Modified Nucleosides in the First Positions of the Anticodons of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ from *Escherichia coli*†

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ABSTRACT: Minor leucine tRNA species, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$, from *Escherichia coli* B have been reported to recognize leucine codons UUA and UUG [Goldman, E., Holmes, W. M., and Hatfield, G. W. (1979) *J. Mol. Biol. 129*, 567−585]. In the present study, these two $tRNA^{Leu}$ species were purified from *E. coli* A19, and the nucleotide sequences were determined by a post-labeling method. $tRNA_5^{Leu}$ was found to correspond to the tRNA gene reported as su°6 tRNA [Yoshimura, M., Inokuchi, H., and Ozeki, H. (1984) *J. Mol. Biol. 177*, 627−644]. The first letter of the anticodon was identified to be 2′-*O*-methylcytidine (Cm). $tRNA_4^{Leu}$ was identified as the minor leucine tRNA that has been sequenced previously ($tRNA_{UUR}^{Leu}$) [Yamaizumi, Z., Kuchino, Y., Harada, F., Nishimura, S., and McCloskey, J. A. (1980) *J. Biol. Chem. 255*, 2220−2225]. There was an unidentified modified nucleoside (N*) in the first position of the anticodon of $tRNA_4^{Leu}$. Nucleoside N* was isolated to homogeneity (1 A_{260} unit). By ^1H NMR spectroscopy, nucleoside N* was found to be a 2′-*O*-methyluridine derivative with a substituent having a $-CH_2NH_2^+CH_2COO^-$ moiety in position 5 of the uracil ring. On the basis of these NMR analyses together with mass spectrometry, the chemical structure of nucleoside N* was determined as 5-carboxymethylaminomethyl-2′-*O*-methyluridine ($cmnm^5Um$). Nucleoside N* was thus found to be a novel type of naturally occurring modified uridine. Because of the conformational rigidity of Cm and $cmnm^5Um$ in the first position of the anticodon, these $tRNA^{Leu}$ species recognize the leucine codons UUA and UUG correctly, but never recognize the phenylalanine codons UUU and UUC.

Many tRNA molecules have modified nucleosides at the first position of the anticodon (position 34), which are supposed to have essential roles in codon recognition (*1*). Particularly, leucine tRNA species that correspond to the UUR codons (R = A and G) often have a modified pyrimidine substituted at position 5 and/or 2′-hydroxyl group, such as 2′-*O*-methyluridine (Um;[1] soybean chloroplast $tRNA^{Leu}$) (*2*), 5-carboxymethylaminomethyluridine ($cmnm^5U$; yeast mitochondrial $tRNA_{UUR}^{Leu}$) (*3*), 5-carbamoylmethyl-2′-*O*-methyluridine (ncm^5Um; yeast cytoplasmic tRNA) (*4*), 2′-*O*-methylcytidine (Cm), (*2*, *5*, *6*), 5-methylcytidine (m^5C; yeast $tRNA_3^{Leu}$) (*7*), and 5-formyl-2′-*O*-methylcytidine (f^5Cm; cytoplasmic tRNAs from bovine liver) (*8*). Other nucleosides have been found from various organisms, but their chemical structures remain unidentified (*9*, *10*).

Escherichia coli has two leucine isoacceptors, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$, that read the UUR codons in an in vitro protein synthesis system (*11*). On the other hand, a tRNA, named as "$tRNA_{UUR}^{Leu}$", was sequenced and was found to have an unidentified modified nucleoside in position 34, which was proposed to be a modified adenosine (*12*). Later, two UUR-reading leucine tRNA genes, su°6 (= *supP*, *leuX*) (*13*, *14*) and *leuY* (*15*), were sequenced. The anticodon of the *leuX* gene is CAA, while the modified nucleosides of

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[1] Abbreviations: Cm, 2′-*O*-methylcytidine; $cmnm^5U$, 5-carboxymethylaminomethyluridine; $cmnm^5Um$, *N**,5-carboxymethylaminomethyl-2′-*O*-methyluridine; D, dihydrouridine; Gm, 2′-*O*-methylguanosine; HPLC, high-performance liquid chromatography; ms^2i^6A, 2-methylthio-N^6-isopentenyladenosine; PDase, snake venom phosphodiesterase; PMase, *Escherichia coli* phosphomonoesterase; s^4U, 4-thiouridine; TLC, thin-layer chromatography; Um, 2′-*O*-methyluridine.

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this tRNA have not been identified. The *leuY* gene was found to correspond to $tRNA^{Leu}_{UUR}$, which indicated that the unidentified nucleoside is encoded by a T (*15*). On the other hand, the unidentified nucleoside of $tRNA^{Leu}_{UUR}$ was indirectly concluded to be Cm (*16*).

In the present study, we purified $tRNA^{Leu}_4$ and $tRNA^{Leu}_5$ from *E. coli* and determined the nucleotide sequences by a post-labeling method. It was revealed that $tRNA_4^{Leu}$ and $tRNA^{Leu}_5$ are the *leuY* and *leuX* products, respectively, and that the nucleosides in position 34 are 5-carboxymethylaminomethyl-2′-*O*-methyluridine (cmnm^{5}Um) and Cm, respectively.

EXPERIMENTAL PROCEDURES

Isolation of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ from E. coli. E. coli A19 cells were harvested in the late logarithmic phase of growth, and crude tRNA was prepared from them as described by Zubay (*17*) except that treatment with alkali was omitted. $tRNA^{Leu}_4$ and $tRNA^{Leu}_5$ were purified by successive chromatography on columns of DEAE-Sephadex A-50 (Pharmacia) at pH 7.5 (*18*, *19*), benzoylated DEAE-cellulose (BD-cellulose, Boehringer-Mannheim) (*20*), and Sepharose 4B (Pharmacia) using a reverse salt gradient (*21*) and DEAE-Sephadex A-50 (Pharmacia) at pH 4.0 (*22*). Assay of amino acid acceptor activity of tRNA using crude *E. coli* aminoacyl-tRNA synthetase was carried out according to Nishimura et al. (*18*), where 5−10 μL of each fraction was used for the measurement of the acceptor activity of each amino acid species.

DEAE-Sephadex A-50 Column Chromatography at pH 7.5. The crude *E. coli* tRNA (110 000 A_{260} units) was dialyzed against 20 mM Tris·HCl buffer (pH 7.5) containing 8 mM $MgCl_2$ and 0.365 M NaCl (buffer A). The dialysate was loaded on a DEAE-Sephadex A-50 column (4.5 × 145 cm) equilibrated with buffer A. Elution was performed, at a flow rate of 67 mL/h, with 20 L of a linear gradient of NaCl (from 0.375 to 0.525 M) and $MgCl_2$ (from 8 to 16 mM) in buffer A, and fractions of 30 mL were collected. Fractions containing $tRNA^{Leu}_4$ and/or $tRNA^{Leu}_5$ were pooled as several fractions, and tRNAs were precipitated by the addition of 2.5 volumes of ethanol.

BD-Cellulose Column Chromatography. Each of precipitated tRNA fractions of the DEAE-Sephadex A-50 column chromatography (pH 7.5) was dissolved in 20 mM CH_3-COONa buffer (pH 6.0) containing 0.35 M NaCl (buffer B), and loaded on a BD-cellulose column (1.5 × 140 cm) equilibrated with buffer B. Elution was performed, at a flow rate of 10 mL/h, first with 1 L of a linear gradient of NaCl (from 0.5 to 1.0 M) in buffer B, and second with 1 L of a linear gradient of NaCl (from 1.2 to 2.5 M) and ethanol (from 0% to 20%) in buffer B, and fractions of 5 mL were collected. Fractions containing $tRNA^{Leu}_4$ and/or $tRNA^{Leu}_5$ were pooled, and tRNAs were precipitated by the addition of 2.5 volumes of ethanol.

Sepharose 4B Column Chromatography. Precipitated tRNAs of the pooled fractions of the BD-cellulose column chromatography were dissolved in 10 mM CH_3COONa buffer (pH 4.5) containing 6 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 1.3 M $(NH_4)_2SO_4$ (buffer C), and loaded on a Sepharose 4B column (0.8 × 60 cm) equilibrated with buffer C. Elution was performed with 220 mL of a linear reverse salt gradient of $(NH_4)_2SO_4$ (from 1.3 to 0 M) in buffer C at the flow rate of 4.6 mL/h, and fractions of 2 mL were collected. Fractions containing $tRNA^{Leu}_4$ or $tRNA^{Leu}_5$ were pooled.

DEAE-Sephadex A-50 Column Chromatography at pH 4.0. Pooled fractions of Sepharose 4B column chromatography were dialyzed against 10 mM CH_3COONa buffer (pH 4.0) containing 10 mM $MgCl_2$ and 390 mM NaCl (buffer D). The dialysate was loaded on a DEAE-Sephadex A-50 column (0.7 × 140 cm) equilibrated with buffer D. Elution was performed, at a flow rate of 5 mL/h, first with 480 mL of a linear gradient of NaCl (from 0.4 to 0.8 M) in buffer D, and fractions of 2 mL were collected. Fractions containing $tRNA^{Leu}_4$ and/or $tRNA^{Leu}_5$ were pooled, and tRNAs were precipitated by the addition of 2.5 volumes of ethanol.

Sequencing of tRNA by Post-labeling Method. Nucleotide sequences of tRNAs were analyzed by the post-labeling method (*23*−*25*). $tRNA^{Leu}_4$ and $tRNA^{Leu}_5$ as obtained by column chromatography were further purified by electrophoresis on 10% and 20% polyacrylamide gels and on a 15% polyacrylamide gel in the presence of 7 M urea. These tRNA species were treated with formamide, and the 5′-termini of degradation fragments were labeled with ^{32}P (*25*). For obtaining 5′-^{32}P-labeled mononucleotides, these labeled fragments were digested by nuclease P1 (0.5 mg/mL) in 1 mM CH_3COONH_4 buffer (pH 5.4), and, in some cases, by snake venom PDase (0.2 mg/mL) in 50 mM Tris·HCl buffer (pH 8.0).

Two-Dimensional TLC Analyses of Nucleotides. These 5′-^{32}P-labeled nucleotides were identified by autoradiography of two-dimensional thin-layer chromatograms (5 × 5 cm) with solvent system I [the first dimension, isobutyric acid/concentrated ammonia/water (50:1:29 v/v/v); the second dimension, 2-propanol/concentrated HCl/water (70:15:15 v/v/v)]. Furthermore, for the band corresponding to position 34, the 5′-^{32}P-labeled nucleotide was examined by autoradiography of two-dimensional thin-layer chromatograms (10 × 10 cm) with solvent system I and with solvent system II [the first dimension, isobutyric acid/concentrated ammonia/water (66:1:33 v/v/v); the second dimension, 100 mL of 0.1 M sodium phosphate buffer (pH 6.8) containing 2 mL of 1-propanol and 60 g of ammonium sulfate] (*26*).

*Preparation of Dinucleotide pN*pA from E. coli $tRNA_4^{Leu}$.* Purified $tRNA^{Leu}_4$ (160 A_{260} units) was digested by nuclease P1 (1 mg/mL) in 10 mM CH_3COONH_4 buffer (pH 5.4) at 37 °C for 14 h. The digest was spotted at the end of a cellulose TLC plate (20 × 20 cm) and developed with a solvent system, isobutyric acid/concentrated ammonia/water (50:1:29 v/v/v). pN*pA was extracted from a peak band between those of pU and pC and loaded on a DEAE-Sephadex A-25 column (0.8 × 20 cm) equilibrated with 0.01 M trimethylammonium bicarbonate buffer (pH 8.0). Elution was performed with 100 mL of a linear gradient of trimethylammonium bicarbonate buffer (pH 8.0) from 0.01 to 2.0 M, and fractions of 1 mL were collected. pN*pA was eluted as the main peak, which was confirmed by two-dimensional TLC analyses.

Analytical and Preparative HPLC of Nucleosides. The purified $tRNA^{Leu}_4$ or $tRNA^{Leu}_5$ (about 1 A_{260} unit) or the purified dinucleotide pN*pA (0.03−3 A_{260} units) was digested by bovine pancreatic RNase A (1 mg/mL), 0.2 mg/

mL snake venom PDase (0.2 mg/mL), and *E. coli* PMase (0.04 mg/mL), in 50 mM Tris·HCl buffer (pH 8.0) at 37 °C for 4−14 h. HPLC was performed using a Shimadzu LC-4A chromatography system equipped with a UV detector. A Shimadzu Shim-pack PC18 column (5.0 × 50 mm) and a Shimadzu Shim-pack CLC-ODS column (6.0 × 150 mm) were used for analytical HPLC and preparative HPLC, respectively. Elution was performed isocratically with 2.5 mM ammonium formate buffer (pH 5.1) containing 5% methanol at a flow rate of 0.9 mL/min.

^{1}H NMR Spectroscopy. Nucleoside N* (0.4 A_{260} units) or dinucleotide pN*pA (0.5 A_{260} units) was dissolved in 0.4 mL of ^2H_2O (99.6%), evaporated in an NMR tube (5 mm), and dissolved in 0.35 mL of ^2H_2O (100%). The pH of the sample solution was measured as a direct pH meter reading (not corrected for the difference from the p^{2}H value) and adjusted by the addition of 0.5 M ^{2}HCl or 0.5 M NaO^{2}H. ^{1}H NMR spectra (400 MHz) of nucleoside N* at 25 °C were recorded with a Bruker WM400 spectrometer. Chemical shifts (ppm) of proton resonances of nucleoside N* relative to the methyl proton resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate were obtained by the use of an internal standard of *tert*-butyl alcohol (1.231 ppm). Chemical shifts and spin-coupling constants of nucleoside N* at pH 8.5 were determined by spectral simulation using a computer program NMRSIM (*27*). pK_a values together with their standard deviations were obtained by the analyses of pH dependences of proton chemical shifts by a nonlinear least-squares method. These pK_a values are corrected neither for the difference between direct pH meter readings and p^{2}H values nor for the ^{1}H-^{2}H isotope effect on pK_a. Usually these two factors compensate largely with each other, so that these pK_a values obtained here may be regarded as approximate values of pK_a in ^1H_2O solution.

Mass Spectrometry. The purified nucleoside N* (0.1 A_{260} units) or an authentic cmnm^{5}U (1 A_{260} unit) was dissolved in a solution of *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane/pyridine (99:1:10), and then heated at 100 °C for 1 h. After removal of silylation reagents under vacuum, the sample was introduced by direct probe. A mass spectrum was recorded with a JEOL JMS-DX300 instrument with ionization voltage of 70 eV and ionization current of 300 μA.

MS2 RNA-Directed Protein Synthesis in vitro. $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ were purified finally by electrophoresis on 10% and 20% polyacrylamide gels and on a 15% polyacrylamide gel in the presence of 7 M urea. tRNAs were aminoacylated with ^{14}C-leucine (Amersham, 330 mCi/mmol) and used in MS2 RNA-directed protein synthesis in vitro as described (*28*). Reactions (0.2 mL) contained 2−3 nmol/mL of each purified ^{14}C-Leu-$tRNA^{Leu}$ species, or 7 nmol/mL of bulk tRNA aminoacylated with ^{14}C-leucine, as well as 1 mM nonradioactive leucine. Following incubation at 37 °C for 15 min, samples were digested with trypsin, subjected to electrophoresis at pH 3.5 (*28*), and autoradiographed. Bands were excised from the pH 3.5 separation, sewn onto a fresh sheet of Whatman 3MM paper, subjected to the second-dimension electrophoresis at pH 1.9 (*28*), and autoradiographed. Spots were cut out and counted in a scintillation counter.

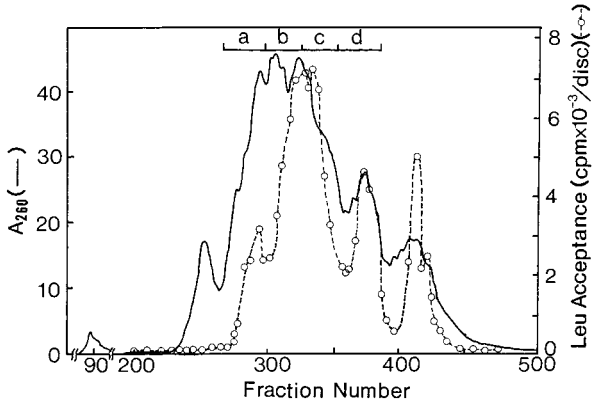

FIGURE 1: DEAE-Sephadex A-50 column chromatography of the *E. coli* tRNA fraction at pH 7.5.

RESULTS

Unambiguous Isolation of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ from E. coli. For the purpose of the present study, it was necessary to obtain authentic samples of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ from *E. coli*. First, crude tRNA (110 000 A_{260} units) was loaded on a column of DEAE-Sephadex A-50 at pH 7.5 (*18*, *19*). Leucine-accepting activity was eluted as four peaks (Figure 1). It has already been reported that $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ are eluted in the middle of the gradient in the same column chromatography (*29*). Therefore, fractions 272−390 were pooled as four samples a, b, c, and d (Figure 1), and each of these was fractionated by BD-cellulose column chromatography (*20*), where both $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ require ethanol to be eluted from the column (*29*). In fact, for each of samples a and b, the leucine-accepting activity was detected in fractions eluted from the BD-cellulose column by a buffer with 20% ethanol (data not shown). Such fractions probably containing $tRNA_4^{Leu}$ and/or $tRNA_5^{Leu}$ were pooled (samples aBD and bBD from samples a and b, respectively).

Each of these pooled samples (aBD and bBD) was fractionated by Sepharose 4B column chromatography with a reverse salt gradient (*21*) as shown in Figure 2. The major $tRNA^{Leu}$ species in sample aBD was eluted at the beginning of the gradient (Figure 2B), while the major $tRNA^{Leu}$ species in sample bBD was eluted at the end of the gradient (Figure 2A). In the same column chromatography, $tRNA_4^{Leu}$ is eluted last and $tRNA_5^{Leu}$ is eluted first, among five $tRNA^{Leu}$ species from *E. coli* (*29*). Accordingly, fractions 100−115 in Figure 2A were pooled as the $tRNA_4^{Leu}$ fraction and fractions 30−60 in Figure 2B were pooled as $tRNA_5^{Leu}$. These $tRNA^{Leu}$ species were further purified by chromatography on a column of DEAE-Sephadex A-50 (pH 4.0) (*12*, *22*). Finally, about 200 A_{260} units each of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ were obtained. The purity of each of them was more than 95%, judging from their leucine-accepting activity and the patterns of electrophoresis on 10% and 20% polyacrylamide gels and on a 15% polyacrylamide gel in the presence of 7 M urea.

On the basis of each of the three chromatographic patterns described by Hurd et al. (*29*), we identified our $tRNA^{Leu}$ species unambiguously to be $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$, respectively. In fact, there has been some confusion in the literature concerning the numbering of five isoaccepting

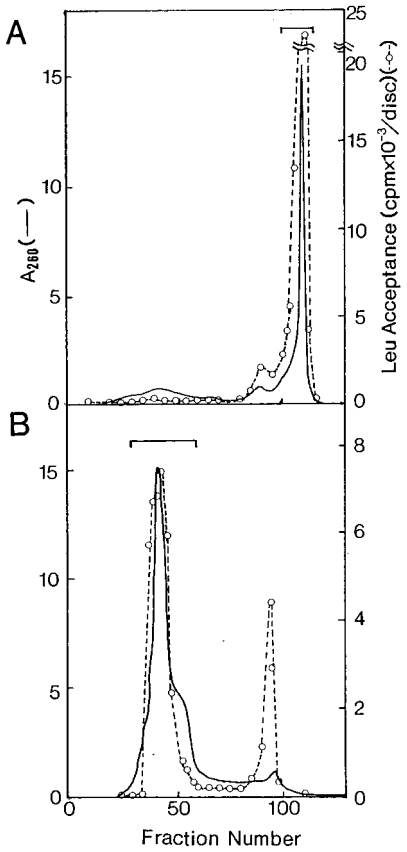

FIGURE 2: Sepharose 4B column chromatography: $tRNA_4^{Leu}$ fraction (A) and $tRNA_5^{Leu}$ fraction (B).

$tRNA^{Leu}$ species (*12*, *14*, *21*, *29*−*32*). However, the numbering of the $tRNA^{Leu}$ species here is unambiguous, since the elution times of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ are remarkably different from each other in the Sepharose 4B column chromatography (*21*, *29*) as shown in Figure 2. Therefore, the present identification of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ is consistent with the original numbering by Kelmers et al. (*31*) using RPC-5 columns.

Nucleotide Sequence of E. coli $tRNA_4^{Leu}$. The nucleotide sequence of $tRNA_4^{Leu}$ except for the first and second positions of the anticodon was determined by the post-labeling method (*23*) as shown in Figure 3A. As far as the determined sequence is concerned, $tRNA_4^{Leu}$ is identical with $tRNA_{UUR}^{Leu}$ which had been sequenced and considered to be $tRNA_5^{Leu}$ (*12*). Modified nucleoside residues, $s^4U(8)$, D(16), Gm(18), D(20), Ψ(32), $ms^2i^6A(37)$, Ψ(39), T(54), and Ψ(55) (according to the universal numbering of residues in tRNA) (*33*), were identified by the positions of spots in TLC analyses in the course of the post-labeling sequencing.

Nucleoside in Position 34 of E. coli $tRNA_4^{Leu}$. As for position 34, an unidentified spot (spot-34) was found in the TLC autoradiogram (Figure 4A), which is close to the authentic sample of queuosine 5′-monophosphate, pQ (*34*). On the other hand, the band corresponding to position 35 was missing in the first sequencing PAGE autoradiogram (data not shown). The phosphodiester bond between nucleosides in positions 34 and 35 is resistant against the formamide treatment. This ^{32}P-labeled nucleotide (spot-34) was not digested at all by further incubation with nuclease P1 even for as long as 10 h at 37 °C (Figure 4A,B). However, by treatment with PDase for 4 h at 37 °C, spot-34 completely disappeared and a new spot was observed in the TLC autoradiogram (Figure 4C). Therefore, spot-34 is not due to a mononucleotide pN(34) but due to a dinucleotide pN(34)-pN(35), which was completely resistant against nuclease P1 but readily digested by PDase resulting in the formation of ^{32}P-labeled pN(34) (Figure 4B,C). This was confirmed with a different solvent system for TLC (solvent system II) (Figure 4D,E). In TLC autoradiograms with these two solvent systems, the positions of the spots due to pN(34) do not agree with those of any modified nucleotides identified so far. Therefore, the nucleoside in position 34 of $tRNA_4^{Leu}$ from *E. coli* is a novel naturally occurring modified nucleoside (designated hereafter as N*).

Nucleoside in Position 35 of E. coli $tRNA_4^{Leu}$. To identify the nucleoside in position 35, which could not be labeled with ^{32}P by the post-labeling method, $tRNA_4^{Leu}$ (2.0 A_{260} units) was digested by nuclease P1 and applied to two-dimensional TLC (10 × 10 cm). The dinucleotide pN*(34)-pN(35) was extracted from the TLC spot, digested with PDase, and then applied to TLC again. Two spots were clearly detected by UV absorption; one was the spot of pN* as found in Figure 4D, and the other was identified to be pA (Figure 5). Accordingly, nucleosides in positions 34 and 35 were found to be an unidentified nucleoside N* and an unmodified A, respectively (Figure 3A). Thus, the anticodon of $tRNA_4^{Leu}$ is now determined to be N*AA.

Large-Scale Preparation of Nucleoside N from E. coli $tRNA_4^{Leu}$*. The nuclease P1 digest of purified $tRNA_4^{Leu}$ (160 A_{260} units) was fractionated by one-dimensional TLC. The dinucleotide pN*pA (about 4 A_{260} units) was extracted from the spot and purified to homogeneity by DEAE-Sephadex A-25 column chromatography. For trial, 0.03 A_{260} units out of this purified pN*pA was digested by PMase and PDase, and subjected to HPLC with an ODS column (Figure 6). In the chromatogram, only two peaks were observed (Figure 6B). The second peak was clearly assigned to adenosine, and, therefore, the first peak was assigned to nucleoside N* (Figure 6B). In the chromatogram of PDase/PMase digest of $tRNA_4^{Leu}$ under this condition (Figure 6A), a peak was observed at the same elution volume as that of the peak of nucleoside N* as prepared from the purified pN*pA (Figure 6B). This indicates that nucleoside N* was not degraded during the course of the purification of pN*pA. Thus, pN*pA (3 A_{260} units) was digested by PMase and PDase and fractionated by HPLC under the same condition as above, and each of the two peaks was collected. The nucleoside of the second peak was confirmed to be unmodified adenosine by ^1H NMR spectroscopy. From the absorbance at 260 nm of separated nucleoside N* and adenosine (Figure 6B) and the molecular extinction coefficient at 260 nm (ϵ_{260}) of adenosine (14.9×10^3), ϵ_{260} of nucleoside N* at pH 5.1 was estimated to be 9.4×10^3. Thus, 0.9 A_{260} unit (ca. 1 μmol) of nucleoside N* was purified to homogeneity.

UV Absorption Spectra of Nucleoside N from E. coli $tRNA_4^{Leu}$*. UV spectra of nucleoside N* were measured in the pH range 2.0−12.0 (Figure 7). The UV spectra at pH 2.0−6.5 are nearly the same as one another, where the absorption maximum is at 265 nm and the minimum is at 232 nm. As pH is raised to 12.0, UV spectra change with a

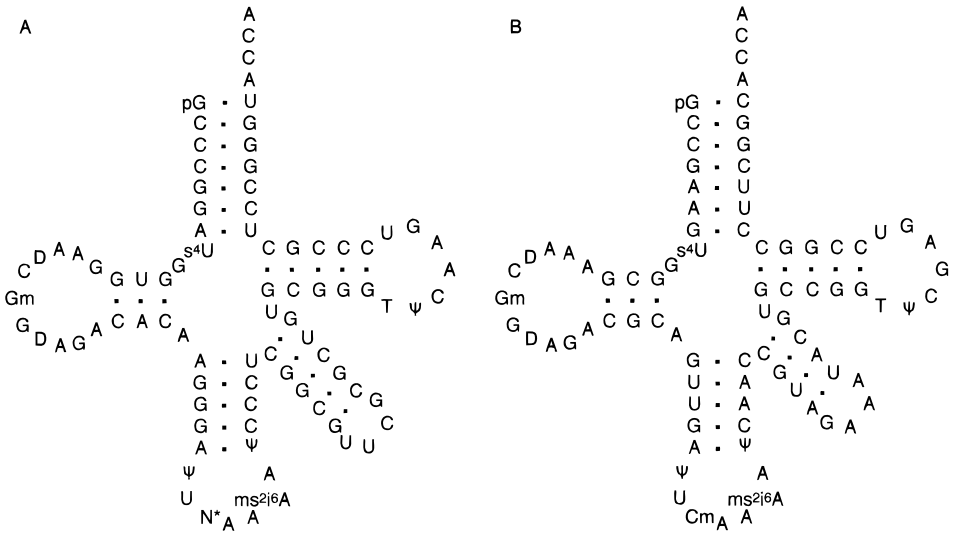

FIGURE 3: Nucleotide sequences of $tRNA_4^{Leu}$ (A) and $tRNA_5^{Leu}$ (B) from *E. coli* A19. Nucleoside N* in the first position of the anticodon (position 34) of $tRNA_4^{Leu}$ has been identified in the present study to be cmnm^{5}Um.

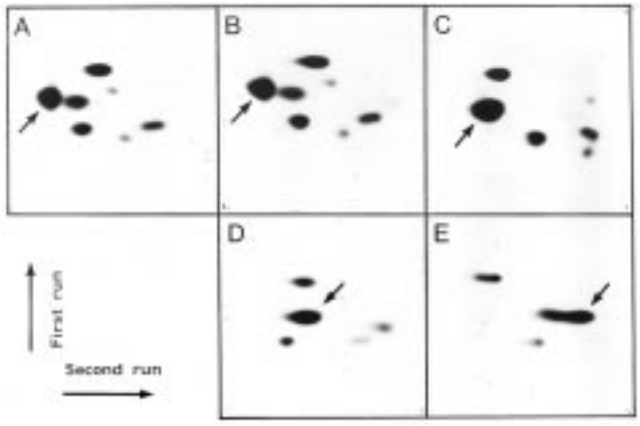

FIGURE 4: Autoradiography of two-dimensional thin-layer chromatograms of 5′-^{32}P-labeled nucleotides from *E. coli* $tRNA_4^{Leu}$. The 5′-^{32}P-labeled fragment terminating in position 34 was digested with nuclease P1 for 1 h (A) and for 10 h (B and D), and also digested with PDase (C and E), and then analyzed with solvent system I (A, B, and C) and solvent system II (D and E). Arrows indicate the spots of pN*pA (A, B, and E) and pN (C and D).

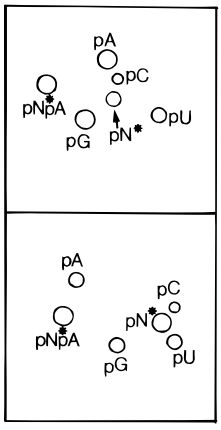

FIGURE 5: Spots of pN*, pN*pA, pCm, and pCmpA in two-dimensional thin-layer chromatography with solvent systems I (upper) and II (lower).

pseudo-isosbestic point around 247 nm. At pH 12.0, the absorption maximum is shifted to 262 nm with a lower molecular extinction coefficient, and the minimum is significantly shallow. These properties of nucleoside N* are similar to those of T (5-methyluridine), suggesting that, for nucleoside N*, a methyl or a methylene group is linked directly to position 5 of a uracil ring. Further, Figure 7 suggests that the deprotonation/protonation of uracil basemoiety of nucleoside N* occurs around pH 8.4. Such a low pK_a of protonation/deprotonation of uracil moiety as compared with that of 5-methyluridine (pK_a = 9.7) (*35*) is possibly

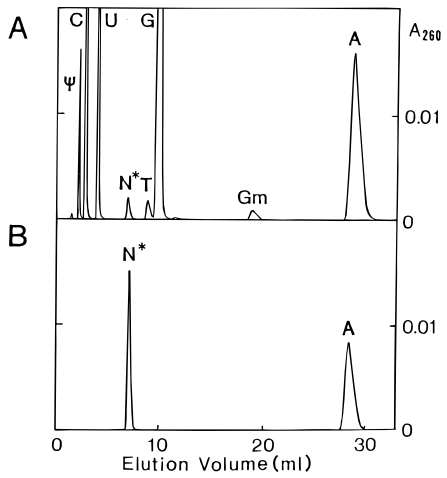

FIGURE 6: Reversed-phase HPLC of PDase/PMase digests of *E. coli* $tRNA_4^{Leu}$ (A) and the purified pN*pA (B).

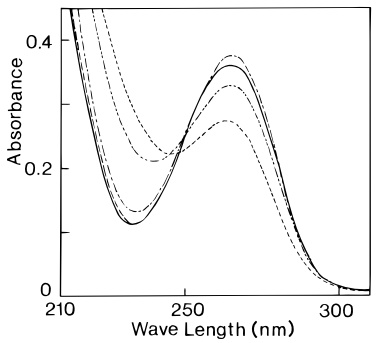

FIGURE 7: UV spectra of nucleoside N* from *E. coli* $tRNA_4^{Leu}$. pH of solutions: 2.0 (— - —), 4.9 (— — —), 6.5 (—), 8.4 (— - - —), 12.0 (- - -).

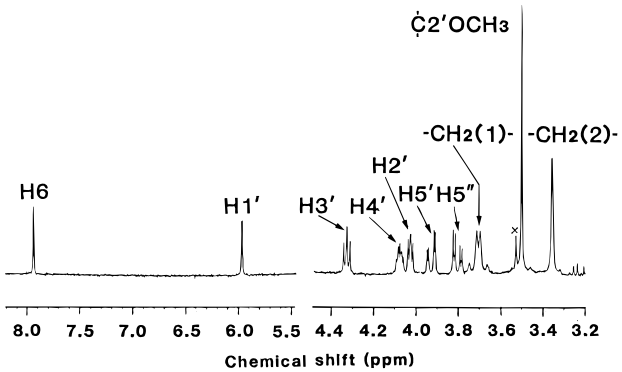

FIGURE 8: ^{1}H NMR (400 MHz) spectrum of nucleoside N* from *E. coli* $tRNA_4^{Leu}$ in ^2H_2O (pH 6.5). Resonances are indicated for the ribose protons [H1′, H2′, H3′, H4′, H5′, and H5″], the 2′-*O*-methyl protons [2′-*O*-CH_3], the H6 proton of the pyrimidine ring [H6], and the methylene protons of the 5-substituent, [CH_2(1) and CH_2(2)].

Table 1: Proton Chemical Shifts and Spin-Coupling Constants of Nucleoside N* and Um[a]

assign	N*	Um
	Chemical Shift	
ribose 1′	5.965	5.977
ribose 2′	4.028	4.052
ribose 3′	4.328	4.342
ribose 4′	4.077	4.122
ribose 5′	3.928	3.922
ribose 5″	3.807	3.813
base 5	nd	5.904
base 6	8.006	7.908P
CH_2(1)	3.688	nd
	3.726	nd
CH_2(2)	3.358	nd
CH_3	3.503	3.509
	Coupling Constant	
ribose $J_{1'2'}$	3.745 (0.066)	4.0
ribose $J_{2'3'}$	5.645 (0.066)	5.4
ribose $J_{3'4'}$	6.179 (0.066)	5.9
ribose $J_{4'5'}$	2.831 (0.067)	2.9
ribose $J_{4'5''}$	3.752 (0.067)	4.3
ribose $J_{5'5''}$	−12.825 (0.068)	−12.8
CH_2(1) J_{AB}	−13.42 (0.62)	nd

[a] Standard deviations are listed in parentheses.

because of the presence of a positively charged group (or groups) in the 5-substituent.

^{1}H NMR Spectroscopy of Nucleoside N from E. coli $tRNA_4^{Leu}$*: Ribose Moiety *2′-O-Methylated.* The 400 MHz ^{1}H NMR spectrum of nucleoside N* in ^2H_2O (pH 8.5) was measured (Figure 8). All of the ribose proton resonances (H1′, H2′, H3′, H4′, H5′, H5″) were assigned as indicated in Figure 8 by spin decoupling. The chemical shifts and spin-coupling constants for these ribose proton resonances of nucleoside N* were compared with those of unmodified uridine at 23 °C (*36*) (Table 1). The H2′ resonance (4.039 ppm) is upfield shifted by as much as 0.301 ppm from that of U, while the H1′ and H3′ resonances (5.961 and 4.327 ppm, respectively) are downfield shifted by 0.060 and 0.106 ppm, respectively, from those of U. These chemical shifts of ribose proton resonances of nucleoside N* are much the same as those of 2′-*O*-methyluridine (Um) (*37*) (Table 1). In fact, there is a methyl proton resonance at a chemical shift of 3.503 ppm, which agrees with that of the 2′-*O*-methyl proton resonance of Um (3.509 ppm at 28 °C) (*37*). All of these indicate that the 2′-hydroxyl group of the ribose moiety of nucleoside N* is methylated.

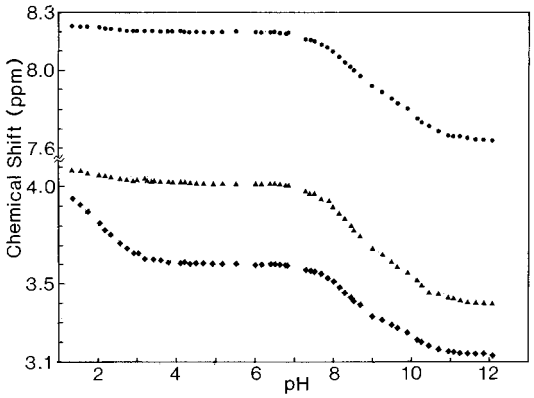

FIGURE 9: pH dependence of proton chemical shifts of nucleoside N* from *E. coli* $tRNA_4^{Leu}$. Resonances of the H6 proton of pyrimidine moiety and the CH_2(1) and CH_2(2) methylene protons of the 5-substituent are indicated as ●, ▲, and ◆, respectively.

Pyrimidine Moiety and 5-Substituent of Nucleoside N from E. coli $tRNA_4^{Leu}$*. As shown in Figure 8, there is no pair of doublet resonances corresponding to protons in positions 5 and 6 of the pyrimidine ring (H5 and H6) of uridine, while there is a singlet resonance at 8.006 ppm (pH 8.5). This singlet resonance is probably due to the H6 proton, because its chemical shift is similar to those of H6 protons of 5-methyluridine derivatives (*27*). Therefore, it is concluded that position 5 of the pyrimidine moiety of nucleoside N* is substituted possibly with a methylene group. In fact, in Figure 8, there are two pairs of methylene proton resonances at 3.688/3.726 ppm [AB pattern, CH_2(1)] and at 3.358 ppm [singlet, CH_2(2)] at pH 8.5. These two pairs of methylene protons are not spin-coupled with each other. One of these two methylene groups is probably bonded directly to position 5 of uracil ring. No other nonexchangeable proton resonance was observed for nucleoside N*. The same set of proton resonances was observed for the nucleoside N* moiety of dinucleotide pN*pA (data not shown).

pH Dependence of Proton Chemical Shifts of Nucleoside N from E. coli $tRNA_4^{Leu}$*. Figure 9 shows the pH dependence of chemical shifts of these resonances of the H6 proton of the pyrimidine moiety and the CH_2(1) and CH_2(2) methylene protons of the 5-substituent in a range of pH from 1 to 12. The proton NMR spectra at neutral pH were identical before and after the NMR measurements at pH 1 or 12, indicating that nucleoside N* is stable even at pH 1 and 12. In this pH range, all these three pH profiles indicate the presence of three titratable groups. By the least-squares analysis, the three pK_a values were obtained to be 2.13, 8.28, and 10.02 with their standard deviations of 0.01, 0.01, and 0.02, respectively. The intrinsic chemical shifts of four ionization states were also obtained as listed in Table 2.

Chemical Structure of 5-Substituent of Nucleoside N.* As shown in Figure 9, only the CH_2(2) methylene proton resonance is shifted significantly around pK_a (2.13), suggesting that a carboxyl group is directly bonded to this methylene carbon. Further, this pK_a value of 2.13 is close to that of the carboxyl group of glycine (pK_a = 2.35), and the chemical shift of this methylene proton resonance is close to that of the methylene proton resonance of glycine (3.94 ppm at acidic pH, 3.55 ppm at neutral pH, and 3.22 ppm at

Table 2: pK_a Values of Nucleoside N* and Synthetic cmnm^{5}U (A) and Intrinsic Chemical Shifts of the Four Ionization States of N* (B) Derived from pH Dependence of Proton Chemical Shifts[a]

pK_a Values of N* and cmnm^{5}U

assign	pK_a N*	pK_a cmnm^{5}U
$CH_2NH_2CH_2$	10.018 (0.016)	10.185 (0.048)
base 3H	8.283 (0.010)	8.151 (0.041)
COOH	2.126 (0.012)	2.129 (0.048)

Intrinsic Chemical Shifts of the Four Ionization States of N* and cmnm^{5}U

nucleoside	intrinsic chemical shift H6	CH_2(1)	CH_2(2)
N*	7.645 (0.002)	3.400 (0.002)	3.139 (0.001)
	7.902 (0.003)	3.660 (0.004)	3.319 (0.003)
	8.201 (0.001)	4.022 (0.001)	3.609 (0.001)
	8.237 (0.002)	4.091 (0.002)	3.995 (0.003)
cmnm^{5}U	7.615 (0.005)	3.398 (0.005)	3.139 (0.005)
	7.881 (0.010)	3.662 (0.010)	3.319 (0.009)
	8.143 (0.004)	4.022 (0.004)	3.607 (0.005)
	8.190 (0.007)	4.092 (0.007)	3.987 (0.011)

[a] Standard deviations are indicated in parentheses.

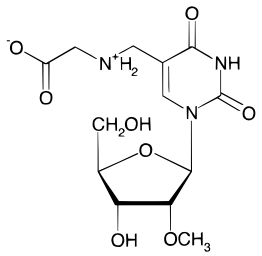

FIGURE 10: Structure of nucleoside N* from *E. coli* tRNA$_4^{Leu}$.

alkaline pH) (*38*). These data indicate the presence of $-NH_2^+CH_2(2)COO^-$ (at neutral pH) in the 5-substituent. Thus, the pK_a of 8.28 may be assigned to the deprotonation of the uracil moiety, which is lower than that of 5-methyluridine (pK_a = 9.7) (*35*) probably because of the presence of the positively charged amino group in the 5-substituent as discussed above. On the other hand, the pK_a of 10.02 may be assigned to the deprotonation of this amino group in the 5-substituent. Accordingly, the 5-substituent of the uracil moiety of nucleoside N* is likely to be $-CH_2(1)NH_2^+CH_2(2)COO^-$.

Comparison of Proton NMR Spectra between Nucleoside N from E. coli tRNA$_4^{Leu}$ and 5-Carboxymethylaminomethyluridine.* Thus, by ^{1}H NMR spectroscopy, it is suggested that nucleoside N* from *E. coli* tRNA$_4^{Leu}$ is 5-carboxymethylaminomethyl-2′-*O*-methyluridine (designated as cmnm^{5}Um hereafter) (Figure 10). Therefore, the pH dependence of proton resonances [H6, CH_2(1), and CH_2(2)] of nucleoside N* were compared with those of an authentic sample of 5-carboxymethylaminomethyluridine (cmnm^{5}U), which has the same base moiety as cmnm^{5}Um. As listed in Table 2, cmnm^{5}U has three titratable groups with pK_a values that are much the same as those of nucleoside N*. Between nucleoside N* and cmnm^{5}U, the intrinsic proton chemical shifts of all the four ionization states agree within 0.01 ppm for CH_2(1) and CH_2(2) methylene protons, and within 0.06 ppm for H6 proton. Consequently, the base moiety including the 5-substituent of nucleoside N* from *E. coli* tRNA$_4^{Leu}$ is identified to be 5-carboxymethylaminomethyluracil. As compared with these proton resonances of the substituted uracil moiety, the chemical shift differences between the two nucleosides are much more significant for ribose protons, because of the presence of the 2′-*O*-methyl group in nucleoside N*.

Mass Spectrometry of Nucleoside N.* Nucleoside N* was trimethylsilylated and analyzed by mass spectrometry. As shown in Figure 11A, a molecular ion with *m*/*z* 705 was observed. This is consistent with the structure of nucleoside cmnm^{5}Um with five TMS groups (Figure 11B). Ions *m*/*z* 588, 574, 488, 414, and 218 were assigned as shown in Figure 11B, while ions *m*/*z* 291/290, 217, 201, and 159 were found to be consistent with common ions of 2′-*O*-methylribonucleoside (*39*, *40*). Therefore, the chemical structure of nucleoside N* from *E. coli* tRNA$_4^{Leu}$ is now unambiguously identified as cmnm^{5}Um. Furthermore, it was confirmed that the present natural sample of nucleoside N* is identical to a chemically synthesized cmnm^{5}Um (*41*) by comparison of the 400 MHz ^{1}H NMR spectra.

Nucleotide Sequence and Modified Nucleosides of E. coli tRNA$_5^{Leu}$. The nucleotide sequence of tRNA$_5^{Leu}$ was also determined by the post-labeling method (*23*) as shown in Figure 3B. Thus, the present tRNA$_5^{Leu}$ is identified to be Su°6 tRNA^{Leu} corresponding to the Su$^+$6 amber suppressor tRNA^{Leu} although modified nucleosides of Su°6 tRNA^{Leu} had not yet been identified (*13*). All of the modified nucleosides of tRNA$_5^{Leu}$ were identified from the positions of spots in TLC analyses in the course of the post-labeling sequencing. In common with tRNA$_4^{Leu}$, identified bases were s^{4}U(8), D(16), Gm(18), D(20), Ψ(32), ms^{2}i^{6}A(37), Ψ(39), T(54), and Ψ(55). Cm was found in position 34 (the first position of the anticodon) of *E. coli* tRNA$_5^{Leu}$ (Figure 12); the spots of pCm and pCmpA were found in the autoradiogram of the thin-layer chromatogram corresponding to position 34 with the standard condition of nuclease P1 digestion (Figure 12C). Only the spot of pCm was found with the longer time of digestion (10 h) (data not shown). For confirmation, the nucleoside composition of this tRNA$_5^{Leu}$ was analyzed by ODS HPLC; Cm was identified from the retention time in the chromatogram (Figure 13) and the UV spectrum (not shown). Thus, Cm was determined to be about 1 mol/mol of tRNA.

Specificity of Codon Recognition by E. coli tRNA$_4^{Leu}$ and tRNA$_5^{Leu}$ in Protein *Synthesis in vitro Directed by MS2 Phage RNA.* The highly purified and characterized preparations of tRNA$_4^{Leu}$ and tRNA$_5^{Leu}$ were aminoacylated with ^{14}C-leucine and used in protein biosynthesis in vitro directed by bacteriophage MS2 RNA (*28*). The tRNA$_4^{Leu}$ and tRNA$_5^{Leu}$ purified nearly to homogeneity (*28*), and partially purified tRNA$_{UUR}^{Leu}$ (*12*) was also tested for comparison. Amounts of these tRNA species in the reaction mixture (0.2 mL) are listed in Table 3. Radioactive proteins synthesized in vitro were digested with trypsin and subjected to the first-dimension electrophoresis at pH 3.5 as shown in the left panel of Figure 14. Peptide bands were sewn onto a fresh sheet of paper and subjected to the second-dimension electrophoresis at pH 1.9 (*28*). The right panel of Figure 14 shows the analysis of band E containing the peptide fragment of the replicase ("replicase 5") (*28*). The region of MS2 RNA coding for this peptide has only one leucine codon, UUA,

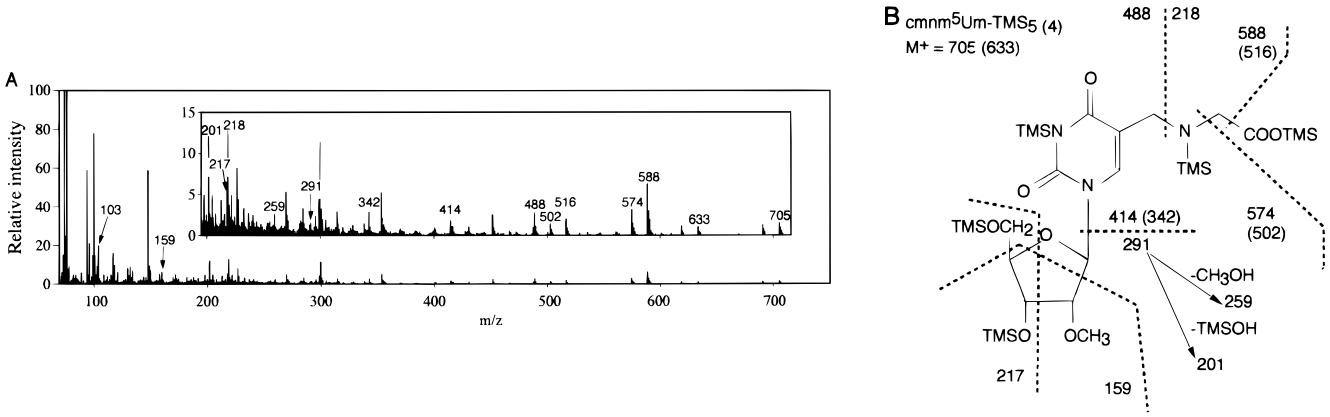

FIGURE 11: Mass spectrum of TMS derivative of nucleoside N* from *E. coli* $tRNA_4^{Leu}$.

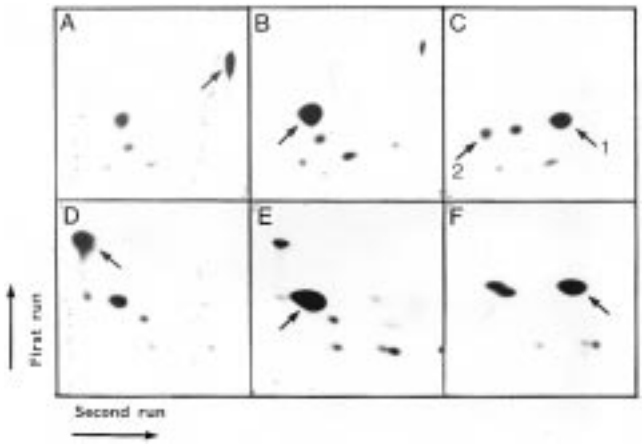

FIGURE 12: Autoradiography of two-dimensional thin-layer chromatograms of 5′-^{32}P-labeled nucleotides from *E. coli* $tRNA_5^{Leu}$. Nuclease P1 digests of the ^{32}P-labeled tRNA fragments terminating in positions 37 (A and D), 36 (B and E), and 34 (C and D) were analyzed in the course of nucleotide sequencing by two-dimensional TLC with solvent system I (A−C) and solvent system II (D−F). Arrows indicate the spots of pms^{2}i^{6}A (A and D), pA (B and E), and pCm (arrow 1) and pCmpA (arrow 2) (C and F).

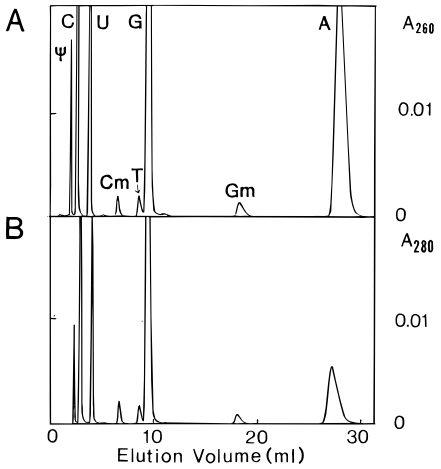

FIGURE 13: Reversed-phase HPLC of PDase/PMase digests of *E. coli* $tRNA_5^{Leu}$ monitored with A_{260} (A) and A_{280} (B).

corresponding to the leucine in position 86 of the replicase. Table 3 lists the radioactivities extracted from the bands in the right panel of Figure 14.

Both of the present preparations of $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ are now shown to recognize the leucine codon UUA; the efficiency of recognition of codon UUA by $tRNA_4^{Leu}$ is twice as high as that by $tRNA_5^{Leu}$(Table 3). This tendency agrees with that in the case of the previously prepared $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ (*28*) (Table 3). $tRNA_{UUR}^{Leu}$ (*12*) appears to have similar properties to those of $tRNA_4^{Leu}$, which is consistent with the above sequencing analysis. As for the recognition of other codons, all of the patterns of electrophoresis indicated that the specificities of the present $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ are much the same as those reported previously (*11*). Leucine codons UUA and UUG are recognized well by both $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$, although, among the four combinations, the recognition of codon UUA by $tRNA_5^{Leu}$ is about half as efficient as the other three cases. By contrast, codons UUU and UUC of phenylalanine are not at all recognized by $tRNA_4^{Leu}$ or $tRNA_5^{Leu}$.

Table 3: Quantitation of [^{14}C]Leucine Incorporation into Peptides Isolated in the Right Panel of Figure 14[a]

sample (source) ^{14}C-Leu-$tRNA^{Leu}$	amount added (μCi/0.2 mL)	initial incorporation (cpm × 10^{-3}/0.2 mL)	band E (cpm)
1 (H)	0.075	13.9	75
1 (H) (duplicate)	0.075	13.5	51
4 (H)	0.075	16.6	230
5 (H)	0.075	11.3	105
5 (N-1)	0.075	18.3	264
4 (N-2)	0.116	27.8	409
5 (N-2)	0.116	22.9	206
bulk (S. M.)	0.164	39.7	218
blank-bulk (S. M.) (no added MS2 RNA)	0.164	9.9	45

[a] Sources: H = G. W. Hatfield. These were tested for coding by Goldman et al. (1979) *J. Mol. Biol. 129*, 567−585. N-1 = S. Nishimura, first sample (1983). N-2 = S. Nishimura, second samples (1985). S. M. = Schwarz-Mann *E. coli* B tRNA.

DISCUSSION

The nucleotide sequence of $tRNA_4^{Leu}$ from *E. coli* A19 was found to be identical to that of the species previously named $tRNA_{UUR}^{Leu}$ (and also tentatively called $tRNA_5^{Leu}$), except for the first position of the anticodon (*12*). The nucleotide sequence of $tRNA_5^{Leu}$ indicated that this species is the product of the su°6 (*supP*, *leuX*) gene (*13*, *14*). The codon-reading properties of each tRNA were similar to those of the corresponding species from *E. coli* B. In this study, we unambiguously identified the modified nucleosides in position 34 of these tRNA species as cmnm^{5}Um and Cm, respectively. The modified nucleoside N in position 34 of $tRNA_{UUR}^{Leu}$ had incorrectly been concluded to be a modified

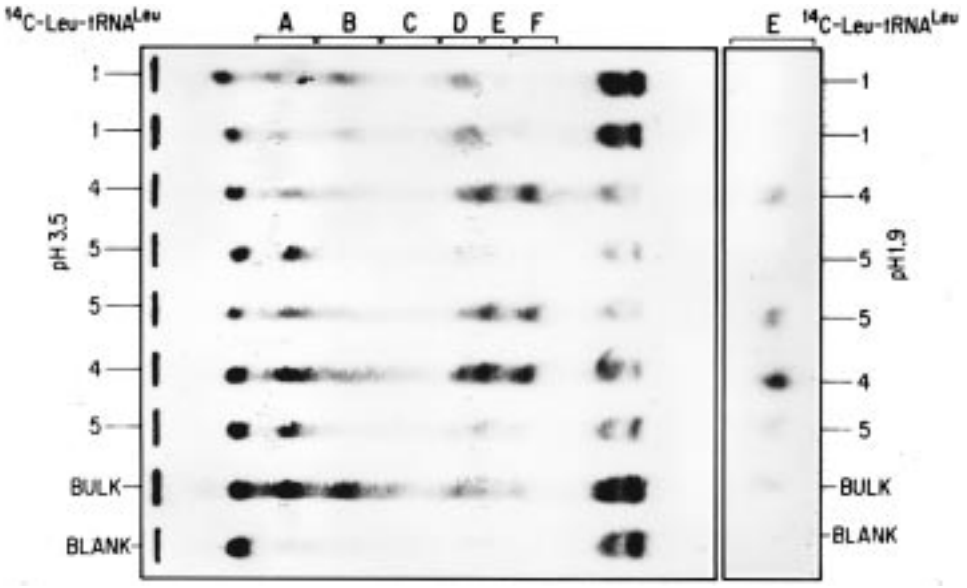

FIGURE 14: Autoradiogram of the tryptic digest of MS2 RNA-directed proteins synthesized in vitro and labeled with various ^{14}C-Leu-tRNA^{Leu} species. See Table 3 for identification of sources of each tRNA^{Leu} isoacceptor used: left panel, the first-dimension electrophoresis at pH 3.5; right panel, the second-dimension electrophoresis at pH 1.9 of band E obtained by the first-dimension electrophoresis.

adenosine; the dinucleotide, pNpA (actually pcmnm^{5}UmpA), was confused with pN because of the resistance to nuclease P_1 digestion, and therefore was regarded as a modified adenosine on the basis of the UV absorption spectra with a large contribution from the adenosine residue of the dinucleotide. Moreover, the modified nucleoside of tRNA$^{Leu}_{UUR}$ had also been suggested to be Cm (*16*); the tRNA sample used in the mass spectrometry analysis was fractionated by BD-cellulose column chromatography, but not by Sepharose 4B column chromatography, and therefore was a mixture of tRNA$^{Leu}_4$ and tRNA$^{Leu}_5$. The present work has clarified the identities of both tRNA$^{Leu}_4$ and tRNA$^{Leu}_5$ and their modified nucleosides in position 34.

The modification of U to cmnm^{5}Um in position 34 of tRNA$^{Leu}_4$ seems to affect the conformational property of this nucleotide residue. Through NMR analyses, we have already shown that two types of uridine modifications in position 34 regulate the conformational rigidity/flexibility of the anticodon, thus facilitating accurate and efficient codon recognition (*1, 42*). The modification from U to 5-methyl-2-thiouridine derivatives (xm^{5}s^{2}U) makes the ribose ring preferentially adopt the C3′-endo conformation. It seems that, in this case, the thiolation at position 2 of the uracil ring has a larger contribution to the conformational rigidity than the addition of the long side chain at position 5 (*42*). However, the long side chain can also substantially contribute to the conformational preference (*1*). In fact, minor arginine tRNA reading codons AGA and AGG have 5-methylaminomethyluridine (mnm^{5}U) in position 34, which is modified only at position 5 of the uracil ring, and this modification causes significant conformational rigidity of the anticodon loop (*43, 44*). In the case of tRNA$^{Leu}_4$, the uridine in position 34 is modified to a 5-methyluridine derivative, cmnm^{5}Um. 5-Carboxymethylaminomethyl moiety, while it has an extra carboxyl group as compared to 5-methylaminomethyl moiety in tRNA$^{Arg}_4$, is expected to have a significant contribution to the conformational rigidity of the anticodon.

In addition, the 2′-hydroxyl group of the U(34) is substituted by the methoxy group in tRNA$^{Leu}_4$. In the xm^{5}s^{2}U-type modified uridines, thiocarbonyl moiety forces the ribose conformation to the C3′-endo form through steric interaction with the 2′-hydroxyl group in the C2′-endo form (*1, 42*). 2′-*O*-Methylation enforces the same type of interaction through the introduction of a bulky group on the part of the ribose ring (*45*). The 2′-*O*-methyl group is forced to orient to the 2-carbonyl moiety in the C2′-endo form due to steric repulsion with 3′-phosphate (*1, 45*). The fact that tRNA$^{Leu}_4$ has a 2′-*O*-methylated uridine derivative supports the theory that the interaction between position 2 of the pyrimidine ring and position 2′ in the ribose ring (*42, 45*) controls codon recognition through regulation of the ribose conformation. Both the substitution at position 5 of the uracil ring and 2′-*O*-methylation regulate codon recognition by controlling conformational rigidity of the anticodon.

The nucleotide sequences of UUR-reading leucine tRNAs from many organisms have been analyzed, but chemical structures of the nucleosides in the first position of the anticodon have been determined for only a small number of tRNA species. The nucleoside at position 34 of leucine tRNA species from *Mycoplasma caplicolum* (*46*) and *Bacillus subtilis* (Murao, K., personal communication) has been determined as cmnm^{5}Um through comparison with our sample. Bean chloroplast tRNA$^{Leu}_1$ (*9*) and potato mitochondria tRNA^{Leu}(NAA) (*10*) have a modified nucleotide with TLC profiles similar to those of pcmnm^{5}Um. A cytoplasmic leucine tRNA species from *Saccaromyces cerevisiae* has 5-carbamoylmethyl-2′-*O*-methyluridine (ncm^5-Um) (*4*). *S. cerevisiae* mitochondria have a tRNA species which has cmnm^{5}U at position 34 (*3*), while one of the soybean chloroplast tRNA^{Leu} species has Um (*2*). Accordingly, all the modified uridines previously identified in the first position of the anticodon of UUA/G-reading leucine tRNAs are 5-methyl- or 2′-*O*-methyluridine derivatives. Only one exception, halobacterium tRNA^{Leu}, has unmodified uridine in the wobble position. For most organisms one or

both of the modifications in position 5 of the uracil ring and position 2′ of the ribose ring are needed in tRNA species with the UAA anticodon in order to minimize the level of misreading of the phenylalanine codons.

The modification of C(34) in $tRNA_5^{Leu}$ also has some contribution to conformational rigidity of the anticodon, as the interaction of the 2′-methoxy group with the keto group of the base ring and 3′-phosphate also occurs in the case of cytidine derivatives (*1*, *45*). In many cases where a conformationally rigid modified cytidine is present in the first position of the anticodon, the tRNA is not allowed to recognize adenosine as the third position of the codon, as in the case of $tRNA^{Met}$ and $tRNA^{Trp}$ (*47*). Many studies have presented the potentiality of cytidine to pair with adenosine (*48*, *49*). Thus, it was natural to assume that the Cm modification prevents unorthodox pairing with A in the third position of the codon. Therefore, it was puzzling that $tRNA_5^{Leu}$ recognizes codon UUA while it has a conformationally rigid modified nucleoside Cm at position 34. Recently, it was found in our laboratory that $tRNA_5^{Leu}$ reads codon UUA when a relatively small amount of Leu-$tRNA_4^{Leu}$ is available but that it never recognizes the UUU/C phenylalanine codons even when Phe-tRNA is scarcely available (*50*). Thus, the primary role of the Cm modification may be prevention of misrecognition of pyrimidines as the third letter of the codons. Further analysis is needed to clarify whether the Cm modification (and other types of cytidine modifications) has significant effects on the recognition of codons terminating in A.

Cytidine derivatives previously identified in position 34 of some UUR-reading leucine tRNA species are unmodified C (*47*), Cm (*2*, *5*, *6*), m^5C (*7*), and f^5Cm (*8*). The 5-formylation of C causes a drastic conformational rigidity (*1*, *45*, *51*), while how the methylation at position 5 of the cytosine ring affects the ribose puckering is not yet clear.

In summary, the first positions of the anticodon of both *E. coli* $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ are occupied by conformationally rigid modified pyrimidines, and, by virtue of the modifications, these leucine tRNAs do not recognize noncognate phenylalanine codons.

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