs which phylogenetic support is weak (5). Finally, evidence is presented that protein SmpB helps to process tmRNA precursors. This work also highlights the important role of helix 5, for which phylogenetic support is weak (5). Finally, evidence is presented that protein SmpB helps to process tmRNA precursors. Further studies will investigate how protein SmpB facilitates maturation of tmRNA, why pk4 is especially important for tmRNA maturation, why disruption of pk3 inhibits synthesis of truncated protein, and why pk2 is more important for protein S1 than the other pseudoknots. Powerful assay systems to address these critical questions are now available.

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Contributions of Pseudoknots and Protein SmpB to the Structure and Function of tmRNA in trans-Translation
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