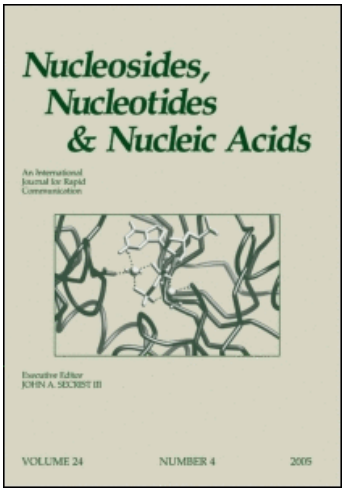

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Recognition of the Anticodon Loop of $tRNA^{Ile}_1$ by Isoleucyl-tRNA Synthetase from *Escherichia coli*

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RECOGNITION OF THE ANTICODON LOOP OF $tRNA_1^{Ile}$ BY ISOLEUCYL-tRNA SYNTHETASE FROM *ESCHERICHIA COLI*

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ABSTRACT: For *Escherichia coli* $tRNA_1^{Ile}$ having anticodon G34-A35-U36, two variants with substitution and/or insertion in the anticodon loop were prepared by *in vitro* recombinant RNA method. A variant with replacement of the *N*-((9-β-D-ribofuranosylpurine-6-yl)carbamoyl)threonine (t^6A) residue at position 37 with an unmodified adenosine exhibited a drastic reduction in isoleucine-accepting activity. This shows that t^6A37 plays a crucial role in the recognition by isoleucyl-tRNA synthetase (IleRS). Into this A37 variant, unmodified A was further inserted at position 36 so that a sequence of GAAUA was created. This insertion did not show further reduction in isoleucine-accepting activity, indicating that IleRS recognizes the three anticodon residues, which have already been found to be identity elements, individually but not as a whole.

INTRODUCTION

Aminoacyl-tRNA synthetases strictly select their cognate tRNAs and amino acid, which is required for the correct decoding of the genetic code. It is considered that each synthetase recognizes a small number of nucleotide residues (identity determinants) of the cognate tRNAs in order to discriminate them from non-cognate ones [1-7]. For most tRNA species, the anticodon residues are identity determinants [5-7]. As for *Escherichia coli* $tRNA_2^{Ile}$, a modified nucleoside (lysidine) at the first position of the anticodon was found to be a major identity determinant, which is the first finding of crucial role of modification on tRNA identity [8]. In the case of another isoleucine $tRNA^{Ile}$ from *E. coli* ($tRNA_1^{Ile}$, FIG. 1), the nucleoside at the first position of the anticodon (unmodified guano-

This paper is dedicated to Dr. Morio Ikehara on the occasion of his 70th birthday.

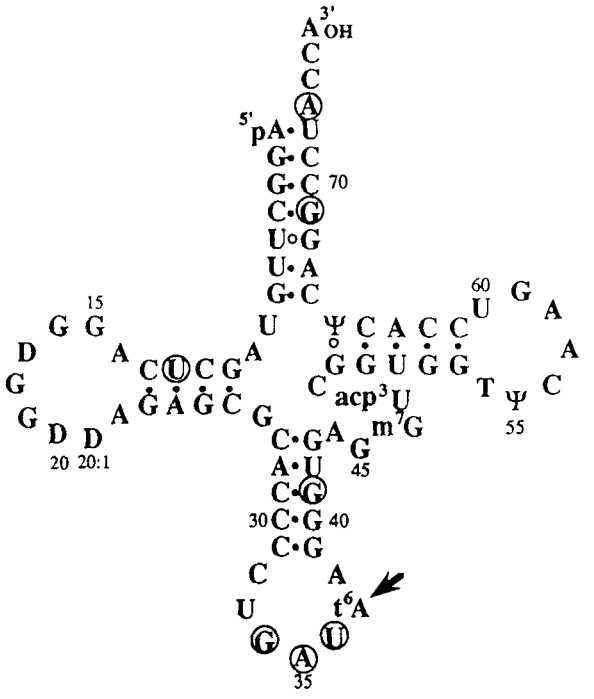

FIG. 1. The secondary structure of $tRNA_I^{Ile}$. D, dihydrouridine; m^7G, 7-methylguanosine; acp^3U, 3-(3-amino-3-carboxypropyl)uridine; T, 5-methyluridine; Ψ, pseudouridine. Circled residues have been found to be identity determinants [10]. The t^6A residue is indicated with an arrow.

sine) is a major identity determinant [9, 10]. The $tRNA^{Ile}$ identity depends also on the second and third letters of the anticodon [10-12]. Further, the completely-unmodified $tRNA_I^{Ile}$ prepared by *in vitro* transcription showed a significantly lower isoleucine-accepting activity than that of the natural fully-modified $tRNA_I^{Ile}$ [9, 10], indicating that the post-transcriptional modification is essential to the recognition of $tRNA_I^{Ile}$ by isoleucyl-tRNA synthetase (IleRS). In this report, by *in vitro* recombinant RNA techniques [8, 13-23], we investigated roles of the anticodon and the adjacent modified residue in the recognition of $tRNA_I^{Ile}$ by IleRS.

EXPERIMENTAL SECTION

Materials. Tetraribonucleotide AUAA was synthesized with a DNA/RNA synthesizer (Milligen/Biosearch). T7 RNA polymerase was purified from an overproducing strain kindly provided by W. Studier (Stonybrook, New York). T4 polynucleotide kinase and

T4 RNA ligase were purchased from Takara Shuzo (Kyoto, Japan). T4 polynucleotide kinase (3'-phosphatase free) and RNase CL3 were from Boehringer-Mannheim. RNase U_2 was from Sankyo (Tokyo, Japan). RNase PhyM was from Pharmacia. *E. coli* tRNA nucleotidyltransferase was a kind gift from Dr. R. Giegé (IBMC, CNRS, Strasbourg). A recombinant *E. coli* IleRS was prepared as described [24, 25]. The natural fully-modified $tRNA_1^{Ile}$ from *E. coli* was prepared as described [26]. The completely-unmodified $tRNA_1^{Ile}$ was prepared with T7 RNA polymerase as described [9].

Construction of $tRNA_1^{Ile}$ variants. The construction scheme is summarized in FIG. 2. $tRNA_1^{Ile}$ 5'-half molecules (A1-G34) and (A1-A35), and $tRNA_1^{Ile}$ 3'-half molecule (G39-A73) were prepared by partial digestion of $tRNA_1^{Ile}$ (0.1 mM) with RNase U_2 (70 units/ml) in 50 mM sodium acetate (pH 4.5), 100 mM $MgCl_2$ at 0 °C for 5 hr. These fragments were purified by 8 M Urea/ 20 % polyacrylamide gel electrophoresis (PAGE). The purified 3'-half molecule (16 μM) was phosphorylated with a 3'-phosphatase-free T4 polynucleotide kinase (100 units/ml) in 50 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM ATP, followed by ligation with AUAA in the same condition as described [13]. The elongated 3'-half molecule was purified by PAGE. The 5'-half molecule and the elongated 3'-half molecule were annealed, phosphorylated at the 5' terminus, dephosphorylated at the 3' terminus and ligated as described [13]. The CCA terminus was repaired by incubation of the truncated tRNA molecule (7 μM) with tRNA nucleotidyltransferase (10 μg/ml) in 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM ATP and 0.05 mM CTP at 37 °C for 30 min. The product was finally purified by PAGE, and its nucleotide sequence was determined by the Donis-Keller's method with 3' labeling using [5'-^{32}P]pCp [27].

Analysis of isoleucine acceptance. Aminoacylation reactions were performed in 100 mM Tris-HCl (pH 7.5), 10 mM KCl, 20 mM $MgCl_2$, 2 mM ATP, 0.1 mM [U-^{14}C] isoleucine (315 mCi/mmol), 0.8 μM IleRS and 0.8 μM tRNA. The other experimental details were as described [9].

RESULTS AND DISCUSSION

Isoleucine-accepting activity of a $tRNA_1^{Ile}$ variant with substitution of t^6A37 by an unmodified adenosine. It has been reported that isoleucine-accepting activity of the completely-unmodified *E. coli* $tRNA_1^{Ile}$ molecule is much lower than that of the natural fully-modified $tRNA_1^{Ile}$ [9, 10]. Also in the condition of the present study, the activity of the completely-unmodified $tRNA_1^{Ile}$ molecule was confirmed to be remarkably low as compared with the fully-modified one (FIG. 3). We prepared the $tRNA_1^{Ile}$ molecule with t^6A37 substituted by A37 ($tRNA_1^{Ile}$(A37)) according to the scheme shown in

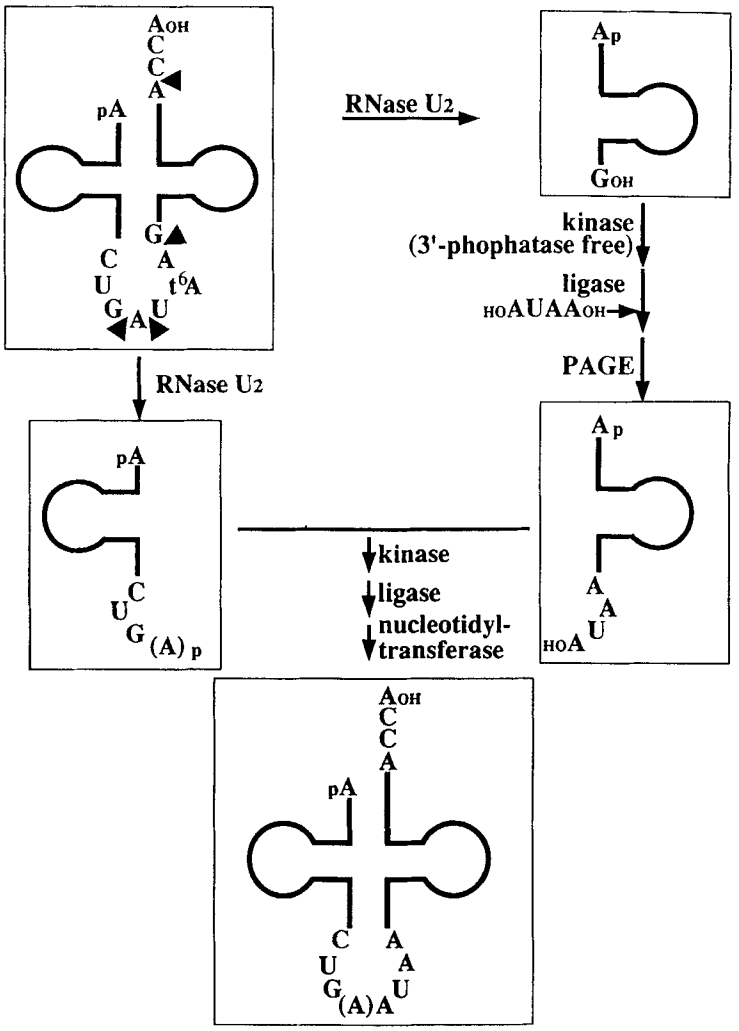

FIG. 2. The scheme for construction of $tRNA_1^{Ile}$ variants. RNase U2 cleavage sites are indicated with closed triangles.

FIG. 2. This variant tRNA exhibited a significantly reduced isoleucine-accepting activity to the same level as that of the completely-unmodified $tRNA_1^{Ile}$ (FIG. 3). Therefore, the t^6A residue, rather than other modified residues (FIG. 1), predominantly contributes to the recognition of $tRNA_1^{Ile}$ by IleRS. So far, it is at position 34 that the modified nucleotide residue has been identified to be a major "positive" determinant for tRNA identity; lysidine of *E. coli* $tRNA_2^{Ile}$ [8], 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) of *E. coli*

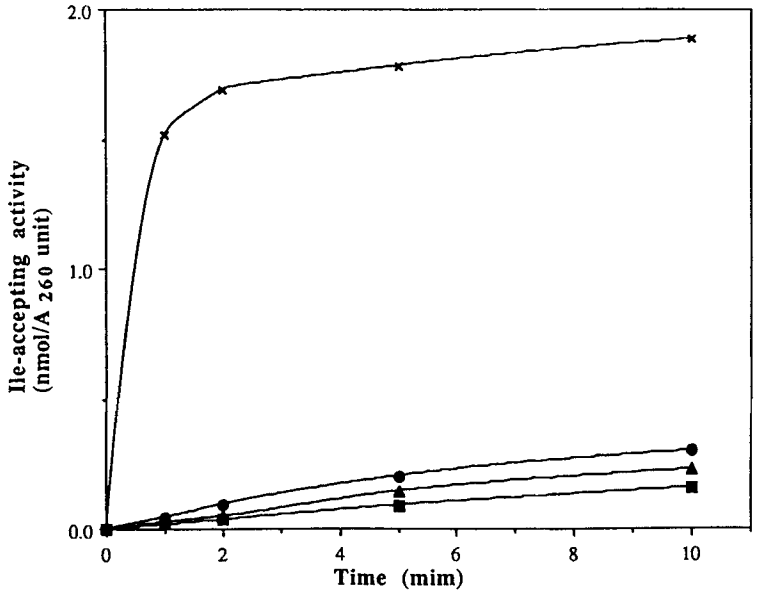

FIG. 3. Time courses of aminoacylation of native $tRNA_1^{Ile}$ (x-x), completely-unmodified $tRNA_1^{Ile}$ (■-■), $tRNA_1^{Ile}$(A37) (●-●), and $tRNA_1^{Ile}$(GAAUA) (▲-▲).

$tRNA^{Glu}$ [23], and possibly mnm^5s^2U of *E. coli* $tRNA^{Lys}$ [28, 29]. As for position 37, it is suggested that 1-methylguanosine (m^1G) of yeast $tRNA^{Asp}$ serves as a "negative" determinant ("antideterminant") that prevents mischarging by arginyl-tRNA synthetase [30]. However, other modified residues at position 37 have been considered to be important for codon-anticodon interaction but not for tRNA identity [31]. The t^6A residue is commonly present at position 37 of tRNAs having U at position 36 and is considered to stabilize the adjacent base pair between the third letter of the anticodon and the first letter of the codon [31]. This is the first report that the modified nucleoside at this position is a major positive determinant for tRNA identity. In this context, it has been found that the three anticodon residues are major determinants of *E. coli* $tRNA^{Ile}$ [10-12]. Therefore, the t^6A residue appears to constitute a recognition element for the tRNA identity together with the adjacent anticodon residues.

Isoleucine-accepting activity of $tRNA_1^{Ile}$ with four anticodon residues. By using the (A1-A35) fragment as the 5'-half fragment, we prepared a $tRNA_1^{Ile}$ variant having a four-residue anticodon GAAU corresponding to positions 34-36 in addition to the unmodified A37 residue ($tRNA_1^{Ile}$(GAAUA)) (FIG. 2). The isoleucine-accepting activity

of this variant was found to be comparable to that of $tRNA_1^{Ile}$(A37) with GAUA in place of GAAUA (FIG. 3). All the three letters of the anticodon are involved in the isoleucine tRNA identity [10-12]; the activities of unmodified $tRNA_1^{Ile}$ variants with GAAA and AAUA were nearly undetectable as compared with that of the unmodified $tRNA_1^{Ile}$ with GAUA [10, 12]. Therefore, $tRNA_1^{Ile}$(GAAUA) is much more active than the tRNAs with GAAA and AAUA. This indicates that IleRS can recognize the discontinuous three letters, G, A, and U, which are embedded in the four-letter anticodon of $tRNA_1^{Ile}$(GAAUA), as the isoleucine anticodon G-A-U. For the individual recognition of G, A, and U, IleRS may have three separate binding-sites for the anticodon residues of $tRNA_1^{Ile}$, as in the case of *E. coli* glutaminyl-tRNA synthetase [32].

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