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Determinants on tmRNA for initiating efficient and precise *trans*-translation: Some mutations upstream of the tag-encoding sequence of *Escherichia coli* tmRNA shift the initiation point of *trans*-translation in vitro

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ABSTRACT

tmRNA facilitates a novel translation, *trans*-translation, in which a ribosome can switch between translation of a truncated mRNA and the tmRNA's tag sequence. The mechanism underlying resumption of translation at a definite position is not known. In the present study, the effects of mutations around the initiation point of the tag-encoding sequence of *Escherichia coli* tmRNA on the efficiency and the frame of tag translation were assessed by measuring the incorporations of several amino acids into in vitro poly (U)-dependent tag-peptide synthesis. One-nucleotide insertions within the tag-encoding region did not shift the frame of tag translation. Any 1-nt deletion within the span of –5 to –1, but not at –6, made the frame of tag translation heterologous. Positions at which a single base substitution caused a decrease of *trans*-translation efficiency were concentrated within the span of –4 to –2. In particular, an A-4 to C-4 mutation seriously damaged the *trans*-translation, although this mutant retained normal aminoacylation and ribosome-binding abilities. A possible stem and loop structure around this region was not required for *trans*-translation. It was concluded that the tag translation requires the primary sequence encompassing –6 to +1, in which the central 3 nt, A-4, G-3, and U-2, play an essential role. It was also found that several base substitutions within the span of –6 to –1 extensively shifted the tag-initiation point by –1.

Keywords: 10Sa RNA; frameshift; tag peptide; tmRNA; *trans*-translation

INTRODUCTION

Transfer-messenger RNA(tmRNA, also known as 10Sa RNA or SsrA RNA) is widely distributed among eubacteria and has also been found in some chloroplasts and mitochondria (Zwieb et al., 1999; Keiler et al., 2000; Williams, 2000; Zwieb & Wower, 2000). It is a novel molecule with both tRNA and mRNA properties. Both terminal regions of this molecule can be folded into a partial tRNA-like structure comprising a TΨC-arm and an amino acid acceptor stem leading to the universal 3′ terminal CCA sequence (Ushida et al., 1994; Komine et al., 1994). The mimicry of the upper-half structure of tRNA has also been shown by comparative studies (Muto et al., 1996; Felden et al., 1996, 1997; Williams & Bartel, 1996), chemical and enzymatic probing studies (Felden et al., 1996, 1997; Hickerson et al., 1998) and the presence of tRNA-specific modified nucleosides, pseudouridine and 5-methyl U, in the putative TΨC loop (Felden et al., 1998). In addition to such a partial tRNA mimicry, the presence of the major identity determinants of $tRNA^{Ala}$, a G-U base pair at the third base pair position in the acceptor stem and an A at the discriminator position (Hou & Schimmel, 1988;

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Abbreviations: DB: downstream box; EF-Tu: elongation factor Tu; Pu: purine; Py: pyrimidine; RF-1: release factor 1.

McClain & Foss, 1988; Shi & Schimmel, 1991), enables specific aminoacylation of tmRNA by alanyl-tRNA synthetase in vitro (Komine et al., 1994; Ushida et al., 1994).

tmRNA has an additional domain, an mRNA domain, surrounded by four pseudoknot structures in the middle of this molecule. The 11-amino-acid tag sequence (AlaAlaAsnAspGluAsnTyrAlaLeuAlaAla), the last 10 amino acids of which are encoded in this domain, was first found at the C-terminus of a fraction of mouse interleukin-6 expressed in *Escherichia coli* (Tu et al., 1995). Later, it was also found on other polypeptides when they are translated from mRNAs lacking a termination codon (Keiler et al., 1996), possessing a cluster of rare codons (Roche & Sauer, 1999) or from mRNA for *E. coli* LacI (Abo et al., 2000) and on an in vitro translation product in the presence of tmRNA and poly (U) mimicking a truncated mRNA (Himeno et al., 1997).

It has been proposed that the molecular interplay between these two functions of this molecule facilitates an unusual translation reaction—*trans*-translation—in which a ribosome can switch from the translation of a truncated mRNA to the tag-encoded sequence of tmRNA. This would relieve stalled translation from mRNAs lacking a stop codon or possessing a cluster of rare codons with the addition of a specific tag peptide as a degradation signal to the truncated C-termini of polypeptides decoded (Atkins & Gesteland, 1996; Keiler et al., 1996; Muto et al., 1996, 1998; Karzai et al., 2000). This *trans*-translation model has also been supported by several other findings showing that the function as a tRNA is a prerequisite for the function as an mRNA in vitro (Himeno et al., 1997), that histidine is substantially incorporated into the in vitro tag-peptide synthesis directed by a histidine-accepting tmRNA mutant (Nameki et al., 1999c) and that tmRNA binds predominantly to 70S ribosomes (Ushida et al., 1994; Komine et al., 1996) but scarcely to polysomes (Tadaki et al., 1996). A series of these processes may promote recycling of ribosomes as well as prevent accumulation of abortively synthesized polypeptides, providing some advantage to the cell for survival (Huang et al., 2000; Muto et al., 2000).

Despite such extensive structural and functional studies, the mechanism by which tmRNA resumes translation from the first GCA codon on its own is poorly understood. It seems reasonable to assume that some structural unit on tmRNA fits elsewhere on the ribosome to settle the first codon on the A-site after the first translocation event. Indeed, the first pseudoknot structure (PK1) 12 nt upstream of the tag-encoding sequence is important for efficiency of *trans*-translation, but it does not contribute to the correct initiation of the tag translation (Nameki et al., 1999a, 1999b). Besides, extensive structural rearrangements in the three other pseudoknots have no or only marginal effects on the efficiency of tag translation with the reading frame unchanged (Nameki et al., 2000). Thus, the focus should be on the region between the first and the second pseudoknot structures. To address this issue, Williams et al. (1999) have attempted an in vivo selection of molecules active in *trans*-translation from a pool of *E. coli* tmRNAs having randomized sequences around the tag-initiation position. The resulting selectants have strong base preference at positions -4 and $+1$, and the A-to-U mutation at -4 inactivates the *trans*-translation ability in vivo. Considering phylogenetically high conservation of -4 to -2 as UAA or UAG, they proposed a possibility of involvement of release factor 1 (RF-1) during the *trans*-translation processes.

In the present study, we made various mutations around the initiation point of the tag-encoding sequence of *E. coli* tmRNA, and the effects were assessed by measuring the incorporations of amino acids into the in vitro poly (U)-dependent tag-peptide synthesis. This allowed us to specify the region required for correct initiation of *trans*-translation. In the course of this study, we found that several mutations upstream of the tag-encoding region extensively shift the tag-initiation point by -1.

RESULTS

Nucleotide insertions within the coding region

The tag-encoding region starts with G at position 90, 12 nt downstream of PK1 (Fig. 1). In the present study, various mutations were introduced around the tag-initiation point, and the effects on incorporations of alanine, arginine, lysine, serine, and threonine in the in vitro poly (U)-dependent tag-peptide synthesis system were examined. Based on the molar ratio of their amino acids incorporated, we can estimate the reading frame(s) of tag-peptide(s) synthesized in vitro. For example, in the presence of wild-type tmRNA, alanine was incorporated, but arginine, lysine, serine, and threonine were not. This indicates that wild-type tmRNA facilitates translation of a tag peptide of a homologous frame (frame 0) from a definite position (G90) in vitro (Fig. 2, top; Fig. 3A).

We first confirmed an earlier result (Nameki et al., 1999b) that an insertion of one adenosine into the oligo-A stretch just downstream of PK1 (85Ains; Fig. 2A) does not change the frame of tag translation (Fig. 3A). This indicates that the possible determinant for the tag-initiation point resides downstream of A84.

To further specify the region that determines the tag-initiation point, we introduced single-nucleotide insertions at positions 93, 96, and 99 (93Ains, 96Cins, and 99Cins, respectively; Fig. 2A). These single-nucleotide insertions within the tag-encoding region of tmRNA substantially changed the amino acid incorporations (Fig. 3A). For every one of these tmRNA mutants, the

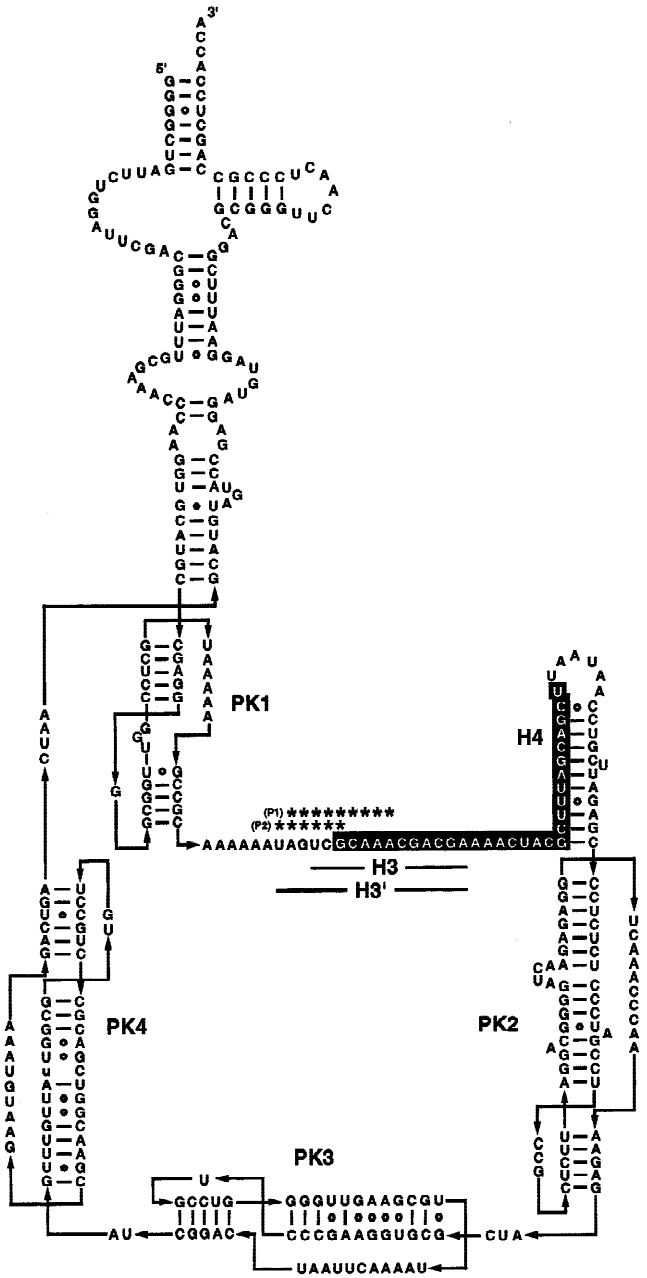


FIGURE 1. A secondary structure model of *E. coli* tmRNA. The tag-encoded sequence highlighted by white with a black background is surrounded by four pseudoknots (PK1–PK4). Asterisks indicate the sequences that can make two different types of potential base pairs with the anti-downstream sequence on 16S rRNA (P1 and P2). Non-Watson–Crick base pairs are shown by open circles. The putative H3 or H3′ helix is shown by a line under the corresponding sequence. The tmRNA sequence used in this study includes a G-to-U substitution at position 256 in PK4 shown by the lowercase character u, although it was found that it affects neither efficiency nor fidelity of tag-peptide translation.

molar ratio of amino acids incorporated for each mutant (the third column of Fig. 3A) matched the theoretical amino acid molar ratio, representing the amino acid composition, of the mutated tag peptide of frame 0 (the fifth column of Fig. 3A). This indicates that these nucleotide insertions did not change the initiation point. The levels of amino acid incorporations indicate that these nucleotide insertions did not significantly change the efficiency of tag-peptide synthesis. We then made an insertion of one guanosine residue just prior to the initiation site (90Gins; Fig. 2A). The molar ratio of amino acids incorporated matches the theoretical amino acid molar ratio of the mutated tag peptide of frame 0 (Fig. 3A). In addition, glycine, which can be uniquely encoded by the GGC triplet initiating with the inserted G, was incorporated in a stoichiometrical fashion (data not shown), indicating that tag-peptide synthesis was initiated from the inserted G. These results are consistent with an earlier in vivo study using insertion mutants at 90 and at 93 (Williams et al., 1999).

These results led to the conclusion that the possible determinant of tag initiation lies in the region upstream of the tag-initiation point and downstream of A84.

Nucleotide deletions around the initiation point of the coding region

We made single-nucleotide deletions at positions 84–89 (Fig. 2B), and the effects on incorporations of five amino acids in the in vitro poly (U)-dependent tag-peptide synthesis system were examined (Fig. 3B). Deletion of A84 (84Adel) caused only a slight decrease in tag-translation efficiency but did not change the frame, which is consistent with the previous results (Nameki et al., 1999b). This result supports the view that the possible determinant of tag initiation lies in the region downstream of A84.

On the other hand, deletions within 85–89 changed the ratio of amino acids incorporated. The ratio in each mutant does not match the theoretical amino acid molar ratio of any one of the three frames of tag peptides. This indicates that the base deletions caused aberrant tag initiation, producing heterologous tag peptides of two or three different reading frames in vitro. Based on the amino acid composition observed, we can roughly estimate the ratio of tag peptides from the three frames (Fig. 3B). The deletion mutant at 85 or 86 (85Udel or 86Adel, respectively) produced tag peptides of three different frames with almost equal proportions. As for the deletion mutant at 87 (87Gdel), the +1 frame product was predominant (~60%) and the −1 frame product was a minor component (~10%). The efficiency of translation of total tag peptides of all three frames for each of these three deletion mutants was estimated to be almost equivalent to that for the wild-type tmRNA. The efficiency of tag-peptide synthesis was slightly affected by deletion at 88 (88Udel), in which about half of the tag peptides were +1 frame products and the −1 frame products were scarcely detected. When deletion was made at 89 (89Cdel), the tag translation became less efficient and the +1 and −1 frame tag peptides were estimated to be about 30% and 10%, respectively.

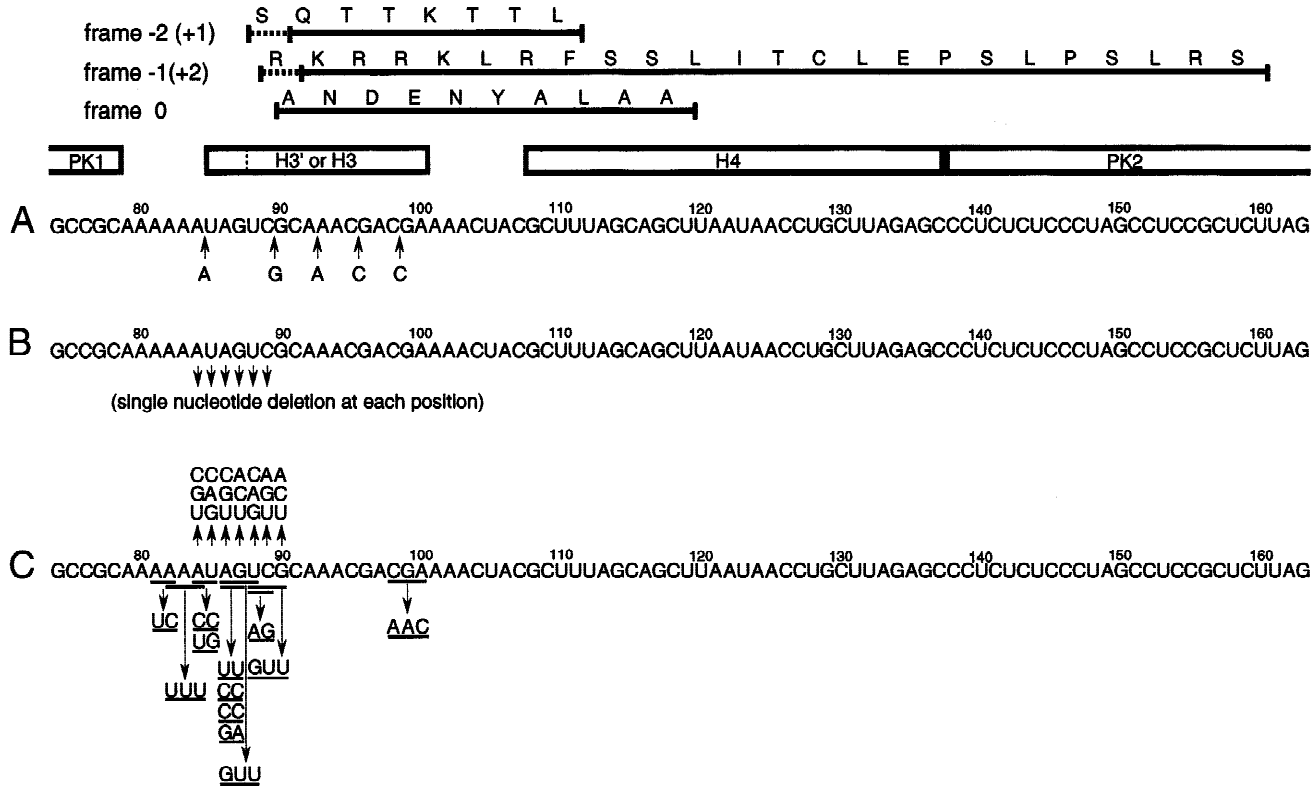


FIGURE 2. Nucleotide insertions (**A**), deletions (**B**), and base substitutions (**C**) made in this study. Not all of the multiple base substitutions are included in **C** (see Fig. 3B). The amino acid sequences of tag peptides translated from five possible starting points and the characteristic secondary structures (PK1, H3, H3′, H4, and PK2) are shown at the top of this figure.

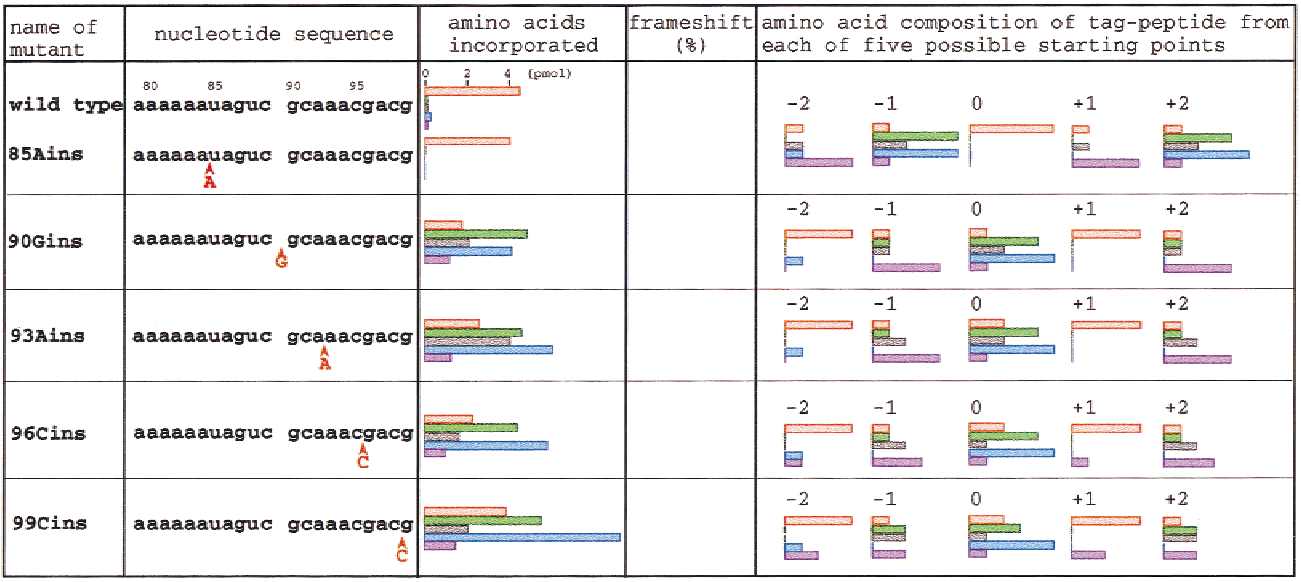
Base substitutions around the initiation point

Next, base substitutions were introduced upstream of the initiation point (Fig. 2C), and the effects on incorporations of five amino acids in the in vitro poly (U)-dependent tag-peptide synthesis system were examined (Fig. 4).

Many single-base substitutions upstream of the tag-initiation point caused a decrease in alanine incorporation (Fig. 4A). The positions 86, 87, and 88 (−4, −3, and −2, respectively, from the initiation point) are the sites in which base substitution most seriously affected the tag-translation efficiency. It has been shown that an A-to-U mutation at position 86 results in no detectable *trans*-translation product in vivo (Williams et al., 1999). In the present study, the *trans*-translation in vitro was decreased but not completely inactivated by an identical mutation. The most prominent effect was observed in the base substitution with C at this position, which caused only a trace level of *trans*-translation activity in vitro, whereas G or U was able to be changed with no or only a small loss of activity. Base substitutions at 84, 85, or 89 had less serious effects on the poly (U)-dependent alanine incorporation in vitro.

Some mutants clearly facilitated incorporations of arginine and serine, which are not included in the tag peptide of the normal frame (Fig. 4A,B). Judging from the apparent lack of threonine incorporation as well as an almost equivalent molar ratio of arginine and serine incorporated, almost all of these products are estimated to be a mixture of normal and −1 (or +2) frame tag peptides. A frameshift occurred more often in multiple point mutants (Fig. 4B). As the most prominent example, up to 60% of total tag translation by 84UG, a double mutant at 84 and 85, was estimated to be −1 (or +2) frameshifted. Frameshift was also observed in the tag translation for 81UC84UG, a four-point mutant at 81, 82, 84, and 85. The extent was similar to that for the 84UG mutant, suggesting that this frameshift was due simply to mutations at 84 and 85. Indeed, the 81UC mutation alone affected neither efficiency nor fidelity of *trans*-translation. It should be noted that neither 84U nor 85G mutation alone caused any detectable frameshift. Another double mutation at these positions, 84CC mutation, also caused no frameshift. Among the single-point mutations at 84 or 85 examined, only one single-point mutation (85A) caused a frameshift with high frequency. A frameshift was also observed in some two-point mutations at 86 and 87 (86UU and 86CC,

A



B

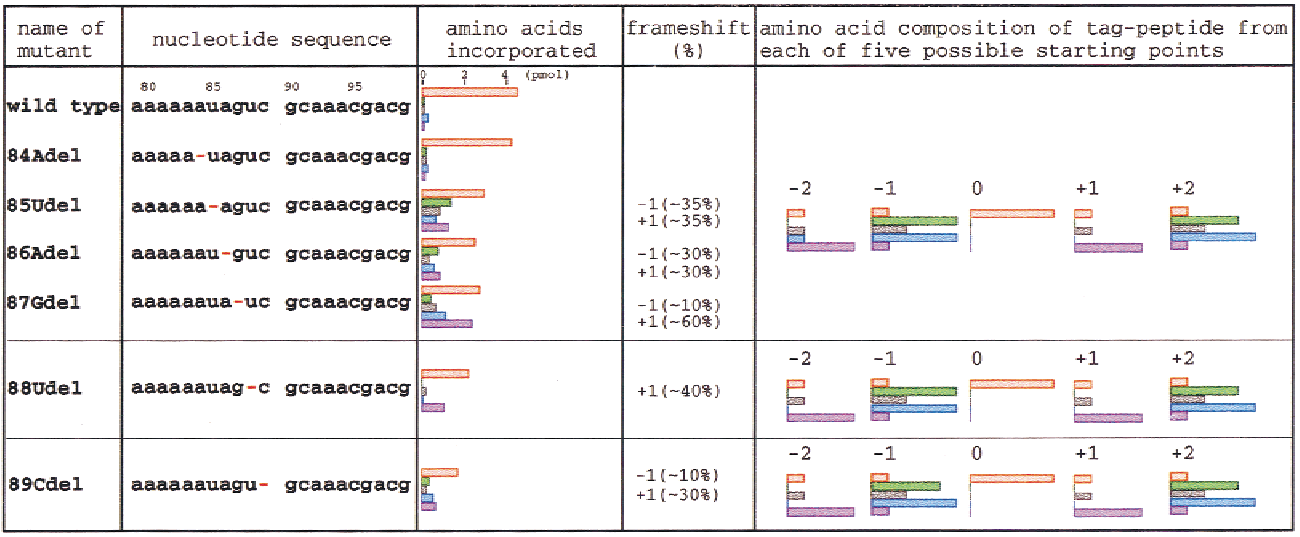


FIGURE 3. The effects of nucleotide insertion (**A**) and nucleotide deletion (**B**) around the tag-initiation region on in vitro poly (U)-dependent incorporations of alanine (red), arginine (green), lysine (grey), serine (blue), and threonine (violet). The positions of nucleotide deletion and the nucleotide insertion are designated by a red bar and a red arrow with a red uppercase character, respectively, in the second column. The length of the bar in the third column indicates the amount (mole) of each amino acid incorporated at 12 min after incubation. The mean value of at least three independent experiments is shown. Standard deviation was within 20%. The theoretical amino acid molar ratio, representing the amino acid composition, of tag peptide translated from each of five possible starting points (−2 to +2) is shown by the length of the bar in the fifth column. The frameshifting percentage shown in the fourth column can roughly be estimated from the data in the third column in consideration of the theoretical molar ratio of five amino acids in the fifth column.

respectively), although it was not detected in any of the single-point mutations at 86 or 87. A single-point mutation at 89 (89G) caused a −1 frameshift, whereas the 89A and 89U mutants almost exclusively produced a normal frame product with relatively higher efficiency.

Base substitution was also made at the starting point (position 90), which is phylogenetically occupied exclusively by G. The 90A mutation had no effect, whereas 90U and 90C mutations decreased the tag-translation activity. Threonine, serine, and proline were stoichiometrically incorporated for 90A, 90U, and 90C mutants, respectively, according to the genetic code rule (Fig. 4A and data not shown for 90C). No frameshift was detected in any single-point mutant at this position.

A

name of mutant	nucleotide sequence	amino acids incorporated	frameshift (%)	amino acid composition of tag-peptide from each of five possible starting points
wild type	aaaaaauaguc gcaaacgacg			
84G	aaaaaGuaguc gcaaacgacg			
84U	aaaaaUuaguc gcaaacgacg			
84C	aaaaaCuaguc gcaaacgacg			
85A	aaaaaaAaguc gcaaacgacg		-1(~45%)	
85G	aaaaaaGaguc gcaaacgacg			
85C	aaaaaaCaguc gcaaacgacg			
86C	aaaaaauCguc gcaaacgacg			
86G	aaaaaauGguc gcaaacgacg			
86U	aaaaaauUguc gcaaacgacg			
87A	aaaaaauaAuc gcaaacgacg			
87C	aaaaaauaCuc gcaaacgacg			
87U	aaaaaauaUuc gcaaacgacg			
88G	aaaaaauagGc gcaaacgacg			
88C	aaaaaauagCc gcaaacgacg			
88A	aaaaaauagAc gcaaacgacg			
89G	aaaaaauaguG gcaaacgacg		-1(~20%)	
89U	aaaaaauaguU gcaaacgacg			
89A	aaaaaauaguA gcaaacgacg			
90A	aaaaaauaguc Acaaacgacg			
90C	aaaaaauaguc Ccaaacgacg			
90U	aaaaaauaguc Ucaaacgacg			

FIGURE 4. The effects of single-point base substitutions (**A**) and multiple base substitutions (**B**) around the tag-initiation region on in vitro poly (U)-dependent incorporations of alanine (red), arginine (green), lysine (grey), serine (blue), and threonine (violet). The base of substitution is designated as a red uppercase character in the second column. The length of the bar in the third column indicates the amount (mole) of each amino acid incorporated at 12 min after incubation. The mean value of at least three independent experiments is shown. Standard deviation was within 20%. The theoretical amino acid molar ratio, representing the amino acid composition, of tag peptide translated from each of five possible starting points (−2 to +2) is shown by the length of the bar in the fifth column. The frameshifting percentage shown in the fourth column can roughly be estimated from the data in the third column in consideration of the theoretical molar ratio of five amino acids in the fifth column. (*Figure continues on facing page.*)

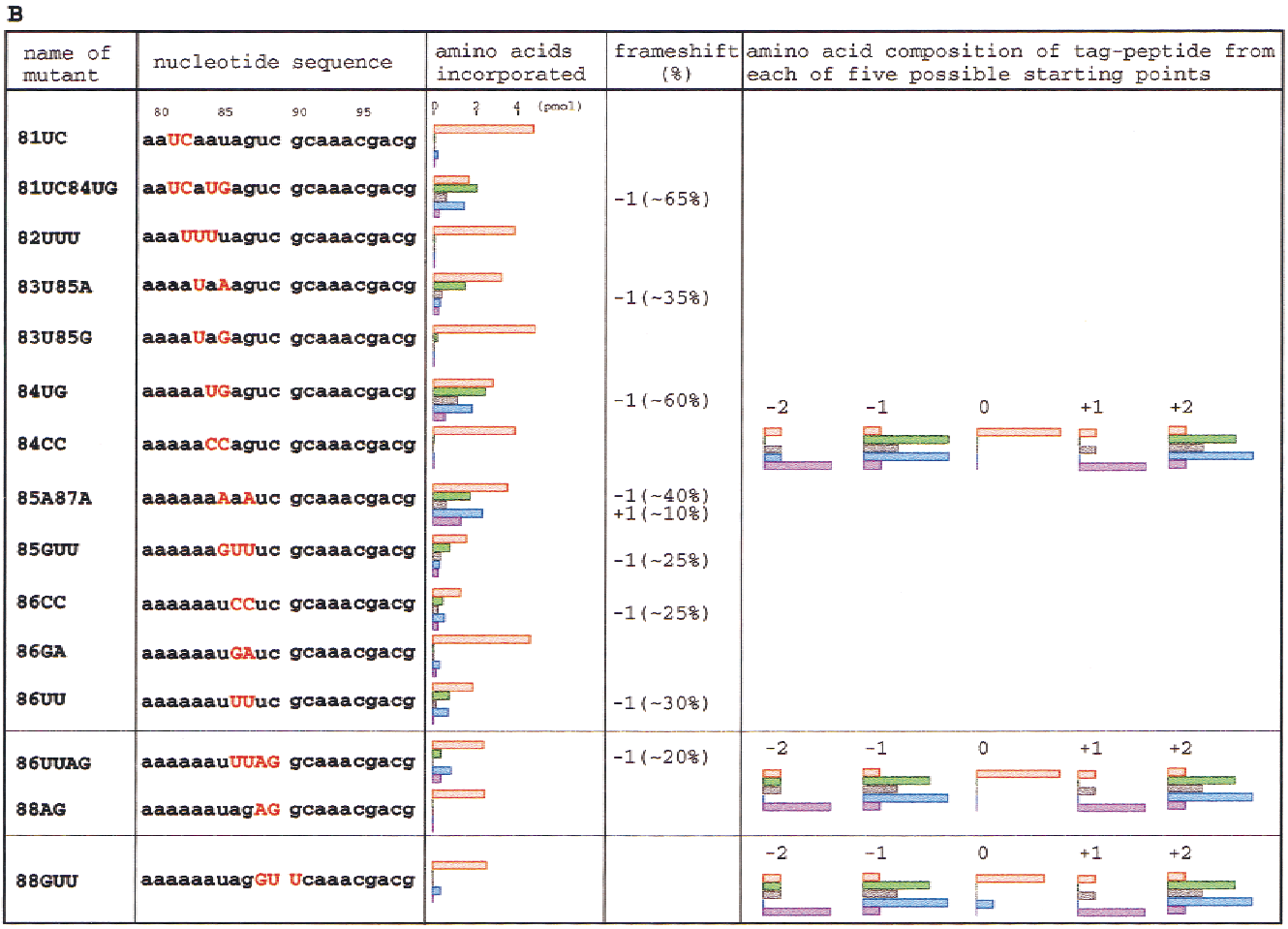


FIGURE 4. *Continued.*

Typical frameshift tag translations started from –1, not from +2

The data shown in Figure 4 suggest that most frameshift tag translations started from −1 rather than from +2. To confirm this, we made an additional G-to-A mutation at 90 into some frameshift mutants (84UG and 85A). This G-to-A mutation affects neither efficiency nor the frame of tag translation by itself (Fig. 4A), although it changes the presumed first arginine codon (C89G90C91) for the −1 frame tag peptide to a histidine codon (C89A90C91), which is a unique histidine codon in all of the possible three frame tag sequences. This makes it possible to identify more unambiguously whether the mutant-directed frameshift tag translation started from −1 or +2. If the frameshift tag translation started from +2, histidine would not be incorporated into the tag peptide. We examined the poly (U)-dependent incorporations of histidine and eight other amino acids for these two mutants, 84UG90A and 85A90A. Histidine incorporation was not detected for 84UG or 85A (data not shown), whereas it was significantly incorporated for 84UG90A or 85A90A (Table 1). The molar ratio of these incorporated amino acids including histidine for each mutant matched well the theoretical amino acid molar ratio of the −1 frame tag peptide. These results unequivocally demonstrate that the frameshift tag translation started from −1, not from +2, at least for the 84UG and 85A mutants. The tag peptide of the normal frame for 84UG90A or 85A90A was estimated to be only a minor component, and the proportion of the −1 frame tag to the total tag peptides (over 80%) was much higher than that for the 84UG or 85A mutant. This suggests that the G-to-A mutation at 90 has a potential to shift the frame of tag translation by −1 in some contexts.

A possible stem and loop structure covering the start point is not involved in *trans*-translation

Chemical and enzymatic probing studies, together with comparative sequence analysis, have suggested the presence of a stem and loop structure including the tag-initiation point (H3; positions 88–100; Figs. 1 and 2; Felden et al., 1997). An alternative, but mutually exclu-

TABLE 1. Incorporations of amino acids for the 84UG90A and 85A90A mutants in the in vitro poly (U)-dependent tag-peptide synthesis.

	Amino acid composition of tag peptide translated from each starting point					Amino acids incorporated (pmol)[a]		
Amino acid	−2	−1	0	+1	+2	Wild type	84UG90A	85A90A
Ala	1	1	4 (5)[b]	1	1	5.1	1.9	1.4
Glu	0	1	1	0	1	1.0	1.2	1.2
His	0	1 (0)[b]	0	0	0	0.1	1.0	1.2
Ile	0	1	0	0	1	0.0	1.2	1.0
Lys	1	2	0	1	2	0.1	2.8	2.3
Pro	0	2	0	0	2	0.1	1.8	1.9
Arg	0	4 (5)[b]	0	0	4	0.1	4.0	3.9
Ser	1	5	0	0	5	0.3	4.9	4.5
Thr	4	1	1 (0)[b]	4	1	0.1	0.8	0.9
Asp	0	0	1	0	0	1.0	0.4	0.4
Asn	0	0	2	0	0	1.9	0.5	0.4
Tyr	0	0	1	0	0	1.1	0.1	0.1

[a]Each value designates the in vitro poly (U)-dependent amino acid incorporation at 12 min after incubation. The mean value of at least three independent experiments is shown. Standard deviation was within 20%.
[b]Parentheses indicate the value for wild-type tmRNA.

sive, stem and loop structure is also possible (H3′; positions 85–100; Figs. 2 and 5A). To study whether such putative stem and loop structures play a role in *trans*-translation, several mutations were made at the stems of their structures (Figs. 2 and 5A).

A triple mutation in the 5′ side of the stem of H3 (88GUU), which is presumed to destabilize this stem, produced a homologous tag peptide of frame 0 with a slightly lower efficiency (Fig. 5B). Another triple mutation in the 5′ side of the stem of H3′ (85GUU), which destabilizes this putative stem, made the tag translation inefficient and heterologous. Approximately 25–30% of the total tag translation was estimated to be −1 frameshifted. The 98AAC mutant, which has a triple mutation in the other side of the stem of either H3 or H3′, produced a homologous tag peptide of frame 0 with an efficiency comparable to that of the wild-type tmRNA.

We then introduced mutations in both sides of the stem of H3 or H3′ (88GUU98AAC or 85GUU98AAC, respectively) to restore the stem and loop structure (Fig. 5A). Each of these compensatory mutations hardly changed the efficiency and frame of the tag-peptide translation directed by the single-side mutant, 88GUU or 85GUU (Fig. 5A,B). Apparently, either 88GUU or 85GUU mutation behaved independent of the 98AAC mutation. These results indicate that efficient and accurate *trans*-translation requires the primary structure upstream of the tag-encoding region but not the stem and loop structure of H3 or H3′, even if it actually exists.

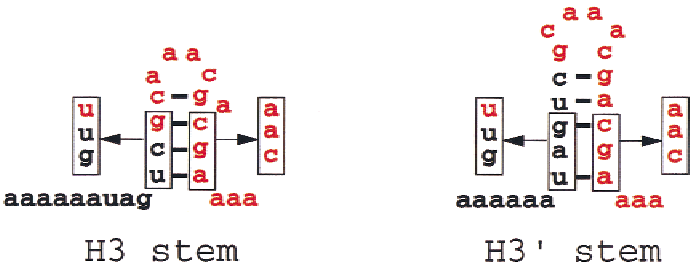
Aminoacylation for some *trans*-translation-defective mutants

Some mutants around the tag-encoding region had a decreased efficiency of tag-peptide synthesis in vitro. The tag translation can be hampered by the blockage of aminoacylation, for example, by the mutation just at the major alanine acceptor identity determinant (Himeno et al., 1997). We examined the ability of aminoacylation by alanyl-tRNA synthetase for several mutants, 84U, 85G, 86C, 87U, 88G, and 89G (Fig. 6). As expected, all of these mutants, including a *trans*-translation-defective mutant (86C), had efficiency of aminoacylation by alanyl-tRNA synthetase comparable to that of the wild-type tmRNA. These results indicate that the deficiency of tag translation is caused by the process after aminoacylation.

Ribosome-binding ability for a *trans*-translation-defective mutant

Next, we examined the ribosome binding properties in vitro for some of these mutants: 86C, having the most damaged *trans*-translation ability, and a typical initiation-shift mutant, 84UG. The reaction mixture of poly (U)-dependent *trans*-translation directed by each tmRNA mutant was fractionated by sucrose density gradient centrifugation, and then the distribution pattern of tmRNA was examined by northern hybridization. As shown previously (Himeno et al., 1997), a tmRNA signal was detected in the 70S ribosomal fraction as well as in the soluble fraction for the wild-type tmRNA, whereas it was found exclusively in the soluble fraction for a mutant (3A) defective in both aminoacylation and *trans*-translation (Fig. 7). The distribution pattern for 84UG was essentially the same as that of the wild-type tmRNA (Fig. 7). The pattern for 86C was also similar, although weak signals were detected over a wide range of fractions between the 70S fraction and the soluble fraction (Fig. 7). When the reaction proceeded under a lower (0.5 mM) magnesium condition in which the 70S ribosomes was dissociated to 50S and 30S subunits, 86C

A



B

name of mutant	nucleotide sequence	amino acids incorporated	frameshift (%)	amino acid composition of tag-peptide from each of five possible start points
wild type	aaaaaauaguc gcaaacgacga			
85GUU	aaaaaaGUUuc gcaaacgacga		-1(~25%)	
88GUU	aaaaaauagGU Ucaaacgacga			
98AAC	aaaaaauaguc gcaaacgaAAC			
85GUU98AAC	aaaaaaGUUuc gcaaacgaAAC		-1(~30%)	
88GUU98AAC	aaaaaauagGU Ucaaacgaac			

FIGURE 5. A: Mutations to disrupt the putative H3 (left) and H3′ (right) helices and compensatory mutations to restore these helices around the tag-initiation region. The tag-encoding sequence is shown in red. Mutations are shown by arrows. **B:** The effects of disruption or compensatory mutation of the putative H3 or H3′ helix on in vitro poly (U)-dependent incorporations of alanine (red), arginine (green), lysine (grey), serine (blue), and threonine (violet). The base of substitution is designated as a red uppercase character in the second column. The length of the bar in the third column indicates the amount (mole) of each amino acid incorporated at 12 min after incubation. The mean value of at least three independent experiments is shown. Standard deviation was within 20%. The theoretical amino acid molar ratio, representing the amino acid composition, of tag peptide translated from each of five possible starting points (−2 to +2) is shown by the length of the bar in the fifth column. The frameshifting percentage shown in the fourth column can roughly be estimated from the data in the third column in consideration of the theoretical molar ratio of five amino acids in the fifth column.

as well as wild-type tmRNA did not bind to any of the dissociated subunits (data not shown). These results indicate that the ribosome binding is not blocked but is affected to some extent by *trans*-translation-defective mutations around the tag-encoding region.

DISCUSSION

The region involved in efficient and accurate translation of tag peptide

In the present study, we made extensive mutations around the initiation point of the tag-encoding sequence of *E. coli* tmRNA. These mutations produced various effects, which are expected to provide a clue for understanding the mechanism underlying efficient and precise *trans*-translation from the specific GCA codon.

A series of base insertions in the 5′ region within the tag-encoding sequence did not shift the initiation site in the present study, explicitly indicating that the possible determinant lies upstream of the tag-encoding region. The present study also confirmed an earlier result that a single-nucleotide deletion or addition in the oligo A stretch just downstream of PK1 did not change the frame of tag translation. These observations allow the possible determinant of the tag-initiation point to be narrowed down to the region spanning 84–90.

Positions at which a single-base substitution caused a decrease in *trans*-translation efficiency were found to be concentrated within the span of 86 to 88. In par-

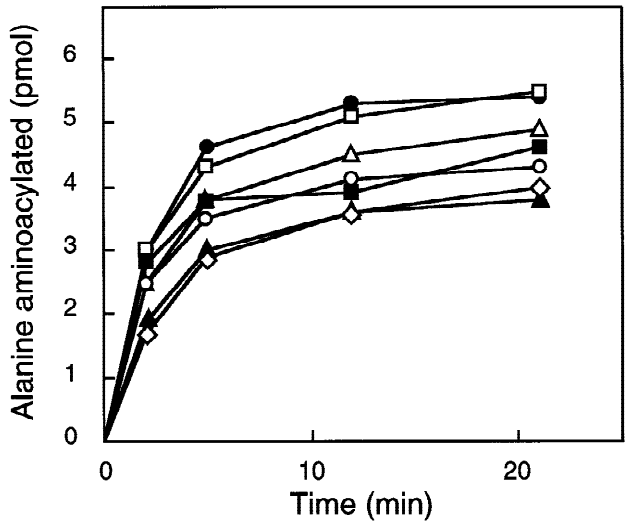


FIGURE 6. Aminoacylation by alanyl-tRNA synthetase for wild-type tmRNA (open circles), 84U (open rectangles), 85G (open triangles), 86C (solid circles), 87U (open diamonds), 88G (solid triangles), and 89G (solid rectangles). The mean value of at least three independent experiments is shown. Standard deviation was within 20%.

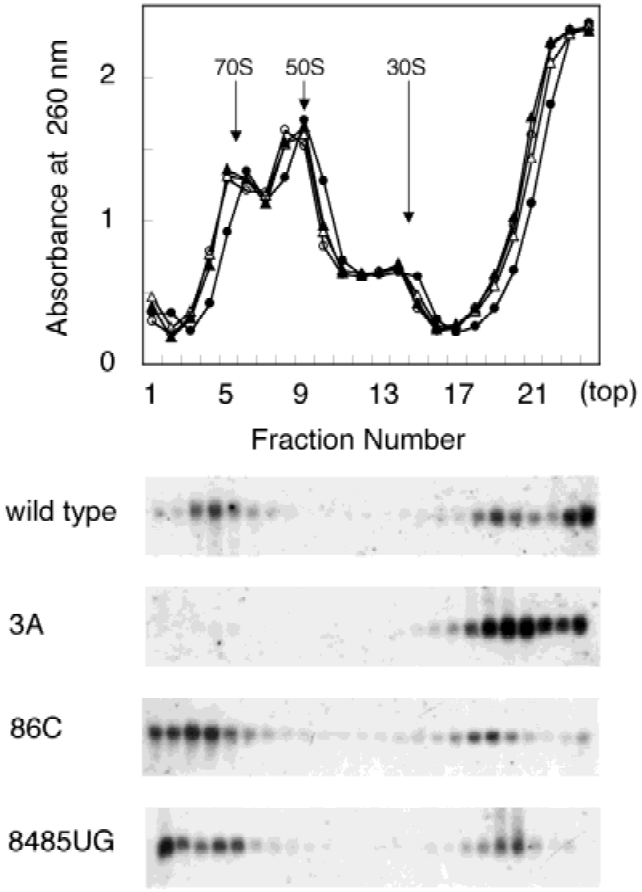


FIGURE 7. Binding of tmRNA variants to the ribosomes. The reaction mixture of in vitro *trans*-translation directed by wild-type tmRNA (solid circles), 3A (open triangles), 86C (solid triangles), or 84UG (open circles) was fractionated by centrifugation on a 5–20% linear sucrose density gradient. UV absorption at 260 nm of each fraction is shown (upper). tmRNA in each fraction was detected by northern hybridization using a 3′-digoxygenin-labeled oligodeoxyribonucleotide complementary to a portion of the tmRNA sequence (lower).

ticular, an A86-to-C86 mutant had only faint *trans*-translation activity. At positions 86 or 87, the other purine base can be substitutable without any significant loss of activity. On the other hand, a relatively wide range of positions (84–89) surrounding A86G87U88 have a potential to shift the starting point by base substitution and sometimes by only a single-base substitution. It is thus concluded that the span of 84 to 89 (-6 to -1) functions as a determinant for initiation of tag translation and that the central 3 nt, A86G87U88 (A-4G-3U-2), occupy the essential part. Initiation shifting was prominent for U85 to A85, A84U85 to U84G85, A86G87 to U86U87, A86G87 to C86C87, and C89 to G89 mutations. Almost all of these initiation shifts caused by base substitution(s) were -1 initiation shifts.

Although no tag peptide except those from *E. coli* and *Caulobacter crescentus* has been sequenced (Tu et al., 1995; Keiler et al., 2000), the reading frame and the initiation point of the tag peptide encoding a sequence from other sources can be deduced from alignment of these tmRNA sequences (Williams et al., 1999; Zwieb et al., 1999; Williams, 2000; Zwieb & Wower, 2000). The A-6U-5A-4G-3U-2C-1G1 sequence, which was identified as the determinant for the initiation point of *E. coli* tmRNA in the present study, is widely conserved as the PuUAPuPuNPyG sequence that can be positioned at -6 to $+1$ when it is aligned in the frame of the C-terminal highly conserved sequence required for proteolysis (Keiler et al., 1996; Gottesman et al., 1998; Herman et al., 1998). The relatively high sequence conservation suggests that this upstream sequence universally functions as a determinant for tag initiation. According to this alignment, the base at 86 is strongly biased to A, while G is almost comparably preferred in the present in vitro system of *E. coli*. U88, one of the core nucleotides indicated by the present study, is less phylogenetically conserved. The start nucleotide G is completely conserved, although it makes no significant contribution to the tag-peptide translation in *E. coli*. The present results demonstrate that $tRNA^{Ala}$ is substitutable with $tRNA^{Ser}$, $tRNA^{Thr}$, $tRNA^{Pro}$, $tRNA^{Gly}$, or $tRNA^{His}$ as the first tRNA to decode the tag sequence according to the genetic code rule.

Williams et al. (1999) have selected 23 molecules active in *trans*-translation in vivo from a pool of *E. coli* tmRNAs having randomized sequences around the tag-initiation position. Although each of these selectants has multiple mutations, base preference appears strongly at positions 86 and 90, and mildly at 85 and 88. At position 85, A is not selected. Note that 85A mutant was found to be the most prominent initiation-shifting mutant in the present study. A is predominant and two selectants have G at position 86, and G has

not been selected at position 88. These are consistent with the present in vitro results. At position 90, G is preferentially but not exclusively selected, which is also consistent with the present in vitro results showing that G is preferable at this position but is not absolutely required.

Possible molecular mechanism of *trans*-translation indicated by the present mutation effects

Even the C86 mutant, the mutant having the most damaged *trans*-translation ability in the present study, retained a normal aminoacylation ability, indicating that the mutations in the mRNA domain affect some processes of *trans*-translation after the aminoacylation step. Alanylated tmRNA has been shown to be specifically recognized by elongation factor Tu (EF-Tu; Rudinger-Thirion et al., 1999; Barends et al., 2000). The present mutations in the mRNA domain, however, do not seem to affect the binding to EF-Tu, as the upper half of the tRNA molecule is sufficient for recognition by EF-Tu (Nissen et al., 1995). On the other hand, the process of binding of the first tRNA into the first codon of tmRNA at the A-site may not be significant, judging from the apparent redundancy of the start nucleotide. Thus, the processes after the EF-Tu binding step and before the first tRNA binding step at the A-site should be focused on. It was found that the 86C mutant still retains the ability to bind 70S ribosomes to a considerable extent and that this mutation also causes a slight increase in tmRNA over a wide range of fractions between the 70S fraction and the soluble fraction in sucrose density gradient centrifugation. This may reflect the accumulation of tmRNA-related complex(es) prior to the ribosome binding due to inefficient association between this complex and ribosome or to inefficiency in the process after ribosome binding. Alternatively, it may merely reflect unstable association between the 86C mutant and ribosome.

The present functional results provide several insights into the yet-to-be identified ribosomal processes. The sequence upstream of the tag starting point, especially the core A86G87U88 sequence, may interact with the decoding region or its periphery of the ribosome directly or via *trans*-acting factor(s). Alternatively, the core sequence may have an intramolecular interaction with another part of tmRNA to make some structural feature, introducing the first GCA codon into the A-site after the first translocation. Some base substitutions or deletions within the core sequence would not allow such a normal intermolecular or intramolecular interaction, resulting in a reduced efficiency of *trans*-translation or a failure of the normal selection of the initiation point. Some mutations around the core sequence would promote an irregular interaction, leading to the shift of the starting point. For example, the preferential use of the −1 shifted frame for the 84UG mutant-directed tag translation can be attributed to the confusion of the altered sequence A83U84G85A86 with the original A84U85A86G87 sequence at the −1 shifted position. As for the 85A mutant, the altered A85A86 sequence can be confused with A86G87.

A direct interaction between the anti-downstream box (anti-DB) sequence around the decoding region on the penultimate stem of 16S rRNA and a sequence around the initiation point on *E. coli* tmRNA has been postulated on the basis of their sequence complementarity (Muto et al., 1996, 1998). The DB sequence downstream or sometimes overlapping the initiation codon on mRNA, which is complementary to the anti-DB (Sprengart et al., 1990), is often required for efficient initiation of translation like the Shine–Dalgarno sequence, whereas the enhancement of translation efficiency involves a yet-unidentified interaction with ribosome rather than base pairing with the anti-DB sequence (O'Connor et al., 1999; La Teana et al., 2000). As for tmRNA, two alternative complementarities, overlapping each other, have been found against the DB sequence of 16S rRNA (Muto et al., 1998). The span of 86–94 on tmRNA can make 9 bp with 1470–1478 on 16S rRNA (type P1), and 85–90 on tmRNA can make 6 bp with 1481–1486 on 16S rRNA (type P2; Fig. 1). Many mutations in the present study should affect these putative base pairings, thereby allowing the evaluation of their validities. Both types of interactions cover the core 3 nt, A86, G87, and U88, significant for tag translation. Some but not all initiation shifts can be explained by unusual and/or unstable association of this base pairing. For example, single-nucleotide deletions in 86–89 can cause a fluctuated base pairing of P1 or P2, consequently leading to heterologous starting points of tag translation. The results of a UV-crosslinking study support the notion of the presence of some interaction between tmRNA and 16S rRNA (Komine et al., 1996). On the other hand, poor phylogenetic conservation of these complementarities argues against this sort of interaction (Williams et al., 1999; Zwieb et al., 1999). A recent review has commented that alteration of the sequence of 1470 to 1481 of 16S rRNA does not affect the tmRNA function in vivo, which can exclude the possibility of P1 (O'Connor et al., 2000). Further in vivo and in vitro studies using this kind of mutant ribosomes will clarify whether the proposed P2 interaction is actually required either for efficient *trans*-translation or for correct *trans*-translation.

The above considerations emphasize the involvement of an alternative and/or additional interaction with ribosomes either directly or via a *trans*-acting factor in determination of the tag-initiation point. Williams et al. (1999) suggested the possibility that the U85A86G87 sequence is recognized by RF-1 during the *trans*-translation processes based on the functional significance of A86 and the high conservation as UAA or

UAG. The present results showed base preference of A or G at either the second or third position, as well as that of U at the position next to this triplet (Poole et al., 1998), supporting the involvement of release factor(s). However, the finding in the present study that U85 is of relatively small significance does not support this possibility, in the light of the high accuracy of recognition of the first U of the termination codons by release factors (Freistroffer et al., 2000).

SmpB, a tmRNA-binding protein essential for *trans*-translation (Karzai et al., 1999, 2000), is a likely candidate as a *trans*-acting factor to determine the tag-initiation point. It has been shown that the depletion of SmpB results in an unsuccessful binding of tmRNA to ribosome, although the mode of interaction between SmpB and tmRNA has not yet been identified. Another candidate is the ribosomal protein S1, which can cross-link with a relatively wide area of the lower half of tmRNA, including PK2, PK3, PK4, and U85 (Wower et al., 2000). It has recently been shown that SmpB and S1 together with several other protein factors form a complex with tmRNA in *E. coli* cell (Karzai & Sauer, 2001).

PK1 is indeed a functional pseudoknot (Nameki et al., 1999a, 1999b). It may be required to introduce the tag-initiation region into the proximity of the decoding region, although correct initiation of tag translation also requires the upstream sequence as revealed by the results of the present study. It has also been shown that none of the other three pseudoknots are involved either in efficiency or in fidelity of *trans*-translation (Nameki et al., 2000). The 3′ half of the tag-encoding region is included in the H4 helix (Fig. 1), which is significant only for the stability of the whole molecule (H. Himeno, N. Nameki, T. Tadaki, & A. Muto, unpubl. results). The present results do not support the notion of involvement of the proposed H3 or H3′ helix just covering the initiation point. Nevertheless, any structural effect should be helpful to rationalize the present findings. Further study on intramolecular tertiary interactions and intermolecular interactions directly or indirectly with ribosomes is needed to elucidate the mechanism that allows the proper arrangement of two apparently distant domains necessary for functional interplay.

MATERIALS AND METHODS

Overproduction of tmRNA mutants

Mutations were introduced by primer-directed polymerase chain reaction, and the amplified DNA fragment was ligated under the T7 RNA polymerase promoter sequence of the plasmid pGEMEX-2. The DNA sequence was confirmed by dideoxy sequencing using a fluorescence DNA sequencer (Hitachi SQ-5500; Messing, 1983). This plasmid was cotransformed with pACYC184 encoding the T7 RNA polymerase gene under the *lac*-promoter sequence into *E. coli* strain W3110 Δ*ssrA*, which lacks the tmRNA gene (Komine et al., 1994). tmRNA induced by the addition of 1.0 mM isopropyl-1-thio-β-D-galactopyranoside was purified as described previously (Ushida et al., 1994).

Preparation of tmRNA mutants

The nucleic acid fraction was extracted with phenol from mid-log-phase cells followed by ethanol precipitation. After performing phenol extraction and ethanol precipitation, the resulting fraction was subjected to differential isopropyl-alcohol precipitations to roughly remove DNA, followed by incubation with RNase-free DNase I (Pharmacia). tmRNA was purified by electrophoresis on a 5% polyacrylamide gel containing 7 M urea. Spectrophotometric measurements were made to determine the concentration of RNA.

In vitro amino acid incorporations in the presence of poly (U)

The preincubated S30 fraction was prepared from middle-log-phase cells of *E. coli* strain W3110 (Δ*ssrA*), as described previously (Oba et al., 1991). The 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 (6.7 GBq/mmol), L-[U-^{14}C]arginine (11.8 GBq/mmol), L-[U-^{14}C]asparagine (7.3 GBq/mmol), L-[U-^{14}C]aspartic acid (7.7 GBq/mmol), L-[U-^{14}C]glutamic acid (10.4 GBq/mmol), [U-^{14}C]glycine (4.2 GBq/mmol), L-[U-^{14}C]histidine (11.1 GBq/mmol), L-[U-^{14}C]isoleucine (12.7 GBq/mmol), L-[U-^{14}C]lysine (11.7 GBq/mmol), L-[U-^{14}C]proline (9.3 GBq/mmol), L-[U-^{14}C]serine (6.3 GBq/mmol), L-[U-^{14}C]threonine (7.7 GBq/mmol), L-[U-^{14}C]tyrosine (16.7 GBq/mmol), or L-[U-^{14}C]valine (10.5 GBq/mmol), and 0.05 mM each of the remaining unlabeled 19 amino acids, 5 μM tmRNA (when 1 A_{260} unit corresponds to 330 pmol), and 20 μL of the S30 fraction, in the presence of 250 μg of poly (U) (50 to 100 mer, Sigma). Each tmRNA variant was used in the reaction without any refolding procedure after purification from the gel. The reaction mixture was incubated at 37 °C. At each time point, a 23-μL aliquot was withdrawn from a 100-μL reaction mixture and spotted on Whatman 3MM filter paper, and radioactivity in the hot trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter. The final value of poly (U)-dependent amino acid incorporation was obtained by subtracting the value in the absence of poly (U) from that in the presence of poly (U).

Aminoacylation with alanine

Alanyl-tRNA synthetase was partially purified from *E. coli* strain Q13 with DEAE-Toyopearl 650 (Tosoh, Tokyo) and subsequent hydroxyapatite column chromatography (Gigapite, Seikagaku Corporation, Tokyo; Tamura et al., 1991). The final enzyme fraction from *E. coli* had a specific activity of 9.1 U/mg, when 1 U 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, 1.0 μM tmRNA mutants, and 9.1×10^{-2} U of alanyl-tRNA synthetase. At the times specified, a 12-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.

Interaction with ribosomes

After 12-min incubation at 37 °C, 100 μL of reaction mixture containing 50 mM Tris-HCl, pH 7.8, 7 mM magnesium acetate, 60 mM ammonium chloride, 300 mM potassium chloride, 2.5 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 5 mM phosphoenol pyruvate, 2.5 μg pyruvate kinase, 0.05 mM each of 20 amino acids, 3.2 μg tmRNA, 250 μg poly (U) and 20 μL of the S30 fraction were immediately loaded on a 5–20% linear sucrose density gradient containing 10 mM Tris-HCl, pH 7.8, 10 mM magnesium chloride and 300 mM potassium chloride, and were centrifuged at 25,000 rpm for 5 h at 4 °C. Nucleic acids prepared from each fraction by phenol extraction and ethanol precipitation were separated by electrophoresis on a 1.5% agarose gel containing 6.3% formaldehyde, and were then blotted onto a nylon membrane. tmRNA was detected by northern hybridization using a 3′-digoxygenin-labeled oligodeoxyribonucleotide (Ushida et al., 1994) complementary to a portion of the tmRNA sequence (nt 251–nt 280).

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