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IDENTITY DETERMINANTS OF *E. coli* THREONINE tRNA

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SUMMARY: To investigate the identity determinants of *E. coli* threonine tRNA, various transcripts were prepared by *in vitro* transcription system with T7 RNA polymerase. Substitutions of the anticodon second letter G_{35} and the third letter U_{36} to other nucleotides led to a remarkable decrease of threonine charging activity. Charging experiments with a series of anticodon-deletion transcripts also suggest the importance of the $G_{35}U_{36}$ sequence. A mutation at either the G_1-C_{72} or C_2-G_{71} base pair in the acceptor stem seriously affected the threonine charging activity. These results indicate that the second and third positions of the anticodon and the first and second base pairs in the acceptor stem are the recognition sites of *E. coli* $tRNA^{Thr}$ for threonyl-tRNA synthetase. Discriminator base, A_{73}, is not involved in threonine charging activity. © 1992 Academic Press, Inc.

Precise molecular recognition of tRNAs by cognate aminoacyl-tRNA synthetases is essential to insure the fidelity of the translation system. Anticodons of many tRNAs are the crucial sites for discrimination by cognate and noncognate aminoacyl-tRNA synthetases (1-4, and cited therein) except $tRNA^{Ala}$ (5-7) and $tRNA^{Ser}$ (8). By exchanging the anticodon from CAU to GGU, *E. coli* elongator $tRNA^{Met}$ acquires threonine charging activity *in vitro* (9), indicating that the anticodon of *E. coli* $tRNA^{Thr}$ is a major recognition site for threonyl-tRNA synthetase. Although four isoacceptor threonine tRNAs are encoded in the *E. coli* genome (10,11), only one species, $tRNA^{Thr}_3$, has been sequenced at the RNA level (12). Consensus bases in all the four $tRNA^{Thr}$ isoacceptors including the three tRNA genes are localized on the acceptor stem and anticodon. Considering these facts, we attempted to examine the identity determinants of *E. coli* $tRNA^{Thr}$. To evaluate the contributions of the anticodon, the acceptor stem and the discriminator base, we constructed *E. coli* $tRNA^{Thr}$ variants using an *in vitro* transcription system with T7 RNA polymerase.

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MATERIALS AND METHODS

Materials

Native *E. coli* $tRNA^{Thr}_3$(GGU) having a specific activity of 1600 pmol/A_{260} was obtained from Subriden RNA. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. T7 RNA polymerase was purified from *E. coli* strain BL21 (13). Threonyl-tRNA synthetase was partially purified from *E. coli* strain Q13.

Plasmid construction and transcription

Ligation product from eight overlapping oligodeoxyribonucleotides was inserted into plasmid pUC19 and the resulting DNA was used to transform *E. coli* strain JM109 as described previously (14-16). The template DNA sequences were confirmed by dideoxy sequencing (17). Each template DNA of the acceptor stem base pair- or discriminator base-substituted variant was prepared from the plasmid carrying the normal $tRNA^{Thr}$ sequence and synthetic primers, by mutation by polymerase chain reaction (PCR) (18). Plasmid DNA digested with BstNI was used as the transcription template (14-16). The transcripts were purified by 20% polyacrylamide gel electrophoreses.

Aminoacylation assay

Aminoacylation was performed at 37 °C in 50 μl of a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 20 μM L-[U-^{14}C]threonine (231 mCi/mmol), 2.5 mM ATP, 10 mM magnesium chloride, 2 mM dithiothreitol and 0.1 mg/ml bovine serum albumin, and various concentrations of transcript tRNA and partially purified threonyl-tRNA synthetase. Kinetics were studied using various concentrations of threonyl-tRNA synthetase and tRNAs (0.04-1.0 μM for native $tRNA^{Thr}_3$, 0.04-2.0 μM for the wild type and N_{73} replaced transcripts, 0.1-10 μM for the other transcripts).

RESULTS AND DISCUSSION

The transcripts of the *E. coli* $tRNA^{Thr}_3$ derivative are summarized in Figure 1(A). The unmodified transcript having the wild type sequence of native $tRNA^{Thr}_3$(GGU) showed almost the same level of threonine acceptance and similar kinetic parameters as native *E. coli* $tRNA^{Thr}_3$ (Table 1 and Figure 2). Schulman and Pelka demonstrated that alteration of the anticodon CAU by GGU changes the aminoacylation specificity of *E. coli* elongator $tRNA^{Met}$ from methionine to threonine (9). This result indicates that the second and/or first letters of anticodon are a primary recognition site for threonyl-tRNA synthetase as well as for methionyl-tRNA synthetase. In the methionine system, the first letter C is also a decisive base (19). The first letter of $tRNA^{Thr}$ seems unlikely to be involved in recognition by threonyl-tRNA synthetase, since it varies among *E. coli* $tRNA^{Thr}$ isoacceptors (G, U or C) (10,11). To elucidate the involvements of the second and third letters of the anticodon in threonine charging activity, various anticodon deletion- and substitution-mutant transcripts of $tRNA^{Thr}$ were constructed (Figure 1(A) and Table 1). Transcripts lacking all three or any two of the anticodon nucleotides had no threonine acceptance (data not shown), whereas that lacking only one nucleotide in either position of the anticodon retained a significant level of threonine acceptance, although the kinetic parameters were seriously affected (Table 1). One nucleotide deletion in the anticodon was expected to cause considerable alteration of tertiary structure of the anticodon loop. However, the single nucleotide deletion-transcripts retained threonine charging activity, although at low activity (Table 1). The single nucleotide deletion at the first or second position yielded a UGU sequence in the new unusual anticodon loop comprising six

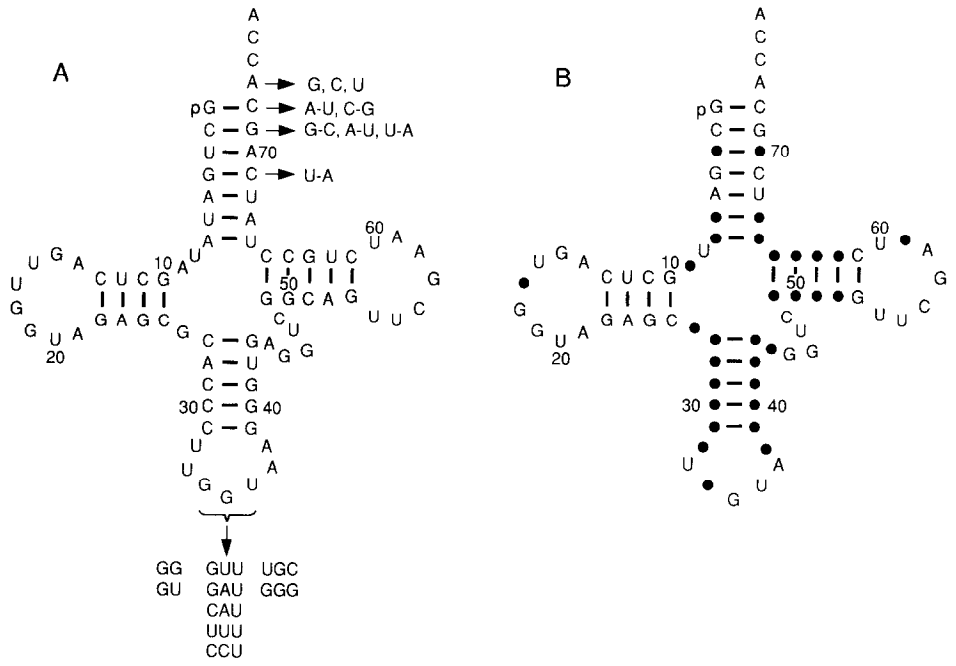

Figure 1. (A) Transcripts of *E. coli* $tRNA^{Thr}_3$ derivatives. Arrows indicate the substitutions and deletions made in this study. Numbering of nucleotides is according to (10). (B) Composite structure of nucleotides common to *E. coli* threonine tRNAs including the threonine tRNA genes (10,11). Dots indicate position of sequence variation. Base modifications are ignored in compiling the conserved sites.

nucleotides, whereas that at the third position did not. This would produce a difference in threonyl-tRNA synthetase recognition between the two mutants. This study shows that all seven nucleotides of the anticodon loop in $tRNA^{Thr}$ are not required for recognition by threonyl-tRNA synthetase. A similar result has been reported in the anticodon deletion-mutants of *Bacillus subtilis* $tRNA^{Thr}$ (anticodon, mo^5UGG) constructed by molecular microsurgery (20). For these one letter deletion-transcripts, the increase of Km value is more apparent than the decrease of Vmax.

The contribution of the anticodon sequence for recognition by threonyl-tRNA synthetase was examined using various anticodon substituted variants of $tRNA^{Thr}$ (Figure 1). Replacement of the anticodon of $tRNA^{Thr}$ from GGU to either GUU, GAU, CAU, UUU or CCU seriously affected threonine charging activity (Table 1). Only a transcript having GUU had faint but measurable activity. Because the sequence alignment of the isoacceptors can exclude the first letter of the anticodon from the recognition element, it is concluded that the second letter G_{35} of the anticodon of $tRNA^{Thr}$ is a powerful recognition site for threonyl-tRNA synthetase. Exchanging the anticodon of $tRNA^{Thr}$ from GGU to UGC also affected its activity, but replacement by GGG was less effective (Table 1 and Figure 2). These results suggest that the third letter U_{36}, was recognized by threonyl-tRNA synthetase with some ambiguity. Similar observations were reported for *E. coli* $tRNA^{Arg}$ and $tRNA^{Lys}$ (K. Tamura *et al.*, submitted for publication), and also for the *E. coli* elongator $tRNA^{Met}$ transcript, where the CAU to CAG change at the anticodon retained a significant level of methionine acceptor activity (21). Presumably, the U and G in the third position

Table 1. Kinetic parameters of aminoacylation for the transcripts with *E. coli* threonyl-tRNA synthetase

Transcripts	Km (μM) (apparent)	Vmax (relative)	Vmax/Km (relative)
Native $tRNA^{Thr}_3$ (GGU)	0.059	1.0	1.0
Transcripts			
$tRNA^{Thr}$(GGU)	0.060	1.0	1.0
$tRNA^{Thr}(G_{73})$	0.053	0.89	1.0
$tRNA^{Thr}(C_{73})$	0.045	0.75	1.0
$tRNA^{Thr}(U_{73})$	0.030	0.71	1.4
$tRNA^{Thr}(G_{34}$ or G_{35} deletion)	7.3	0.040	3.3×10^{-4}
$tRNA^{Thr}(U_{36}$ deletion)	21	0.044	1.3×10^{-4}
$tRNA^{Thr}$(GUU)	1.7	0.013	4.6×10^{-4}
$tRNA^{Thr}$(GAU)	-	-	$<1.0 \times 10^{-5}$
$tRNA^{Thr}$(CAU)	-	-	$<1.0 \times 10^{-5}$
$tRNA^{Thr}$(UUU)	-	-	$<1.0 \times 10^{-6}$
$tRNA^{Thr}$(CCU)	-	-	$<1.0 \times 10^{-6}$
$tRNA^{Thr}$(UGC)	-	-	$<1.0 \times 10^{-6}$
$tRNA^{Thr}$(GGG)	0.8	0.16	0.012
$tRNA^{Thr}(G_2\text{-}C_{71})$	2.0	0.060	1.8×10^{-3}
$tRNA^{Thr}(A_2\text{-}U_{71})$	6.5	0.027	2.5×10^{-4}
$tRNA^{Thr}(U_2\text{-}A_{71})$	2.2	0.23	6.0×10^{-3}
$tRNA^{Thr}(U_4\text{-}A_{69})$	0.21	0.64	0.18
$tRNA^{Thr}(A_1\text{-}U_{72})$	0.74	0.15	0.012
$tRNA^{Thr}(C_1\text{-}G_{71})$	1.9	0.064	2.0×10^{-3}
$tRNA^{Thr}$(GGG, $A_2\text{-}U_{71}$)	-	-	$<1.0 \times 10^{-6}$
$tRNA^{Thr}$(GUU, $A_2\text{-}U_{71}$)	-	-	$<1.0 \times 10^{-6}$

Each parameter was determined from a Lineweaver-Burk plot.

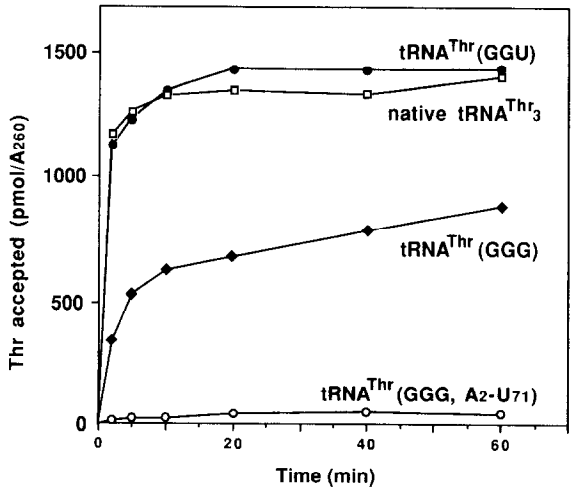

Figure 2. Aminoacylation of native $tRNA^{Thr}_3$ and the $tRNA^{Thr}$ transcripts possessing the wild type sequence (GGU), the altered anticodon (GGG), and the altered anticodon and acceptor stem (GGG, $A_2\text{-}U_{71}$) with *E. coli* threonyl-tRNA synthetase.

may present a common structural feature such as the 4-keto and 3-imino groups of U, and the 6-keto and 1-imino groups of G for recognition by synthetase. Theobald *et al.*, by means of chemical and enzymatic footprinting analyses, demonstrated that the first and second GG sequence of the anticodon of *E. coli* $tRNA^{Thr}_3$ was strongly protected by threonyl-tRNA synthetase, but that the third U was not. (22).

All conserved bases within the four $tRNA^{Thr}$ isoacceptors including the three tRNA genes are summarized in Figure 1(B). Several conserved base pairs within the $tRNA^{Thr}$ isoacceptors are localized on the acceptor stem. The contribution of these base pairs to the threonine acceptor identity was determined as listed in Table 1. Replacement of the C_2-G_{71} base pair with either G-C, A-U or U-A markedly affected the threonine acceptor activity. The Vmax/Km values of all the substituted transcripts were decreased by two to four orders of magnitude, indicating that the C_2-G_{71} base pair is responsible for recognition by threonyl-tRNA synthetase. Replacement of the G_1-C_{72} base pair with A-U or C-G also resulted in a marked decrease of Vmax/Km. In *E. coli*, up to fifteen amino acid-specific tRNAs possess this G_1-C_{72} base pair. $tRNA^{Gly}$, $tRNA^{Phe}$, $tRNA^{Leu}$, $tRNA^{Arg}$ and $tRNA^{Val}$ also possess C_2-G_{71} base pair in addition to G_1-C_{72} (10,11). The difference in the second letter of the anticodon would significantly contribute to threonyl-tRNA synthetase discrimination against these five tRNA species. Exchange of the G_4-C_{69} base pair with U-A less seriously affected than substitution of the C_2-G_{71} or G_1-C_{72} pairs. Substitution of A_5-U_{68}, another conserved base pair in the acceptor stem of *E. coli* $tRNA^{Thr}$ isoacceptor, with U_5-A_{68} did not affect its activity (data not shown). This is consistent with a previous observation that *B. subtilis* $tRNA^{Thr}$ having G_5-C_{68} is efficiently charged with threonine by *E. coli* threonyl-tRNA synthetase (23). These results clearly indicate that the precise recognition of $tRNA^{Thr}$ by threonyl-tRNA synthetase requires these two base pairs, C_2-G_{71} and G_1-C_{72}, besides the anticodon.

The $tRNA^{Thr}$ transcript which substituted the anticodon third letter from GGU to GGG retained significant threonine acceptor activity as described above (Table 1 and Figure 2). The introduction of an A_2-U_{71} base pair into this mutant transcript resulted in the almost total loss of threonine charging activity (Figure 2). Similarly, the introduction of this base pair did not introduce any detectable threonine acceptor activity into the $tRNA^{Thr}$ transcript having the anticodon GUU. These results confirm that the C_2-G_{71} base pair of $tRNA^{Thr}$ is a distinct recognition site, besides the anticodon second letter G_{35}, for threonyl-tRNA synthetase. The importance of C_2-G_{71} and G_{35} has already been pointed out by statistical multiple-position analysis (24). The present study shows that the G_1-C_{72} base pair is additionally required for recognition by threonyl-tRNA synthetase. It has also been reported that the Vmax/Km for the mutant methionine tRNA possessing the threonine anticodon GGU was much lower than that for the transcript of the wild-type $tRNA^{Thr}$(GGU) sequence, suggesting a requirement for additional elements (9). Here, we demonstrated that the C_2-G_{71} and G_1-C_{72} base pairs are the additional recognition sites.

In many tRNAs, the discriminator base, N_{73}, is involved in recognition by cognate aminoacyl-tRNA synthetases (7,8,15,16,25-32). The effects of discriminator base substitution from A to the other three bases on aminoacylation were examined. There were no significant variations in threonine charging activity among all the transcripts (Table 1), indicating that the discriminator base A_{73} of *E. coli* $tRNA^{Thr}$ is not involved in recognition by threonyl-tRNA synthetase. In *E. coli* $tRNA^{Ser}$, replacing the discriminator base G_{73} with other bases does not affect its charging activity (M. Shimizu *et al.*, submitted for publication), but G_{73} may act as a negative recognition element

for discrimination by tyrosyl-tRNA synthetase (8). The discriminator base is generally well conserved within every amino acid-specific tRNA among many organisms (10). Exceptionally, the base at this position of $tRNA^{Thr}$ is variable among many organisms, such as A for coliphage, yeast and most eubacteria, and U for archaebacteria, drosophila and mammals, and is also variable even within a single eubacterium such as *Pseudomonas aeruginosa* and *B. subtilis* (10). Such phylogenetic fluidity would be closely related to the present finding that A_{73} is not involved in recognition by *E. coli* synthetase.

It has been reported that expression of *E. coli* threonyl-tRNA synthetase is regulated at the level of translation by binding to its mRNA that resembles the anticodon loop and stem of threonine tRNAs (33,34). Point mutations of the anticodon-like GU sequence lead to a loss of regulation (35,36). Thus the product, threonyl-tRNA synthetase, is supposed to bind to this sequence of its own mRNA (36). The secondary structure of the 5' leader region of this mRNA possesses G-C and C-G which apparently correspond to G_1-C_{72} and C_2-G_{71}, respectively, in the acceptor stem of $tRNA^{Thr}$. It should be of interest to clarify whether threonyl-tRNA synthetase additionally recognizes these G-C and/or C-G.

In conclusion, recognition sites of *E. coli* $tRNA^{Thr}$ by threonyl-tRNA synthetase are dispersed within the second and third positions of the anticodon, $G_{35}U_{36}$, and within the two base pairs in the extremity of the acceptor stem.

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