# Identity elements of *Thermus thermophilus* $tRNA^{Thr}$

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**Abstract In this study, we identified nucleotides that specify aminoacylation of $tRNA^{Thr}$ by *Thermus thermophilus* threonyl-tRNA synthetase (ThrRS) using in vitro transcripts. Mutation studies showed that the first base pair in the acceptor stem as well as the second and third positions of the anticodon are major identity elements of *T. thermophilus* $tRNA^{Thr}$, which are essentially the same as those of *Escherichia coli* $tRNA^{Thr}$. The discriminator base, $U_{73}$, also contributed to the specific aminoacylation, but not the second base pair in the acceptor stem. These findings are in contrast to *E. coli* $tRNA^{Thr}$, where the second base pair is required for threonylation, with the discriminator base, $A_{73}$, playing no roles. In addition, among several mutations at the third base pair in the acceptor stem, only the $G_3$-$U_{70}$ mutant was a poor substrate for ThrRS, suggesting that the $G_3$-$U_{70}$ wobble pair, which is the identity determinant of $tRNA^{Ala}$, acts as a negative element for ThrRS. Similar results were obtained in *E. coli* and yeast. Thus, this manner of rejection of $tRNA^{Ala}$ is also likely to have been retained in the threonine system throughout evolution.**

*Key words:* tRNA; Aminoacyl-tRNA synthetase; T7 transcript; tRNA identity

## 1. Introduction

The correct recognition of tRNAs by their cognate aminoacyl-tRNA synthetases is crucial for the accurate transmission of genetic information. Technical advances have revealed how aminoacyl-tRNA synthetases distinguish cognate tRNAs from a pool of various tRNA species sharing a similar tertiary structure [1–3]. A small set of nucleotides of tRNA, which often includes anticodon nucleotides and the discriminator base at position 73, are major identity elements governing specific aminoacylation [4–7]. In addition to positive recognition by cognate aminoacyl-tRNA synthetase, negative elements prevent a tRNA from being recognized by a non-cognate synthetase [8,9]. Recently, systematic studies of recognition sets in several organisms have enabled comparisons of the tRNA recognition between prokaryotes and eukaryotes, which have indicated not only similarities, but also differences [10–18]. Recognition by threonyl-tRNA synthetase (ThrRS) has been extensively studied in *Escherichia coli* and *Saccharomyces cerevisiae* [19,20]. The first base pair in the acceptor stem and the second and third positions of the anticodon are retained as identity elements by both ThrRSs from *E. coli* and *S. cerevisiae*. In contrast to these commonly identified elements, the second base pair in the acceptor stem is required for threonylation by *E. coli* ThrRS, but not by the *S. cerevisiae* counterpart. Unlike many tRNAs, the discriminator base of *E. coli* $tRNA^{Thr}$ does not contribute to the tRNA identity, whereas that of the *S. cerevisiae* counterpart is involved in specific aminoacylation. Furthermore, in *S. cerevisiae*, the $G_3$-$U_{70}$ mutant is a poor substrate for *S. cerevisiae* ThrRS, suggesting that the $G_3$-$U_{70}$ wobble pair, which is a major identity determinant of $tRNA^{Ala}$, acts as a negative element for ThrRS. These findings indicate conservation and differences in tRNA recognition between prokaryotes and eukaryotes.

Although the aminoacylation of *E. coli* has been sufficiently studied for a pattern of tRNA recognition to emerge, little attention has been given to that of other prokaryotes. The question has arisen as to whether the recognition mode of *E. coli* is true of other prokaryotes. *Thermus thermophilus* is an extremely thermophilic bacterium. According to a cross-aminoacylation study of *E. coli* and *T. thermophilus*, ThrRS from *E. coli* can aminoacylate $tRNA^{Thr}$ from *T. thermophilus* as efficiently as homologous $tRNA^{Thr}$, whereas $tRNA^{Thr}$ from *E. coli* is a poor substrate for ThrRS from *T. thermophilus* [21]. This result suggested a difference in the identity elements of $tRNA^{Thr}$ between mesophilic and thermophilic prokaryotes, although the $tRNA^{Thr}$ sequences are similar between *E. coli* and *T. thermophilus*, particularly the nucleotides of the D stem, the anticodon arm, and the variable loop (Fig. 1) [22].

We examined the identity elements of $tRNA^{Thr}$ towards *T. thermophilus* ThrRS, using in vitro transcripts. The current data for the *E. coli* and yeast threonine systems allowed a comparison of identity elements of $tRNA^{Thr}$ between prokaryotes as well as between a prokaryote and a eukaryote, revealing the evolutionary divergence of tRNA identity in the threonine system.

## 2. Materials and methods

### 2.1. Preparation of template DNAs and in vitro transcripts

Synthetic DNA oligomers carrying the T7 promoter and tRNA genes were ligated into pUC19 and transformed into *E. coli* strain JM109 [3,23,24]. The template DNA sequences were confirmed by dideoxy sequencing [25]. Each template DNA for the discriminator base-substituted mutant was prepared from a plasmid carrying the normal tRNA sequence and two synthetic primers by mutation using the polymerase chain reaction [26]. Transcripts of the tRNA genes were prepared in a reaction mixture containing 40 mM Tris-HCl (pH 8.1), 5.0 mM dithiothreitol, 2.0 mM spermidine, 10 mM magnesium chloride, bovine serum albumin (50 μg/ml), 2.0 mM of each NTP, 20 mM 5′ GMP, *Bst*NI-digested template DNA (0.2 mg/ml), 2 units of inorganic pyrophosphatase (Sigma) and pure T7 RNA polymerase (50 μg/ml) [3,23,27]. Transcripts initiated with A were prepared in a reaction mixture containing 20 mM 5′ AMP instead of 5′ GMP [23]. The transcripts were purified by 20% polyacrylamide gel electrophoresis.

### 2.2. Aminoacylation assay

Threonyl-tRNA synthetase, partially purified from *T. thermophilus* strain HB8 by column chromatography with DE52, was provided by Prof. Kimitsuna Watanabe and Dr. Takahiro Nojima at the Univer-

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sity of Tokyo. The final enzyme fraction had a specific activity of 9.4 U/mg (1 unit of threonyl-tRNA synthetase catalyzes the formation of 1 nmol threonyl-tRNA per 10 min under the reaction conditions described below), and had no detectable alanyl-tRNA synthetase activity. The aminoacylation reaction proceeded at 65°C in a buffer containing 60 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 30 mM potassium chloride, 5.0 mM dithiothreitol, 2.5 mM ATP and 15 μM L-[U-$^{14}$C]threonine (8.6 GBq/mmol), with various concentrations of tRNA transcripts and *T. thermophilus* threonyl-tRNA synthetase. The initial rates of aminoacylation were determined by using six concentrations of tRNA transcripts ranging from 0.01 to 5.0 μM at a fixed concentration of the synthetase, depending on the mutant tRNA transcripts. $K_m$ and $V_{max}$ values were determined from a plot of [S] against [S]/$v$ ([S], tRNA concentration; $v$, observed initial velocity of threonylation). The $V_{max}/K_m$ values for two or three separate determinations were within ±15%.

## 3. Results and discussion

To examine the identity elements of *T. thermophilus* tRNA$^{Thr}$, we investigated the effects on the aminoacylation kinetics of variants of *T. thermophilus* tRNA$^{Thr}$ constructed using T7 RNA polymerase. Based on *E. coli* and yeast data, substitutions of nucleotides focused upon positions in the acceptor stem and the anticodon region, where the nucleotides are conserved within two tRNA$^{Thr}$ isoacceptors [28] (Fig. 1).

### 3.1. Discriminator base

Discriminator bases at position 73 play important roles in the specific aminoacylation of many tRNAs from *E. coli* [6]. In most prokaryotes, a purine base predominantly appears at this position in seventeen tRNA species, whereas U occupies it in tRNA$^{Gly}$ and tRNA$^{Cys}$, and C in tRNA$^{His}$ [22]. However, *T. thermophilus* tRNA$^{Thr}$ possesses $U_{73}$ at this position (Fig. 1). A base substitution of $U_{73}$ with $C_{73}$ had no effects on aminoacylation, whereas those with $A_{73}$ and $G_{73}$ caused a marked decrease with 210- and 17-fold lower $V_{max}/K_m$ values, respectively (Table 1). This is consistent with the failure of cross-aminoacylation of *E. coli* tRNA$^{Thr}$ possessing $A_{73}$ by *T. thermophilus* ThrRS (Table 1). These results showed that the discriminator base, $U_{73}$, is an important identity element. The absence of damage by the U to C mutation in threonylation suggests the following: a keto group common on the mutation (O-2 of C and O-2 of U) directly interacts with the synthetase; only a purine base at this position is disfavored by the synthetase, behaving as an obstacle to the normal positioning of the CCA end in aminoacylation. This finding is in contrast to *E. coli* tRNA$^{Thr}$, where mutations of the discriminator base, $A_{73}$, have no effects on the specific aminoacylation [19]. In yeast tRNA$^{Thr}$, changing the discriminator base, $A_{73}$, to $G_{73}$ or $C_{73}$ impairs threonine accepting activity by yeast ThrRS, indicating that this position contributes to the discrimination from other tRNAs possessing $G_{73}$ or $C_{73}$, such as tRNA$^{Ser}$ and tRNA$^{Pro}$ [20]. The base preference at position 73 in *T. thermophilus* is U = C > G ≫ A, whereas in yeast it is A = U ≥ C > G, with differences in the quantitative effects of mutations between the two organisms. The mechanism by which the discriminator base is recognized by ThrRS is probably different between *T. thermophilus* and yeast. However, the *T. thermophilus* threonine system is similar to the yeast counterpart, in that ThrRS cannot discriminate all the different nucleotides at position 73.

Analysis of the available tRNA and tRNA gene sequences indicates that the discriminator bases of many tRNA species are phylogenetically well conserved among prokaryotes and/or eukaryotes [22]. However, the discriminator base in the threonine system was not regularly conserved. The nucleotide varies among many organisms, such as A for coliphage, most eubacteria and yeast, and U for archaebacteria, *T. thermophilus* and higher eukaryotes. It is also variable even within a single eubacterium such as *Pseudomonas aeruginosa* and *Bacillus subtilis* [22]. A and/or U, which have no chemical groups in common, appear in this position. Considering the phylogenetic fluidity of the discriminator base sequence and of the importance of the discriminator base in tRNA recognition, the recognition mechanism of the discriminator base by ThrRS may have been acquired at a later stage of evolution, depending on the organism.

### 3.2. Acceptor stem

In the acceptor stem, the first base pair is significantly recognized by both *E. coli* and yeast ThrRSs. The second base pair is important for threonylation by only *E. coli* ThrRS [19,20]. Substitutions of $G_1$-$C_{72}$ by $A_1$-$U_{72}$ and $G_1$-$U_{72}$ resulted in a 21- and a 6-fold decrease in the $V_{max}/K_m$, respectively, and that by $A_1$-$C_{72}$ reduced the $V_{max}/K_m$ 150-fold (Table 1). These findings indicated that the first base pair is recognized by *T. thermophilus* ThrRS. There were no apparent effects on aminoacylation by the mutation of $G_2$-$C_{71}$ to $C_2$-$G_{71}$ (Table 1). In view of the lack of common chemical groups in major or minor grooves between G-C and C-G base pairs, the results showed that the second base pair is not involved in base-specific recognition by *T. thermophilus* ThrRS. The first base pair, $G_1$-$C_{72}$, is indeed conserved as an identity element among the three species, but it would be of little use for discrimination from non-cognate tRNAs, because many tRNAs possess $G_1$-$C_{72}$ as the first base pair irrespective of the organism [22]. Another recognition site that appears helpful to the discrimination is located around the first base pair in the acceptor stem. In *E. coli*, the second base pair is required for threonylation with the discriminator base playing no roles, whereas in *T. thermophilus* and yeast, the discriminator base is involved in the specific aminoacylation instead of the second base pair. In the *T. thermophilus* system, particular impairment by a purine base at the discriminator base position appears to be more effective for discrimination from many other tRNA species possessing a purine base at position 73.

The first and second base pairs are completely conserved among prokaryotic tRNA$^{Thr}$s [22]. Despite the complete conservation within prokaryotes, the second base pair plays an important role on the aminoacylation function for *E. coli* ThrRS, but not for the *T. thermophilus* counterpart. This sequence conservation may be involved in the contribution to discrimination for non-cognate aminoacyl-tRNA synthetase. The occurrence of $C_2$-$G_{71}$ in tRNA$^{Thr}$ would be useful in precluding tRNA$^{Thr}$ from being mischarged by several non-cognate aminoacyl-tRNA synthetases that recognize the second base pair other than the $C_2$-$G_{71}$ of the corresponding tRNAs, for example, in *E. coli*, glutaminyl- [29], glutamyl- [30], and methionyl- [31] tRNA synthetases.

The third base pair in the acceptor stem is not retained within *T. thermophilus* tRNA$^{Thr}$ isoacceptors, which possess either U-A or C-G [28]. Substitution of $U_3$-$A_{70}$ by $A_3$-$U_{70}$ or an $A_3$-$C_{70}$ wobble pair had little effect on threonylation, whereas that to $G_3$-$C_{70}$ resulted in a 7-fold decrease in the

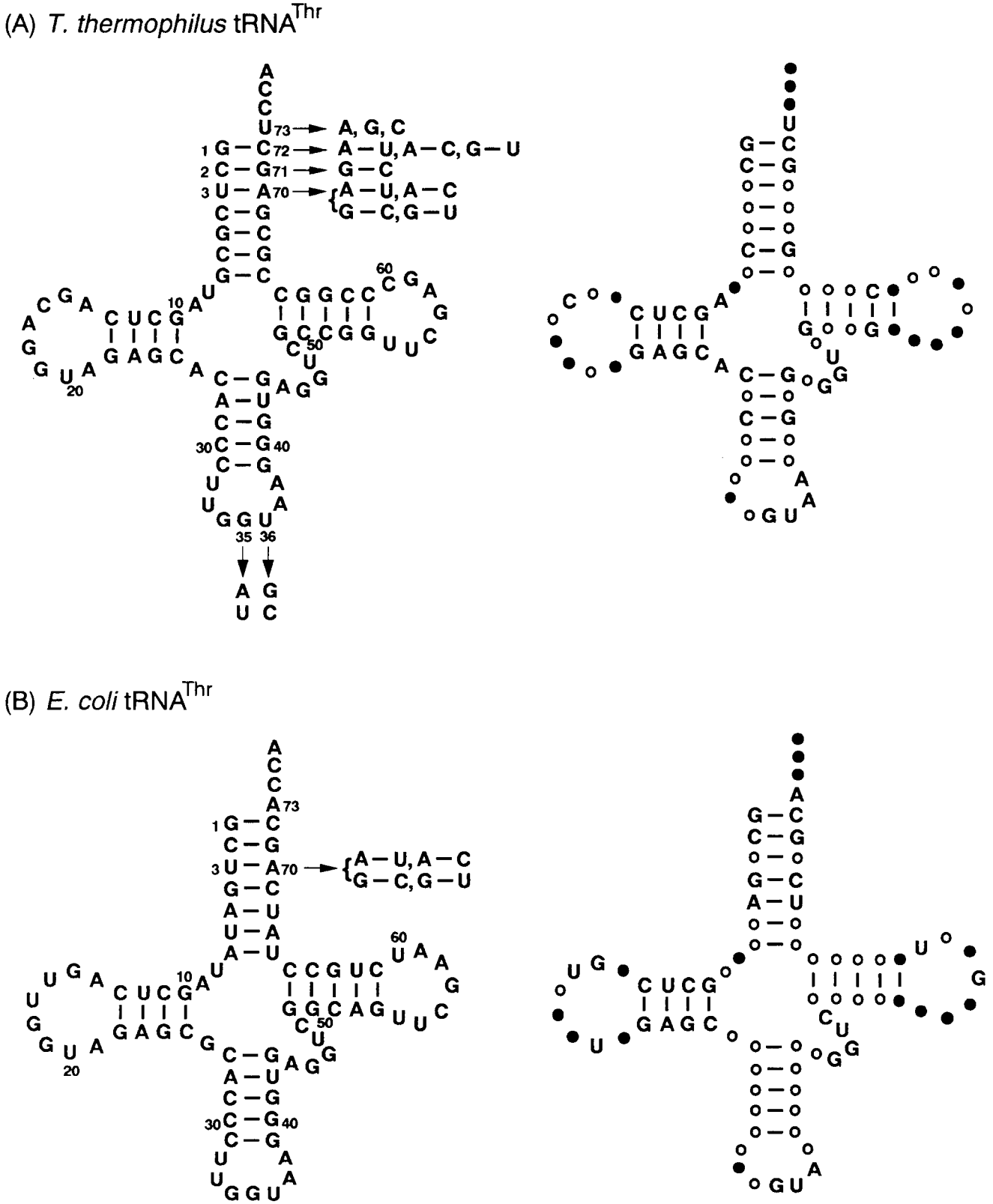


Fig. 1. Schematic cloverleaf structure of *T. thermophilus* $tRNA^{Thr}$ (A) and of *E. coli* $tRNA^{Thr}$ (B) with the base modifications omitted. For *T. thermophilus* $tRNA^{Thr}$, two genes are known but the RNA sequence is not [28]. Numbering is according to [22]. Left, $tRNA^{Thr}$ isoacceptor with the GGU anticodon. Arrows indicate the substitutions in this study. Right, filled circles indicate the invariant bases in tRNAs, and unfilled circles, the position of sequence variation. The indicated nucleotides are absolutely conserved within the isoacceptors.

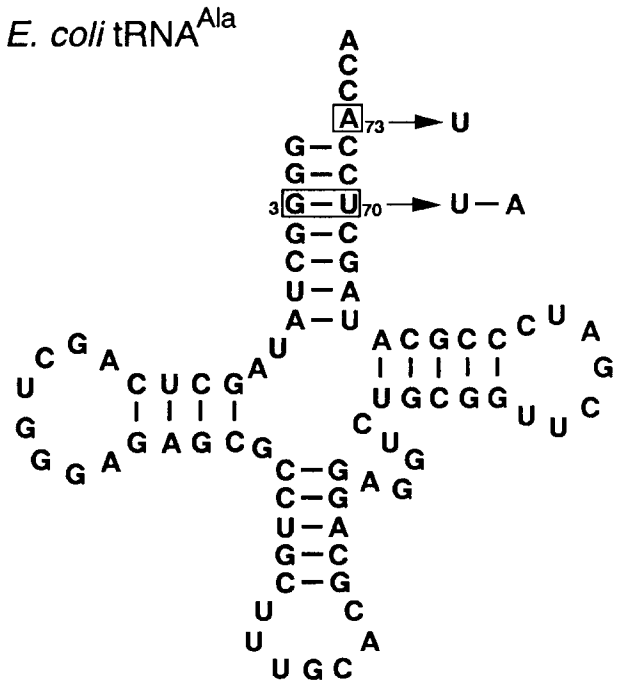


Fig. 2. The *E. coli* $tRNA^{Ala}$ transcript for the aminoacylation specificity conversion from alanine to threonine. Numbering is according to [22]. Arrows indicate the substitutions in this study.

$V_{max}/K_m$. Moreover, changing it to a $G_3$-$U_{70}$ wobble pair caused a marked reduction of threonine accepting activity with a 150-fold lower $V_{max}/K_m$ (Table 1). Irrespective of the organisms, it is only $tRNA^{Ala}$ isoacceptors that possess a wobble pair, G-U, at the third base pair position, which are conserved as major identity elements among distantly related organisms [32,33]. These findings indicated that the third base pair is not involved in base-specific recognition by ThrRS, and that a $G_3$-$U_{70}$ wobble pair, which is the identity determinant of $tRNA^{Ala}$, is disfavored by ThrRS. The bases of a G-U wobble pair that are pushed into the helical grooves can make two hydrogen bonds, creating a distinctive pattern of functional groups that could allow specific protein recognition [4]. Among mutations at position 3-70, the specific reduction in threonylation by G-U implies that the functional group(s) on G-U directly hinders interaction between $tRNA^{Ala}$ and ThrRS to prevent $tRNA^{Ala}$ from being mischarged by ThrRS.

Either $U_3$-$A_{70}$ or $C_3$-$G_{70}$ also occurs in *E. coli* $tRNA^{Thr}$ [22]. To compare the results from *T. thermophilus* and yeast, we prepared substitutions at the third base pair in *E. coli* $tRNA^{Thr}$ (Fig. 1). Similar results were obtained in the *E. coli* system; the $G_3$-$U_{70}$ mutation damaged the threonine accepting activity the most severely among the four mutations (Table 2).

To verify the importance of $A_{73}$ and $G_3$-$U_{70}$ in the acceptor stem of $tRNA^{Ala}$ for prevention of misrecognition by *T. thermophilus* ThrRS, we constructed several mutants of *E. coli* $tRNA^{Ala}$ (Fig. 2). Although neither the DNA nor the RNA sequence of *T. thermophilus* $tRNA^{Ala}$ is yet known, the phylogenetic conservation of $A_{73}$ and $G_3$-$U_{70}$ as well as the anticodon nucleotides among $tRNA^{Ala}$s from various organisms permitted of the use of an *E. coli* $tRNA^{Ala}$ transcript as an analogous *T. thermophilus* $tRNA^{Ala}$ in this study [22]. The wild-type transcript was not aminoacylated with threonine,

Table 1
Kinetic parameters with *T. thermophilus* threonyl-tRNA synthetase for the transcripts

| | | $K_m$ (μM) | $V_{max}$ (relative) | $V_{max}/K_m$ (relative) | Loss of efficiency (*x*-fold) |
|---|---|---|---|---|---|
| *T. thermophilus* $tRNA^{Thr}$ derivatives | | | | | |
| Wild-type in vitro transcript | | 0.13 | 1 | 1 | 1 |
| Discriminator base | | | | | |
| $U_{73}$ | → $A_{73}$ | 1.2 | 0.045 | 0.0047 | 210 |
| | → $G_{73}$ | 0.21 | 0.096 | 0.059 | 17 |
| | → $C_{73}$ | 0.14 | 0.98 | 0.90 | 1 |
| Acceptor stem | | | | | |
| $G_1$-$C_{72}$ | → $A_1$-$U_{72}$ | 0.72 | 0.27 | 0.049 | 21 |
| | → $A_1$-$C_{72}$ | 0.64 | 0.034 | 0.0069 | 150 |
| | → $G_1$-$U_{72}$ | 0.13 | 0.18 | 0.18 | 6 |
| $C_2$-$G_{71}$ | → $G_2$-$C_{71}$ | 0.11 | 0.69 | 0.79 | 1 |
| $U_3$-$A_{70}$ | → $A_3$-$U_{70}$ | 0.11 | 0.98 | 1.1 | 1 |
| | → $G_3$-$C_{70}$ | 0.50 | 0.58 | 0.15 | 7 |
| | → $A_3$-$C_{70}$ | 0.15 | 0.65 | 0.56 | 2 |
| | → $G_3$-$U_{70}$ | 0.64 | 0.042 | 0.0067 | 150 |
| Anticodon | | | | | |
| $G_{35}$ | → $A_{35}$ | 0.19 | 0.0068 | 0.0047 | 210 |
| | → $U_{35}$ | 2.1 | 0.19 | 0.012 | 84 |
| $U_{36}$ | → $G_{36}$ | 2.0 | 0.71 | 0.046 | 22 |
| | → $C_{36}$ | 1.0 | 0.21 | 0.027 | 37 |
| *E. coli* $tRNA^{Ala}$ derivatives | | | | | |
| Wild-type in vitro transcript | | | | $<1\times10^{-4}$ | >10000 |
| $A_{73}$ | → $U_{73}$ | 1.2 | 0.0081 | $8.6\times10^{-4}$ | 1200 |
| $A_{73}$, $G_3$-$U_{70}$ | → $U_{73}$, $U_3$-$A_{70}$ | 1.7 | 0.55 | 0.042 | 24 |
| *E. coli* native $tRNA^{Thr}$ | | 0.33 | 0.022 | 0.0086 | 120 |

The threonyl-tRNA synthetase used here was not fully purified; the apparent $K_m$ values are therefore presented.

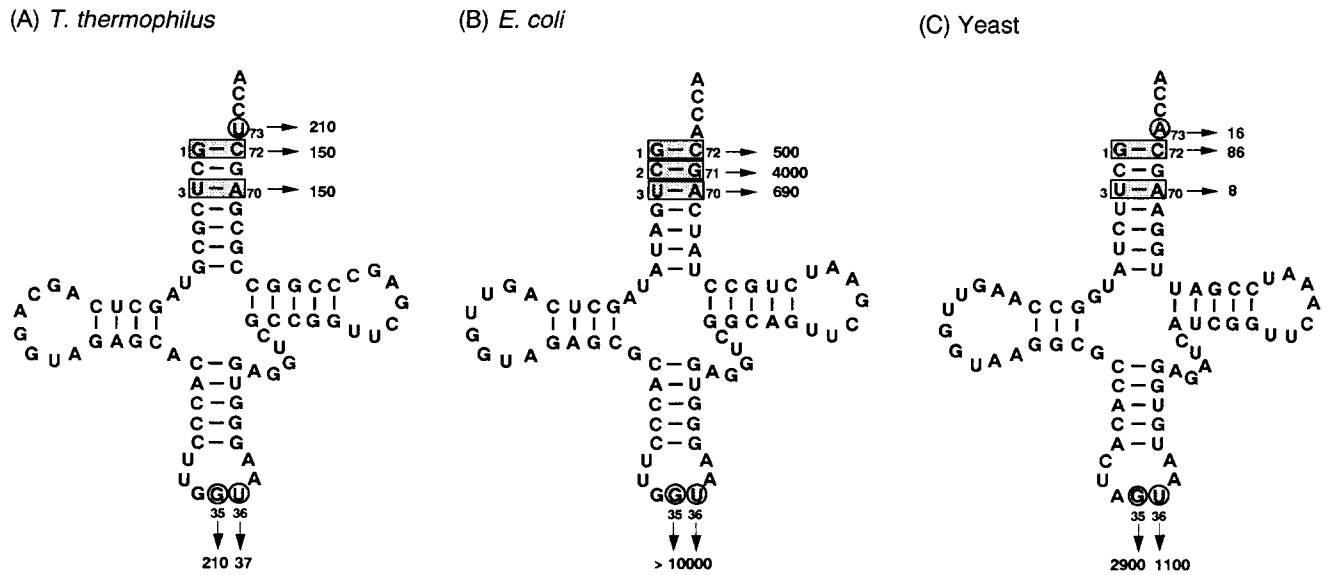


Fig. 3. A summary of the identity nucleotides in *T. thermophilus* $tRNA^{Thr}$ identified in this study (A), those in *E. coli* $tRNA^{Thr}$ identified in this study and taken from [19] (B), as well as those in yeast $tRNA^{Thr}$ taken from [20] (C), with the base modifications omitted. The numbers refer to the -fold reduction in $V_{max}/K_m$ upon substitution at that site. Where there were variable effects of aminoacylation, kinetic data for the base substitution causing the largest defeat by factors of $>5$ are shown. As for the third base pair, only the $G_3$-$U_{70}$ mutation in $tRNA^{Thr}$s from three organisms causes a marked reduction on threonylation.

into which $U_{73}$ and/or $U_3$-$A_{70}$ were introduced. The $A_{73}$ to $U_{73}$ mutant showed threonine accepting activity with a 1200-fold lower $V_{max}/K_m$ (Table 1). Additional mutation of $G_3$-$U_{70}$ to $U_3$-$A_{70}$ elevated the activity to a 24-fold lower $V_{max}/K_m$. This decrease in $V_{max}/K_m$ would be mainly due to the different third letter, $C_{36}$, of the anticodon from that in $tRNA^{Thr}$, considering that the $U_{36}$ to $C_{36}$ mutation in $tRNA^{Thr}$ resulted in a 32-fold decrease in $V_{max}/K_m$ (Table 1). These findings showed that the discriminator base, $A_{73}$, and the $G_3$-$U_{70}$ wobble pair in $tRNA^{Ala}$ contribute to ThrRS discrimination. In *E. coli*, a similar specificity conversion was performed. Unlike *T. thermophilus* $tRNA^{Thr}$, the discriminator base, $A_{73}$, of *E. coli* $tRNA^{Thr}$ is identical to that of $tRNA^{Ala}$. The $tRNA^{Ala}$ mutant with $U_3$-$A_{70}$ also had low, but significant threonine accepting activity (Table 2). This relatively low activity compared with that in the *T. thermophilus* system would be explained by the difference between alanine and threonine tRNAs in the third letter of the anticodon and the second base pair in the acceptor stem. In *E. coli* $tRNA^{Thr}$, mutation of $C_2$-$G_{71}$ to $G_2$-$C_{71}$ or of $U_{36}$ to $C_{36}$ was found to have significant effects on aminoacylation efficiency, showing that both $C_2$-$G_{71}$ and $C_{36}$ are required for aminoacylation for *E. coli* ThrRS unlike the *T. thermophilus* counterpart [19].

Consequently, the $G_3$-$U_{70}$ mutant of $tRNA^{Thr}$ is a poor substrate for all ThrRSs from three species; *T. thermophilus*, *E. coli*, and yeast. The third base pair in the acceptor stem is well conserved as Py3-Pu70 in prokaryotic and eukaryotic $tRNA^{Thr}$s [22]. It is likely that during the evolutionary processes, this conservation as Py3-Pu70 has been useful in precluding $tRNA^{Thr}$ from being mischarged by alanyl-tRNA synthetase, and that the $G_3$-$U_{70}$ pair of $tRNA^{Ala}$ simultaneously functions as a negative element for ThrRS.

### 3.3. Anticodon

The most remarked impairment of aminoacylation has been observed with many tRNAs mutated in the anticodon region

Table 2
Kinetic parameters with *E. coli* threonyl-tRNA synthetase for the transcripts

| | | $K_m$ (μM) | $V_{max}$ (relative) | $V_{max}/K_m$ (relative) | Loss of efficiency (*x*-fold) |
|---|---|---|---|---|---|
| *E. coli* $tRNA^{Thr}$ derivatives | | | | | |
| Wild-type in vitro transcript | | 0.10 | 1 | 1 | 1 |
| Acceptor stem | | | | | |
| $U_3$-$A_{70}$ | → $A_3$-$U_{70}$ | 0.19 | 0.98 | 0.52 | 2 |
| | → $G_3$-$C_{70}$ | 1.3 | 0.57 | 0.045 | 22 |
| | → $A_3$-$C_{70}$ | 0.58 | 1.1 | 0.19 | 5 |
| | → $G_3$-$U_{70}$ | 3.7 | 0.053 | 0.0015 | 690 |
| *E. coli* $tRNA^{Ala}$ derivatives | | | | | |
| Wild-type in vitro transcript | | | | $<1\times10^{-5}$ | $>10^5$ |
| $G_3$-$U_{70}$ | → $U_3$-$A_{70}$ | | | $1.9\times10^{-4}$ | 5200 |

The aminoacylation with *E. coli* ThrRS proceeded at 37°C, the other conditions being in accordance with aminoacylation using *T. thermophilus* ThrRS. The ThrRS used here was partially purified, with a specific activity of 90 U/mg and no detectable alanyl-tRNA synthetase activity. The apparent $K_m$ values are therefore presented.

[4–7]. In $tRNA^{Thr}$, the first position of the anticodon seems unlikely to be involved in base-specific recognition by ThrRS, since $tRNA^{Thr}$ isoacceptors belonging to a four-codon box family require at least two different bases at this position [22,28] (Fig. 1). At the second position of the anticodon, the substitution of $G_{35}$ by $A_{35}$ and $U_{35}$ reduced the $V_{max}/K_m$ 210- and 84-fold, respectively (Table 1). As observed in *E. coli* and yeast $tRNA^{Thr}$s, the $G_{35}$ to $U_{35}$ mutation had less effect on the activity than other mutations. The keto group conserved upon mutation (O-6 of G and O-4 of U) could be a site of direct interaction with the synthetase. In the yeast aspartic acid system, the $U_{35}$ to $G_{35}$ mutation in $tRNA^{Asp}$ had the least effect on aspartylation among three mutations [14]. The O-4 of $U_{35}$ has a direct interaction of aspartyl-tRNA synthetase according to the crystallographic data of the tRNA/synthetase complex [34]. The substitution of $U_{36}$ at the third position by $G_{36}$ and $C_{36}$ resulted in a 22- and a 37-fold decrease in $V_{max}/K_m$, respectively (Table 1). These findings showed that the second and third positions of the anticodon are responsible for recognition by *T. thermophilus* ThrRS, as well as by *E. coli* and yeast ThrRSs. The relatively moderate reduction by mutations in the anticodon region compared with those in the *E. coli* and yeast systems may be involved in the findings that the discriminator base plays even more crucial roles in threonylation than those of the two species.

### 3.4. Concluding remarks

The results from *T. thermophilus* showed that the discriminator base, the first base pair in the acceptor stem, and the second and third positions of the anticodon are major identity elements of *T. thermophilus* $tRNA^{Thr}$, and that the third base pair in the acceptor stem contributes to the discrimination from $tRNA^{Ala}$ (Fig. 3). It was thus found that the first base pair in the acceptor stem, and the second and third positions of the anticodon are common identity elements among *E. coli*, *T. thermophilus* and yeast $tRNA^{Thr}$s. In contrast to the conservation of these identified elements, the substantial differences involve the contribution of the discriminator base and the second base pair in the acceptor stem to the specific aminoacylation. Throughout evolution, the first base pair and the anticodon nucleotides have been conserved as identity elements of $tRNA^{Thr}$, whereas the mechanism by which ThrRS recognizes the vicinity of the usual first base pair seems to have diverged with the species to effectively function for the tRNA discrimination. In addition, it was found that the $G_3$-$U_{70}$ wobble pair of $tRNA^{Ala}$ behaves as a negative element for all the three ThrRSs. Thus, this manner of rejection of $tRNA^{Ala}$ is also likely to have been retained in the threonine system.

During the evolutionary processes, both tRNAs and aminoacyl-tRNA synthetases have undergone extensive mutations, which would have influenced the substrate-enzyme recognition mode under the accurate maintenance of the tRNA recognition and discrimination system. Recently, crystallographic structural studies of aminoacyl-tRNA synthetases have rapidly progressed so that detailed information on the protein side can now be obtained [35]. Accumulating information about tRNA recognition from the perspective of both the tRNA and the enzyme from various organisms will provide a general understanding of the evolutionary divergence in the enzyme-substrate recognition mode.

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