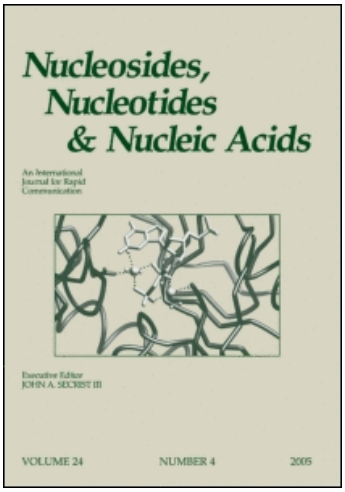

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Tomonari Muramatsu[a]; Tatsuo Miyazawa[ab]; Shigeyuki Yokoyama[a]
[a] Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo, Bunkyo-ku, Japan [b] Protein Engineering Research Institute, Osaka 565, Japan

To cite this Article Muramatsu, Tomonari , Miyazawa, Tatsuo and Yokoyama, Shigeyuki(1992) 'Recognition of the Nucleoside in the First Position of the Anticodon of Isoleucine tRNA by Isoleucyl-tRNA Synthetase from Escherichia Coli', Nucleosides, Nucleotides and Nucleic Acids, 11: 2, 719 — 730
To link to this Article: DOI: 10.1080/07328319208021736
URL: http://dx.doi.org/10.1080/07328319208021736

NUCLEOSIDES AND NUCLEOTIDES, 11(2-4), 719-730 (1992)

RECOGNITION OF THE NUCLEOSIDE IN THE FIRST POSITION OF THE ANTICODON OF ISOLEUCINE tRNA BY ISOLEUCYL-tRNA SYNTHETASE FROM *ESCHERICHIA COLI*

Tomonari Muramatsu, Tatsuo Miyazawa[1] and Shigeyuki Yokoyama*

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Abstract: *Escherichia coli* $tRNA_1^{Ile}$ prepared *in vitro* by T7 RNA polymerase transcription was found to be charged with isoleucine by *E. coli* isoleucyl-tRNA synthetase (IleRS). Replacement of G in the first position of the anticodon with U, C, or A resulted in complete loss of isoleucine-accepting activity. This indicates that IleRS strictly recognizes the structural feature of guanosine in this position of $tRNA_1^{Ile}$ which is common to that of lysidine in *E. coli* $tRNA_2^{Ile}$.

INTRODUCTION

Escherichia coli has two isoleucine tRNA species with the identical sequence of the anticodon loop except for the first position of the anticodon (position 34)[2-5]. In position 34, the major species ($tRNA_1^{Ile}$) has guanosine[2], while the minor species ($tRNA_2^{Ile}$) has a unique modified nucleoside[3]. The chemical structure of this novel nucleoside was determined by NMR spectroscopy, mass spectrometry and chemical synthesis, and this nucleoside was named lysidine (L) (FIG. 1)[5]. From the nucleotide sequence of the tRNA gene, lysidine was found to be derived from cytidine[6]. Further we found that the conversion of lysidine of $tRNA_2^{Ile}$ to cytidine resulted in a loss of the isoleucine-accepting activity[6]. Thus, *E. coli* isoleucyl-tRNA synthetase (IleRS) recognizes guanosine or

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The authors wish to dedicate this paper to the memory of Dr. Tohru Ueda.

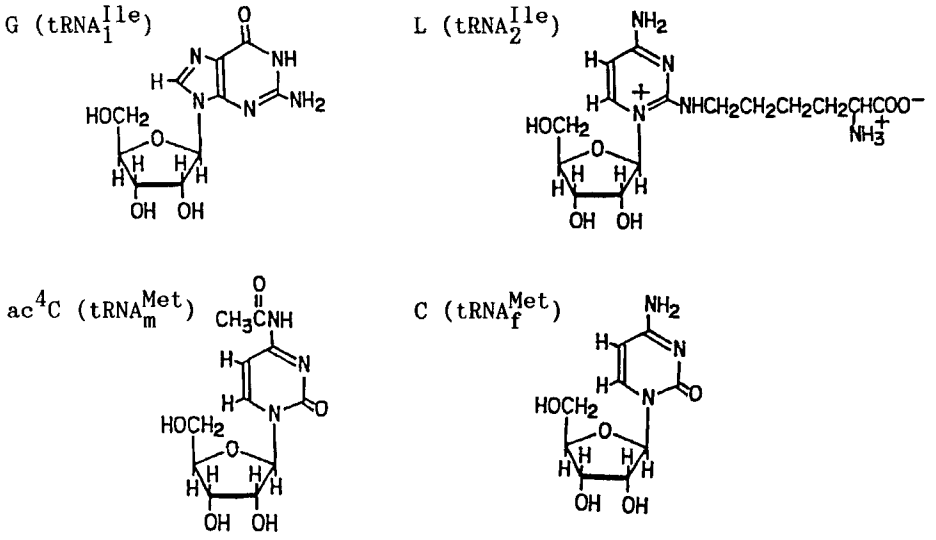

FIG. 1. Nucleosides in the first position of the anticodon of *E. coli* tRNA$_1^{Ile}$, tRNA$_2^{Ile}$, tRNA$_m^{Met}$, and tRNA$_f^{Met}$.

lysidine, rather than cytidine, in position 34 for the aminoacylation of tRNA. Note that the structures of guanosine and lysidine appear to be much different from each other (FIG. 1).

How does IleRS recognize a nucleoside in the first position of the anticodon? There are two possible schemes in discriminating a tRNA with the anticodon CAU from those having the anticodon GAU or LAU. In the first case, IleRS recognizes a structural feature that is common to guanosine and lysidine but not to cytidine. In the second case, IleRS simply discriminates against tRNA species having the methionine anticodon CAU (or ac^{4}CAU). In the present study on the recognition schemes of *E. coli* IleRS, unmodified molecules of tRNA$_1^{Ile}$ with the anticodon GAU and the variants with the anticodon CAU, AAU and UAU were prepared *in vitro* by the use of T7 RNA polymerase. The unmodified tRNA$_1^{Ile}$ accepted isoleucine, while the variants with the methionine anticodon CAU did not. Further, the variant tRNA with the anticodon AAU or UAU did not accept isoleucine either. These indicate that IleRS requires a common feature of guanosine and lysidine in position 34 for the aminoacylation.

EXPERIMENTAL PROCEDURE

Strain and plasmid — *E. coli* MV1184(*Δ(srl-recA)*306::Tn10, *Δ(lac-pro), ara⁻, thi⁻, rpsL⁻, φ80dlacZ M15,* [F'*traD36, proAB, lacI*^{q}*ZΔM15*]), MV1190(*Δ(srl-recA)*306::Tn10, *Δ(lac-pro), thi⁻, supE,* [F'*traD36, proAB, lacI*^{q}*ZΔM15*]), and CJ236(*dut-1, ung-1, thi-1, relA-1*) were used as host cells for M13 mp19, and were also used for mutagenesis. Plasmid pTTQ18 was from Amersham Inc.

Oligonucleotide synthesis — All oligonucleotides were synthesized on the Cyclone Plus DNA synthesizer (MilliGen Biosearch).

E. coli ileV gene and mutagenesis — The two-kilobase fragment containing *ileV* was cloned from *E. coli* K12 W3110 strain by the use of an oligonucleotide probe consisting of 21 nucleotides corresponding to the anticodon region of the tRNA. This fragment was confirmed to be the same as that cloned previously[7] by the comparison of the restriction maps with *Hae*III and *Alu*I. Mutagen Kit (Bio-Rad Laboratories) was used for oligonucleotide directed mutagenesis.

Plasmid construction — A 103-bp fragment containing 3/4 (the 3'-part) of the *ileV* gene was prepared by digestion of the 2.0-kb fragment with *Alu*I and *Nsp*I (FIG. 2). This fragment together with a chemically synthesized oligonucleotides containing the T7 promoter and 1/4 (the 5'-part) of the *ileV* gene was inserted between the *Bam*HI and *Sph*I sites of M13 mp19. Oligonucleotide-directed mutagenesis was performed in the position succeeding the CCA terminus of the *ileV* gene and an *Nsp*I site was formed. In addition, in order to raise the efficiency of transcription with T7 RNA polymerase[8], A1 and T72 of the tRNA genes were replaced with G1 and C72, respectively. Further, G34 (the first position of the anticodon) of the tRNA genes was replaced by C, A and T. Finally, these genes with the T7 promoter (FIG. 3) were cleaved from the M13 vector and inserted into plasmid pTTQ18 (FIG. 2). The *Nsp*I digests of these plasmids were used as templates for the *in vitro* syntheses of $tRNA_1^{Ile}$s with the anticodon GAU, CAU, AAU and UAU.

In vitro transcription of tRNA gene with T7 RNA polymerase — The transcription of the wild-type and variant tRNA genes was performed in a reaction mixture of 0.5 ml containing 40 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 2 mM spermidine, 10 mM $MgCl_2$, bovine serum albumin (50 μg/ml; Takara Shuzo Co., Ltd.), 2.0 mM each NTP (ATP, CTP, GTP and UTP), 20 mM 5'-GMP, *Nsp*I-digested DNA (10 μg), and 80 μg of T7 RNA

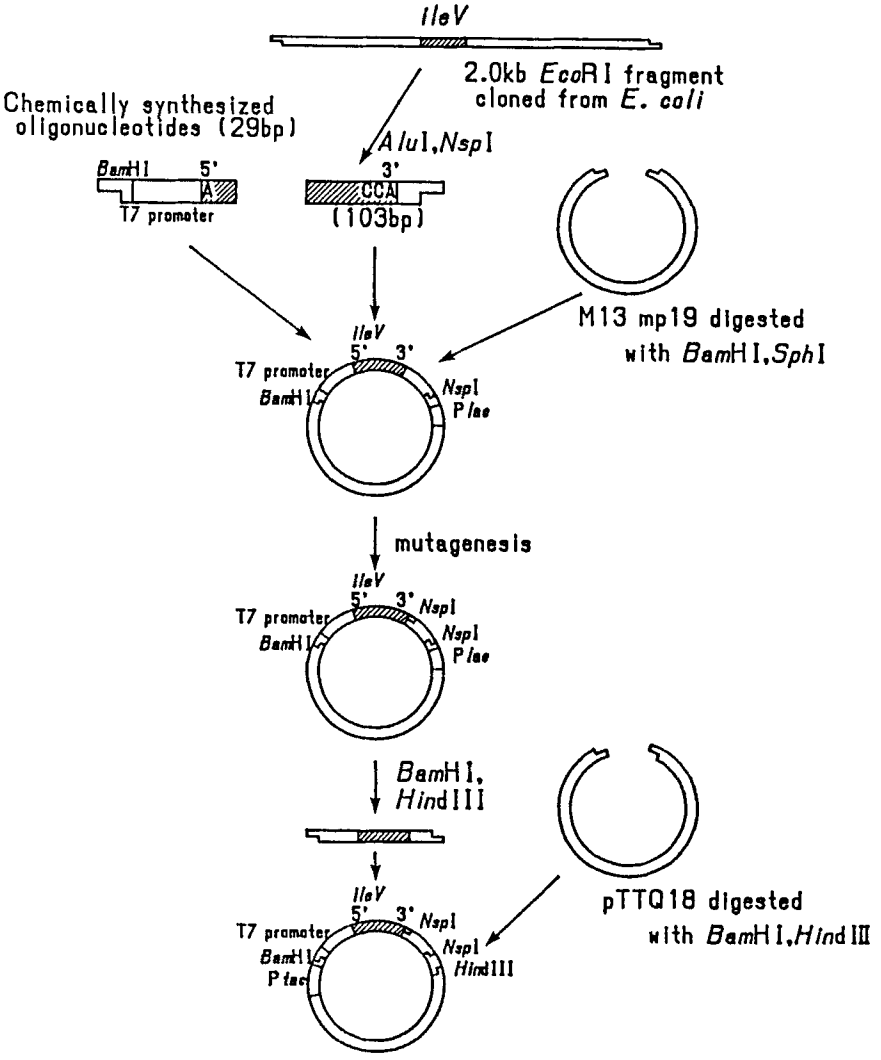

FIG. 2. Construction of the plasmid DNAs bearing the *E. coli* $tRNA_1^{Ile}$ gene (*ileV*) and variant genes.

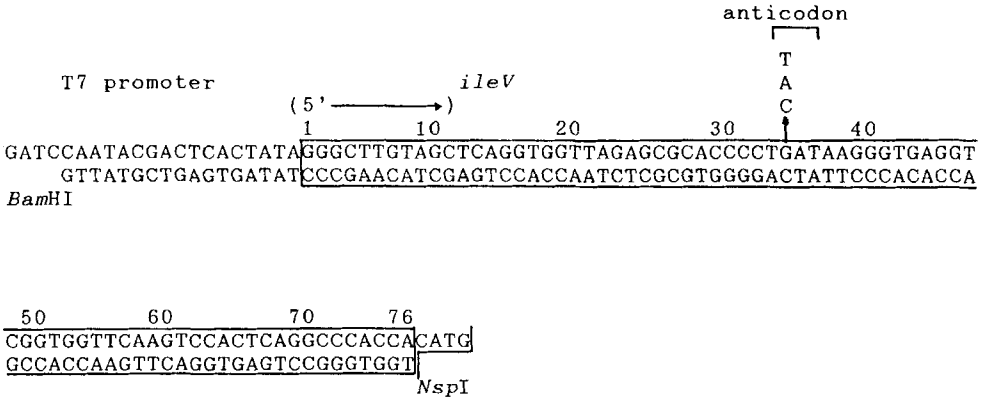

FIG. 3. Nucleotide sequences of the fragments inserted into pTTQ18. The numbering is after Sprinzl[9]. $tRNA_1^{Ile}$ has an extra residue (number 20a).

polymerase as described[10] (a concentration of T7 RNA polymerase as high as 10-200 μg/ml is necessary for the synthesis of short RNAs[10]). After incubation for 1 hr at 37°C, 5 units of inorganic pyrophosphatase (Sigma) and 40 μg of pure T7 RNA polymerase were added, and the incubation was continued for 2 hr. After successive extraction with phenol, phenol/chloroform and chloroform[11], the transcripts were purified by 7 M urea/10 % polyacrylamide gel electrophoresis as described[12].

Assay for isoleucine acceptance — tRNA gene transcripts were finally purified by 7M urea/10% PAGE, dissolved in a solution containing 20 mM Tris-HCl (*p*H 7.5) and 10 mM $MgCl_2$, and annealed by heating at 65°C for 10 min and cooling gradually to room temperature over 2-3 hr. The isoleucine-accepting activity of tRNA transcript (0.5 A_{260} units ml^{-1}, final) was measured in a solution (50 μl) containing 100 mM Tris-HCl (*p*H 7.5), 2 mM ATP, 5 mM $MgCl_2$, 10 mM KCl, 45 μM [U-^{14}C]isoleucine (324 mCi $mmol^{-1}$) and 167 μg ml^{-1} of *E. coli* isoleucyl-tRNA synthetase[13]. After various incubation times at 37°C, an aliquot (10 μl) was transferred on to a Whatman 3MM paper filter. The filter was washed 3 times with cold 5 % CCl_3COOH and twice with ethanol and dried, and the ^{14}C radioactivity was counted with a liquid scintillation counter.

The dependence of isoleucine-accepting activity of tRNA transcript (0.25 A_{260} units ml^{-1}) upon Mg^{2+} concentration was measured in a solu-

tion (40 μl) containing 100 mM Tris-HCl (*p*H 7.5), 2 mM ATP, 10 mM KCl, 9.1 μM [U-^{14}C]isoleucine (324 mCi mmol^{-1}), 84 μg ml^{-1} of *E. coli* isoleucyl-tRNA synthetase[13], and 0, 5, 10, 20, 50, or 100 mM $MgCl_2$. This mixture was incubated at 37°C, and after 1, 2 and 5 min, an aliquot (10 μl) was taken, and the amount of isoleucine charged to tRNA was measured as described above.

RESULTS AND DISCUSSION

T7 RNA polymerase transcription — The plasmids carrying *E. coli* ($tRNA_1^{Ile}$ gene) variants placed under the control of the bacteriophage T7 promoter were constructed (FIGS. 2,3). The replacement of the A1 · T72 base pair of $tRNA_1^{Ile}$ with G1 · C72 is expected to have no effect on the isoleucine-accepting activity, because the other isoleucine tRNA having the G1 · C72 base pair ($tRNA_2^{Ile}$)[4] accepts isoleucine with the same efficiency as native $tRNA_1^{Ile}$ (data not shown). The T7 promoter is placed properly upstream of the tRNA structural genes in these plasmids, so that the transcription with T7 RNA polymerase begins exactly at G1 of tRNA. By the use of *Nsp*I digests of plasmids as templates, transcription exactly terminates at the CCA termini of tRNAs. 0.6 A_{260} unit of transcript was obtained by using 10 μg of an *Nsp*I-digested plasmid encoding $tRNA_1^{Ile}$ with anticodons NAU (N = U, C, A or G).

Isoleucine-accepting activity of the unmodified $tRNA_1^{Ile}$ — Under the normal aminoacylation condition, the isoleucine-accepting activity of the unmodified $tRNA_1^{Ile}$ transcript with the anticodon GAU was much lower than that of mature $tRNA_1^{Ile}$ from *E. coli* cells (data not shown). In the case of yeast $tRNA^{Phe}$, the transcript prepared with T7 RNA polymerase has, under the normal condition, a lower phenylalanine-accepting activity than mature $tRNA^{Phe}$ from yeast cells, and the Mg^{2+} concentration required for efficient aminoacylation of the transcript is higher than that required for aminoacylation of mature $tRNA^{Phe}$.[14] Thus, the post-transcriptional modifications of yeast $tRNA^{Phe}$ contribute slightly to the aminoacylation through stabilization of the tertiary structure[14,15]. This is considered to be the case also for several tRNAs (*E. coli* $tRNA^{Met}$, $tRNA^{Val}$, $tRNA^{Ala}$, $tRNA^{His}$, and $tRNA^{Gln}$, and yeast $tRNA^{Asp}$)[16-21]. By contrast, the initial velocity of aminoacylation of the $tRNA_1^{Ile}$ transcript depends only slightly on Mg^{2+} concentration (FIG. 4), although the stability of the tertiary structure of the *E. coli*

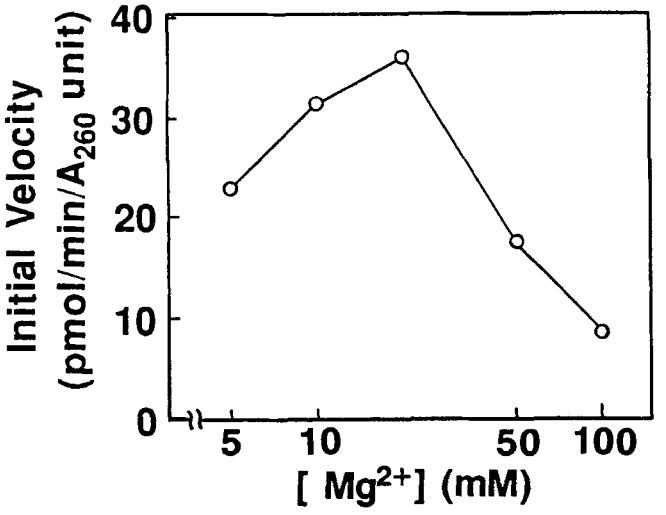

FIG. 4. Initial velocity of aminoacylation of $tRNA_1^{Ile}$ transcript on Mg^{2+} concentration.

$tRNA_1^{Ile}$ transcript probably depends on the concentration of Mg^{2+}. This indicates that some of the modified residues (D20, D20a, t^6A37, m^7G46, acp^3U47, T54, or $\Psi 55$) of *E. coli* isoleucine tRNAs are directly recognized by IleRS as identity determinants. These modifications are also found at the corresponding positions in some *E. coli* tRNAs specific to other amino acids. In general, it is rare that a single residue determines the identity of a tRNA; at least two residues of a tRNA constitute an identity set[22]. For instance, cytidine in the first position of the anticodon (C34) is one of the major identity determinants for *E. coli* methionine tRNAs[23], although there are many tRNAs with C34 which are specific to other amino acids. Thus, as the identity determinants for *E. coli* isoleucine tRNAs, there may be unmodified residues which constitute the "identity set" with the modified residues.

By the use of IleRS at a high concentration (0.167 mg/ml), the time course of aminoacylation of the $tRNA_1^{Ile}$ transcript was measured. The plateau level of isoleucine acceptance of the transcript (FIG. 5) was as low as 1/4 of that of native $tRNA_1^{Ile}$ (data not shown). As described above, modified nucleosides of *E. coli* $tRNA_1^{Ile}$ are directly involved in the interaction of $tRNA_1^{Ile}$ and IleRS. Nevertheless, the isoleucine-

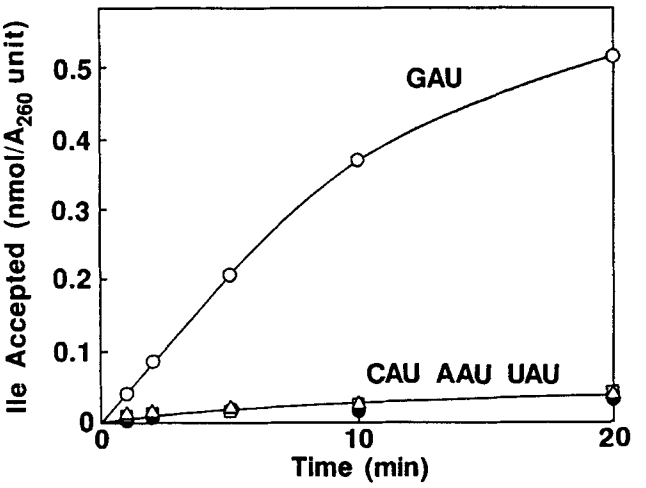

FIG. 5. Time course of aminoacylation of $tRNA_1^{Ile}$s having the anticodon GAU (○-○), CAU (●-●), AAU (△-△), and UAU (□-□).

accepting activity of the unmodified $tRNA_1^{Ile}$ transcript was high enough for further analysis of the substrate specificity in the aminoacylation reaction by IleRS.

Isoleucine-accepting activities of variant tRNA transcripts — The variant tRNA transcript with the anticodon CAU did not accept isoleucine (FIG. 5); which is consistent with the previous result on $tRNA_1^{Ile}$ variant with the same anticodon[6]. Further, the tRNA transcripts with the anticodon AAU and UAU did not accept isoleucine either (FIG. 5). Thus, as for isoleucine acceptance, the tRNA transcript with the native anticodon GAU is active while the variant tRNA transcript with the anticodon AAU, UAU and CAU are inactive.

IleRS recognizes a common feature of guanosine and lysidine — *E. coli* IleRS is now found to distinguish the isoleucine tRNA with the anticodon GAU from those with AAU, UAU and CAU. IleRS also recognizes another isoleucine tRNA, $tRNA_2^{Ile}$, which has the anticodon LAU. Thus, there should be a structure feature common to guanosine and lysidine but not to adenosine, cytidine, or uridine in the first position of the anticodon.

As for lysidine, three tautomeric forms are possible although the abundance ratios of those forms have not been obtained[5]. However, a

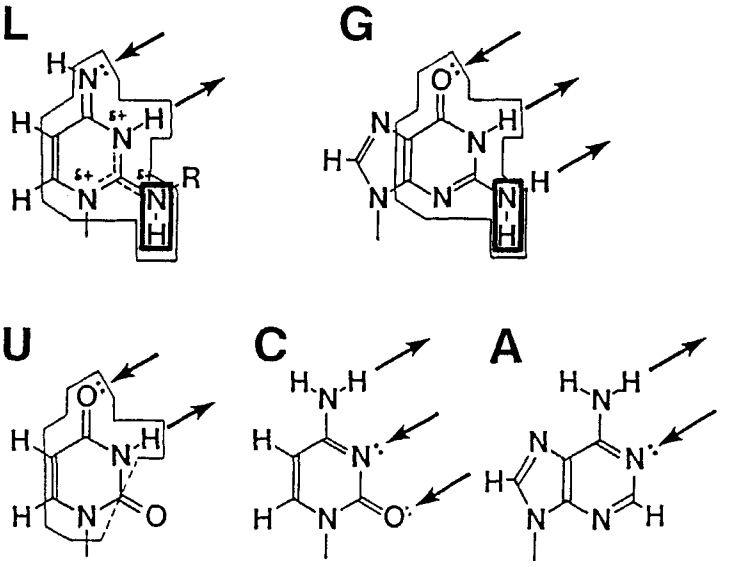

Fig. 6. A tautomeric form of the base of lysidine (L) that shares a common feature with G. The common feature is enclosed by thin lines. Hydrogen donors and acceptors are indicated with arrows ↗ and ↙, respectively. The N-H group enclosed by a rectangle is suggested to be the identity determinant of *E. coli* isoleucine tRNAs.

tautomeric form of lysidine (FIG. 6) shares a common structure feature with guanosine. In this form, lysidine has an -NHR group in position 2, an >NH group in position 3, and an =NH group in position 4 (FIG. 6). The base moiety of lysidine resembles the six-membered ring part of guanosine; the N atom in position 4 of lysidine corresponds to the oxygen atom in position 6 of guanosine. The common feature of lysidine and guanosine is enclosed by thin lines in FIG. 6.

E. coli IleRS probably recognizes this common feature of lysidine and guanosine. Uridine, rather than cytosine and adenosine, shares a part of the common feature (hydrogen donation in position 3 and hydrogen acceptance in position 4), but does not have the N-H group in position 2 (FIG. 6). This N-H group in position 2 (enclosed by thick lines) must be recognized by IleRS in distinguishing lysidine and guanosine from the

other three nucleosides (U, C, or A). Probably, the N-H group in position 2 of the nucleoside in the first position of the anticodon is a positive determinant of isoleucine tRNA identity.

The tautomeric form of lysidine (FIG. 6) is a reasonable structure in the first position of the anticodon of $tRNA_2^{Ile}$, which recognizes the isoleucine codon AUA rather than AUU or AUC[3]. The >NH group (hydrogen donor) in position 3 and the =NH group (hydrogen acceptor) in position 4 of lysidine are involved in the base pair with adenosine in the third position of the codon, while the presence of the bulky substituent (R) in position 2 does not allow the formation of a pair with guanosine, thus preventing the misrecognition of the codon AUG of methionine.

In contrast to *E. coli*, eukaryotic cells have cytoplasmic isoleucine tRNAs with the anticodon IAU[9]. However, inosine does not have the 2-NH group which is one of identity determinants for *E. coli* isoleucine tRNA. Moreover, yeast has another species of cytoplasmic isoleucine tRNA, in which the anticodon is coded by TAT[24]. Thus, the identity determinants of isoleucine tRNAs of yeast are possibly different from those of *E. coli*. This can be examined by aminoacylation experiments of yeast and *E. coli* $tRNA^{Ile}$s by yeast and *E. coli* enzymes.

In conclusion, for the identity of isoleucine tRNA, the determinant must be elucidated in the level of chemical structure of the nucleoside in the first position of the anticodon, rather than in the level of tRNA sequence. $tRNA^{Ile}$s from other species or engineered tRNAs with chemically synthesized nucleoside analogues in the first position of the anticodon will be useful for that purpose.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (01656002 and 02238102) from the Ministry of Education, Science and Culture of Japan, by a Bioscience Grant for International Joint Research Project from the New Energy and Industrial Technology Development Organization, and by a Research Grant from International Human Frontier Science Program Organization.

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Received 9/11/91
Accepted 11/15/91