Three of four pseudoknots in tmRNA are interchangeable and are substitutable with single-stranded RNAs

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Abstract A novel translation, *trans*-translation, is facilitated by a highly structured RNA molecule, tmRNA. This molecule has two structural domains, a tRNA domain and an mRNA domain, the latter including four pseudoknot structures (PK1 to PK4). Here, we show that replacement of each of these pseudoknots, except PK1, in *Escherichia coli* tmRNA with a single stranded RNA did not seriously affect the functions as an alanine tRNA and as an mRNA. Furthermore, these three pseudoknots were interchangeable with only small losses of the two functions. These findings suggest that neither PK2, PK3 nor PK4 interacts in a functional manner with ribosome during the *trans*-translation process. Together with an earlier study showing the significance of PK1, it is concluded that among the four pseudoknots, PK1 is the most functional.

Key words: *trans*-Translation; 10Sa RNA; tmRNA; Pseudoknot; Translation; *Escherichia coli*

1. Introduction

tmRNA (also known as 10Sa RNA) is widely distributed among eubacteria, with the exception of the group of alpha-proteobacteria, and is also found in some chloroplasts [1–4]. This molecule is unique in that it functions both as an alanine tRNA [5,6] and as an mRNA encoding the last 10 residues of the 11 amino acid tag-peptide (AlaAlaAsnAspGluAsnTyrAla-LeuAlaAla). This tag-peptide sequence was initially found in the C-termini of truncated murine interleukin-6 expressed in *Escherichia coli* [7], and was also found in other polypeptides when they were translated from mRNAs lacking a termination codon or possessing a cluster of rare codons [8,9] and in an in vitro translation product in the presence of tmRNA and poly U as a truncated mRNA [10].

It has been proposed that tmRNA facilitates a non-canonical translation reaction named *trans*-translation, in which a ribosome can switch between the translation of a truncated mRNA and the tmRNA-encoded tag sequence to terminate a stalled translation by adding a specific tag-peptide to the truncated C-termini of the polypeptide from mRNA without a stop codon [8,11,12]. This model was also supported by several other findings showing that its function as a tRNA is a prerequisite for its function as an mRNA in vitro [10], that tmRNA binds predominantly to the 70S ribosome but scarcely to the polysome [13], and that histidine is substantially incorporated into the in vitro tag-peptide synthesis directed by the histidine-accepting tmRNA mutant [14].

Extensive sequence alignments and chemical probing studies have revealed the secondary structure model of this molecule (Fig. 1) [3,15–18]. Both terminal sequences mimic a half molecule of the tRNA structure in that they possess an acceptor stem leading to the universal 3′ CCA sequence and a T-arm with two tRNA specific modified nucleotides [19]. It has recently been shown that this tRNA-like region can be recognized by EF-Tu [20]. The central two third of this molecule forms a large loop comprised of four pseudoknots (PK1 to PK4) and two stem and loop structures (H3 and H4).

The tag-peptide translation initiates from an alanine codon, GCA, in the putative H3 helix in the central large loop (Fig. 1). However, it remains unclear as to how a stalled ribosome accommodates tmRNA to start *trans*-translation from this apparently featureless point. It is possible that some structural unit around the tag-encoded region interacts with somewhere on the ribosome directly or via an accessory protein during *trans*-translation.

Here, we focused on the pseudoknot structures on tmRNA. An earlier study has shown the importance of the first pseudoknot (PK1) of *E. coli* tmRNA 12 nucleotides upstream of the tag-initiation point for efficient *trans*-translation, although it is not involved in defining the initiation point of tag-translation [21]. In the present study, each of these four pseudoknots was replaced by a single stranded RNA. Besides, these pseudoknots were exchanged for each other. These drastic structural rearrangements allow us to evaluate the structural and functional significances of these pseudoknot structures.

2. Materials and methods

2.1. Engineering tmRNA variants

Mutations were introduced by primer-directed polymerase chain reactions. Two mutation primers for both directions (M*n*L and M*n*R primers; *n* is any of 1 to 4), in addition to two terminal primers that included the restriction endonuclease sequences as the cloning sites (CL primer and CR primer), were used. Two mutation fragments preliminarily amplified with CL and M*n*R primers and with CR and M*n*L primers using the plasmid encoding the wild-type tmRNA sequence as the template were combined to anneal the region of mutation, from which the total length fragment was amplified again with the addition of excess CL and CR primers. The resulting amplified DNA fragment was ligated under the T7 promoter sequence of pGEMEX-2. This plasmid was co-transformed with pACYC184 encoding the T7 RNA polymerase gene under the *lac*-promoter sequence into the *E. coli* strain W3110 Δ*ssrA*, which lacks the tmRNA gene. tmRNA induced by the addition of 1.0 mM IPTG was purified as described [5,21].

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2.2. In vitro aminoacylation with alanine

Alanyl-tRNA synthetase was partially purified from *E. coli* strain Q13, as described earlier [22]. The final enzyme fraction from *E. coli* had a specific activity of 9.1 units/mg, when one unit of alanyl-tRNA synthetase catalyzed the formation of 1 nmol alanyl-tRNA per 10 min under the reaction conditions described below. The aminoacylation reaction proceeded at 37°C, in a 50 μl reaction mixture containing 80 mM Tris–HCl (pH 7.5), 150 mM ammonium chloride, 2.5 mM dithiothreitol, 2.5 mM ATP, 20 μM L-[U-^{14}C]alanine (6.5 GBq/mmol), 1.0 μM tmRNA variants and 9.1×10^{-2} units of alanyl-tRNA synthetase. At the times specified, a 10 μl aliquot was withdrawn and spotted on Whatman 3MM filter paper, and radioactivity in the trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter.

2.3. In vitro amino acid incorporation

The preincubated S30 fraction was prepared from mid-log phase cells of *E. coli* strain W3110 (Δ*ssrA*) [6], as described previously [23]. A reaction mixture (100 μl) contained 80 mM Tris–HCl (pH 7.8), 5 mM magnesium acetate, 150 mM ammonium chloride, 2.5 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 20 μM L-[U-^{14}C]alanine, 0.05 mM each of the remaining unlabelled 19 amino acids, 0.1 μM each of the tmRNA variants (when 1 A_{260} unit corresponds to 330 pmol), and 20 μl of the S30 fraction, in the presence of 25 μg of poly (U) (50 to 100 mer, Sigma). The reaction mixture was incubated at 37°C, and a 24 μl aliquot was withdrawn at each time point and spotted on Whatman 3MM filter paper, and radioactivity of the hot trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter.

3. Results and discussion

E. coli tmRNA has four pseudoknot structures, PK1 (position 49–78), PK2 (138–196), PK3 (200–245) and PK4 (248 to 298) (Fig. 1). First, we attempted to replace them by single stranded RNAs of 10, 21, 17 and 20 nucleotide lengths, respectively, each possessing a sequence corresponding to the 5′-side sequence of the first stem directly followed by the 3′-side sequence of the second stem of the pseudoknot (pk1L-pk4L; Fig. 2a). The tmRNA mutants with the expected sequences were successfully obtained by using the mutation primer-directed polymerase chain reaction.

The aminoacylation activities with alanine for these tmRNA mutants were measured. As shown in Fig. 3a (left), pk2L, pk3L and pk4L were efficiently aminoacylated. The levels were almost comparable to that of wild-type tmRNA. In contrast, pk1L had only a significantly reduced aminoacylation ability. When a five-fold higher concentration of alanyl-tRNA synthetase was used, we were able to detect a weak aminoacylation activity in pk1L (data not shown). An aminoacylation deficiency has also been observed when either of two stems of PK1 is disrupted [21]. PK1 seems relatively distant from the terminal region mimicking tRNA^{Ala} (Fig. 1). Indeed, tRNA^{Ala} identity elements are concentrated in the acceptor stem, but a footprinting study has shown that the region covered by alanyl-tRNA synthetase extends to the anticodon stem [24]. PK1 may be in close contact with alanyl-tRNA synthetase during aminoacylation, although whether there is direct interaction between them has yet to be clarified. Otherwise, an unexpected global conformation change caused by mutation in PK1 may prevent normal recognition by alanyl-tRNA synthetase.

Next, we measured the alanine incorporation into the in vitro poly (U)-dependent tag-peptide synthesis for the tmRNA mutants (Fig. 3b, left). An almost normal efficiency of alanine incorporation was observed for pk3L or pk4L. The alanine incorporation ability was also retained in pk2L, although with about half the efficiency of the wild-type tmRNA. In contrast, only a faint alanine incorporation was observed for pk1L. We also measured the incorporations of arginine and threonine, which are present in the respective alternative frames, but not in the original frame, of the tag-peptide. Neither arginine nor threonine incorporation was de-

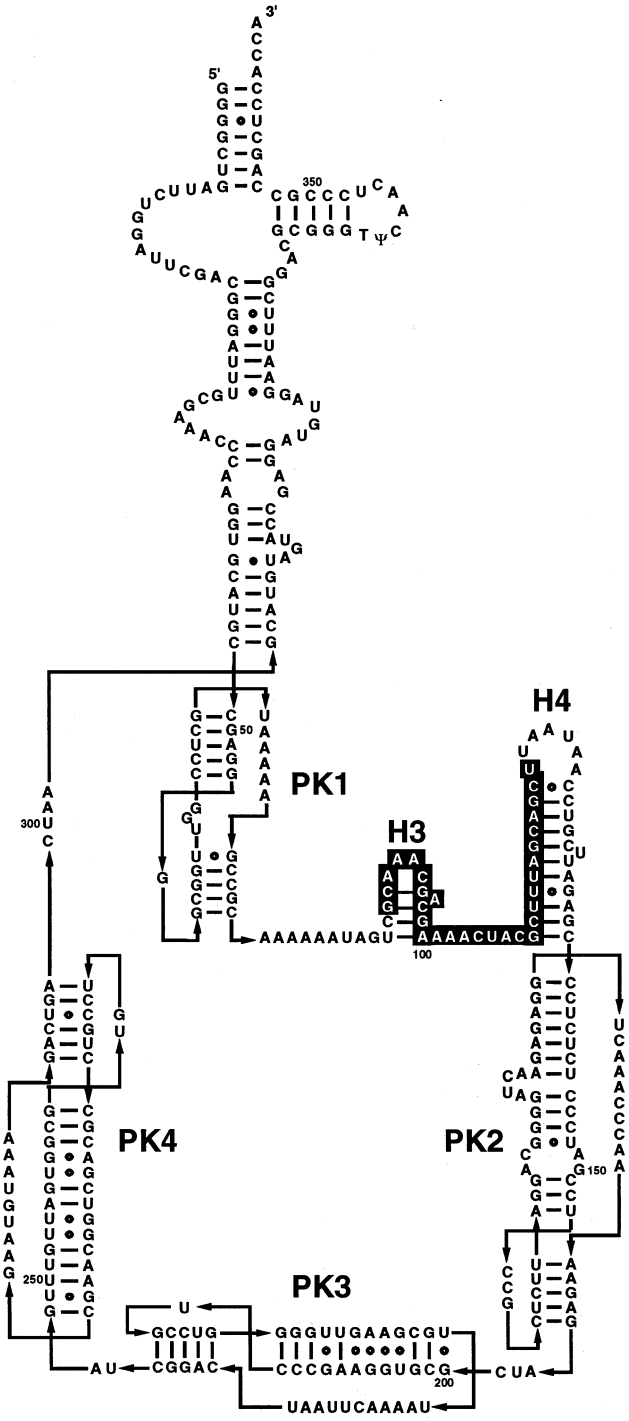

Fig. 1. A secondary structure model of *E. coli* tmRNA. The tag-encoded sequence highlighted in white with a red background is surrounded by four pseudoknots (PK1–PK4). Non-Watson–Crick base pairs are shown by open circles. This RNA has two tRNA specific modified nucleotides, 5-methyl U and pseudouridine in the T-loop [19], indicated as *T* and *Ψ*, respectively.

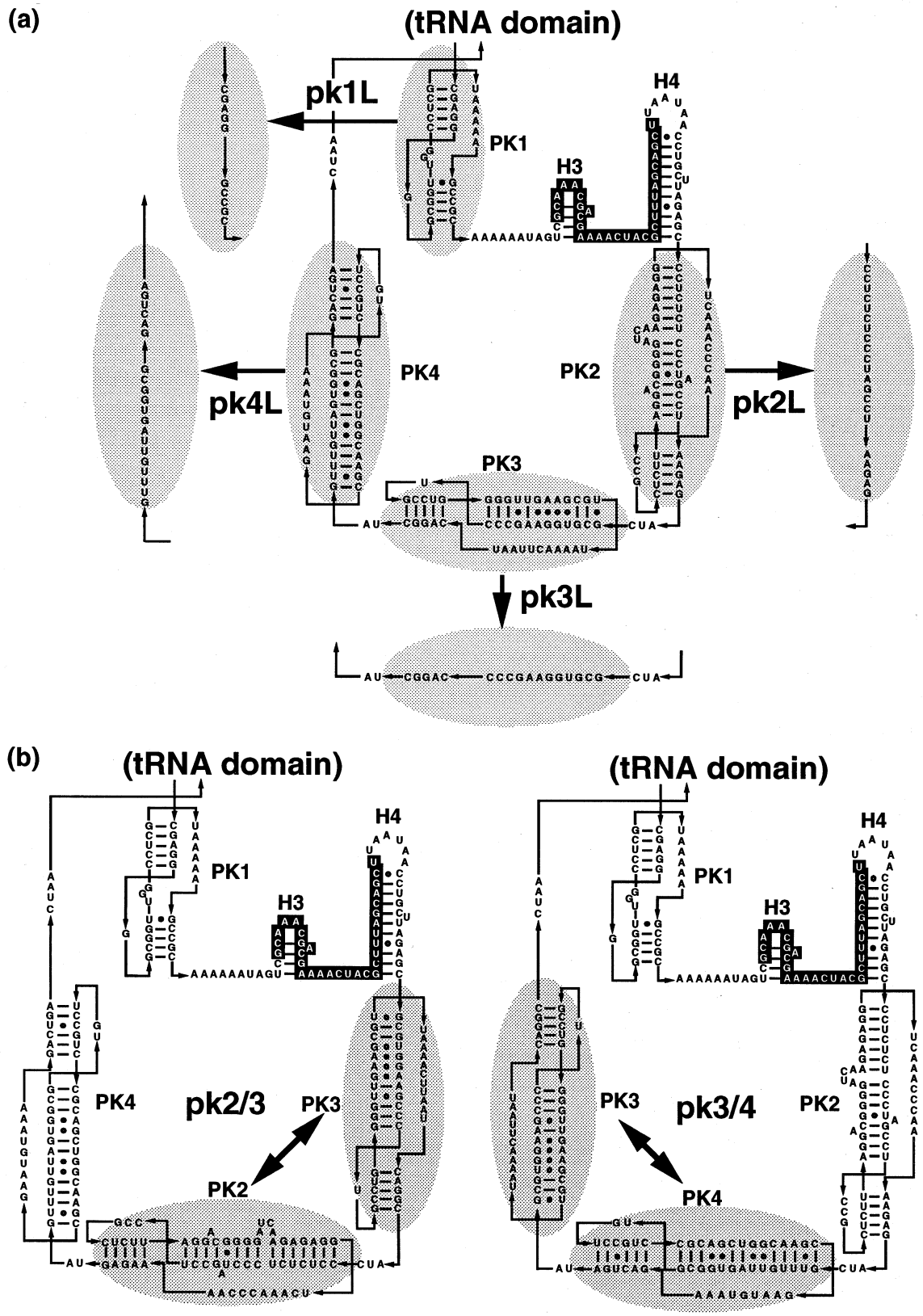

Fig. 2. *E. coli* tmRNA mutants used in this study. a: Each of the four pseudoknots was replaced by a single stranded RNA, shown by an arrow. b: PK2 and PK3 (left), or PK3 and PK4 (right) were interchanged, as shown by bidirectional arrows. Only the central loop region including the four pseudoknots (position 49–302) is shown, and the tag-encoded sequence is highlighted in white with a black background.

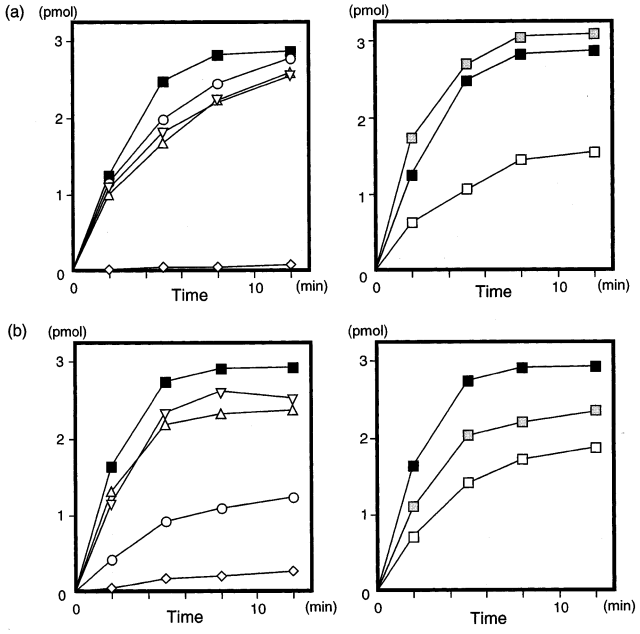

Fig. 3. In vitro aminoacylations with alanine (a) and in vitro poly (U)-dependent alanine incorporations (b), for (left) pk1L (diamond), pk2L (circle), pk3L (triangle) and pk4L (inverted triangle), and for (right) pk2/3 (open square) and pk3/4 (hatched square), as compared to wild-type tmRNA (closed square). The mean value obtained from at least two experiments was plotted.

tected for any of these four mutants (data not shown), indicating that only a normal frame tag-peptide was translated for pk2L, pk3L and pk4L.

These results clearly demonstrate that these pseudoknot structures, except for PK1, are not essential. Next, we attempted to construct tmRNA mutants in which PK2 and PK3, and PK3 and PK4 were interchanged (pk2/3 and pk3/4, respectively; Fig. 2b).

Exchanging PK3 and PK4 did not affect aminoacylation, and exchanging PK2 and PK3 reduced the aminoacylation efficiency by only half (Fig. 3a, right). The efficiency of alanine incorporation was only slightly affected by these interchanges of pseudoknots (Fig. 3b, right). Neither arginine nor threonine incorporation was detected for these mutants (data not shown). These pseudoknot interchange experiments confirm that neither PK2, PK3 nor PK4 is essential.

A sequence alignment has suggested that almost all tmRNAs have a set of four pseudoknots, implying that each pseudoknot structure has some functional role [1–3,25]. However, the present study shows that each of PK3 and PK4 can be replaced by a single stranded RNA without any serious losses of the two functions. The mutant in which PK2 is replaced by a single stranded RNA also substantially retains the functions, although with a slightly affected ability of tag-peptide synthesis. Besides, the interchange of two of these three pseudoknots, which have variations in sequence and length, also failed to affect the functions. These results indicate the functional and/or structural redundancy of the region encompassing PK2 to PK4. The redundancy of this region has also been implied by the poor sequence conservation [1–3]. There may be no or only a few tertiary interactions with other regions. Even if such an interaction exists, it is not critical to the functions. Unlike PK1, which is involved in the global conformation of this molecule [21], PK2, PK3 and PK4 may contribute only to the local conformational stability.

In the 250 nucleotide span of PK2 to PK4, all the nucleotides except five are involved in the pseudoknots (Fig. 1). The present results suggest that this region, particularly from PK3 to PK4, does not interact in a functional manner either directly with the ribosome or with *trans*-acting factors involved in *trans*-translation including SmpB [26]. It can be envisaged that this region is predominantly looped out from the ribosome at the initiation of the *trans*-translation. This seems quite reasonable, when we consider how elaborately the ribosome must accommodate tmRNA possessing a molecular weight over four-fold that of tRNA. Note that the mRNA span covered by the ribosome during the canonical translation is only 39 ± 3 nucleotides in length [27].

Among the four pseudoknots, PK1 is the most functional. It is the smallest pseudoknot and is closest to the tag-initiation point. Our previous mutation and NMR studies have shown that the specific conformation of PK1 as well as its specific loop sequence is important for tmRNA function [21,28]. This pseudoknot may act as a key structural unit to appropriately arrange the two apparently distant domains for the functional interplay on the ribosome. However, it does not serve as a *cis*-element to fine-tune the tag-initiation point. Other structural features as well as the peculiar sequence [29] required for tmRNA function should be focused on in order to clarify this novel translation mechanism.

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