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Unusual anticodon loop structure found in *E.coli* lysine tRNA

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ABSTRACT

Although both $tRNA^{Lys}$ and $tRNA^{Glu}$ of *E.coli* possess similar anticodon loop sequences, with the same hypermodified nucleoside 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) at the first position of their anticodons, the anticodon loop structures of these two tRNAs containing the modified nucleoside appear to be quite different as judged from the following observations. (1) The CD band derived from the mnm^5s^2U residue is negative for $tRNA^{Glu}$, but positive for $tRNA^{Lys}$. (2) The mnm^5s^2U monomer itself and the mnm^5s^2U-containing anticodon loop fragment of $tRNA^{Lys}$ show the same negative CD bands as that of $tRNA^{Glu}$. (3) The positive CD band of $tRNA^{Lys}$ changes to negative when the temperature is raised. (4) The reactivity of the mnm^5s^2U residue toward H_2O_2 is much lower for $tRNA^{Lys}$ than for $tRNA^{Glu}$. These features suggest that $tRNA^{Lys}$ has an unusual anticodon loop structure, in which the mnm^5s^2U residue takes a different conformation from that of $tRNA^{Glu}$; whereas the mnm^5s^2U base of $tRNA^{Glu}$ has no direct bonding with other bases and is accessible to a solvent, that of $tRNA^{Lys}$ exists as if in some way buried in its anticodon loop. The limited hydrolysis of both tRNAs by various RNases suggests that some differences exist in the higher order structures of $tRNA^{Lys}$ and $tRNA^{Glu}$. The influence of the unusual anticodon loop structure observed for $tRNA^{Lys}$ on its function in the translational process is also discussed.

INTRODUCTION

It is now established that almost all tRNAs have similar cloverleaf secondary structures [1, 2], which are folded into the common L-shaped tertiary structure [3–7]. It has been elucidated in crystal [3–7] as well as in solution [8–12] that the anticodon loop region of tRNA is generally single-stranded, and that the anticodon base triplet and the two 3′-adjacent bases at positions 37 and 38 form a helical stack in continuity with the anticodon stem on the 3′-end, which enables the anticodon to base-pair with its cognate codon in mRNA on the ribosomes during the translation process [13–15].

In a comparative study of the tertiary structures of glutamic acid tRNA ($tRNA^{Glu}$) and lysine tRNA ($tRNA^{Lys}$) isolated from *E.coli*, both of which have very similar anticodon loop sequences, with the same hypermodified nucleoside 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) at position 34 [2], we found that the anticodon structure of $tRNA^{Lys}$ is actually different from that of $tRNA^{Glu}$ [16] in terms of circular dichroism (CD), which is known to be a very sensitive method for detecting the conformation of sulfur-containing nucleosides (or nucleotides) such as 4-thiouridine [17], 5-methyl-2-thiouridine [18, 19] and mnm^5s^2U [19, 20]. Since $tRNA^{Glu}$ is known to have a normal anticodon loop structure with respect to CD [19–22], as well as sensitivity towards nucleases [23,24], the anticodon of $tRNA^{Lys}$ appears to be different in its tertiary structure from those of most tRNAs so far studied [3–12].

Here we present several pieces of experimental evidence to confirm the distinctive conformation of the $tRNA^{Lys}$ anticodon, and discuss the possibility that this uncommon feature is related to some unusual phenomena, especially the 'frameshifts' observed in *E.coli* [25–28], in which $tRNA^{Lys}$ is likely to be directly involved.

MATERIALS AND METHODS

Materials

Ribonucleases T_1, T_2 and nuclease S_1 were purchased from Sankyo, Japan. RNase V_1 was from Pharmacia. Polynucleotide kinase derived from T_4-infected *E.coli*, T_4 RNA ligase and bacterial alkaline phosphatase were obtained from Takara, Japan. Hexokinase was from Sigma. Cellulose thin layer plate (Avicel SF cellulose) and X-ray film (AIF new RX-50) were purchased from Funakoshi Pharmaceuticals and Fiji Film, respectively. All

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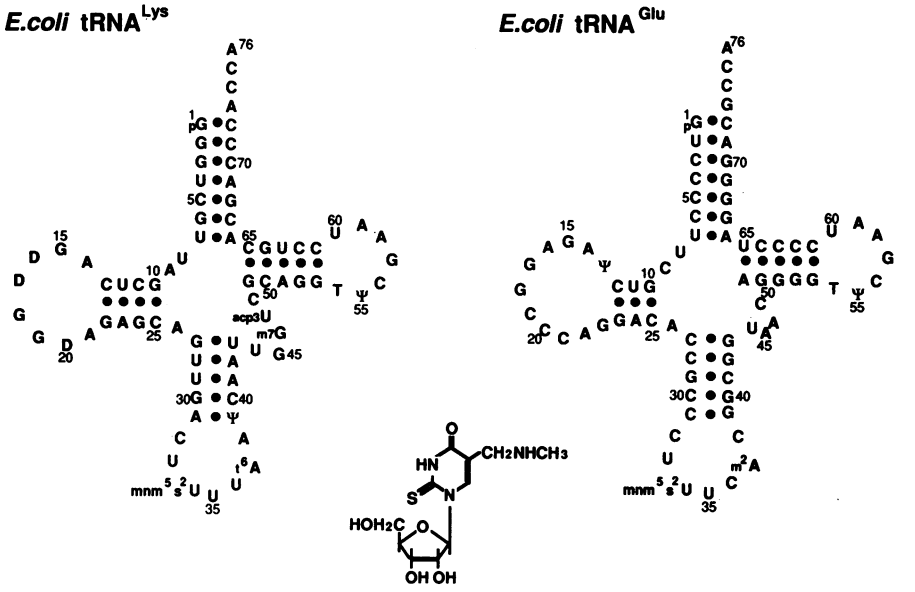

Figure 1. Cloverleaf structures of *E.coli* $tRNA^{Lys}$ and $tRNA^{Glu}$ containing the mnm^5s^2U residue at the first position of the anticodon, and the chemical structure of mnm^5s^2U. The numbering of each residue conforms to the proposal of Sprinzl *et al.* [2].

chemicals (analytical grade) were obtained from Wako Pure Chemicals. Culture media for *E.coli* were obtained from Difco Laboratories and Kyokuto Pharmaceutical Industries. [U-^{14}C]-amino acids were purchased from Amersham, Japan.

Methods

Purification of tRNAs. Crude *E.coli* tRNA mixture (100,000 A_{260} units ~5 g) was prepared from *E.coli* A19 cells (2kg) harvested at the late log phase, according to Zubay's method [29].

$tRNA^{Lys}$ was purified from the tRNA mixture by successive column chromatographies of DEAE-Sephadex A50 (pH 7.5), Sepharose 4B (pH 4.5), BD-cellulose (pH 6.0) and hydroxyapatite (pH 6.8) as described in the literature [30, 31]. Finally, 200 A_{260} units of *E.coli* $tRNA^{Lys}$ was purified. The tRNA isolated in this way accepted 1.5 nmoles of lysine per 1 A_{260} unit of tRNA, so that is was estimated to be more than 90% pure, since 1 A_{260} unit of fully purified tRNA is thought to accept 1.67 nmoles of the cognate amino acid [32]. The $tRNA^{Lys}$ gave a single band on a polyacrylamide gel electrophoretogram and the same nucleotide sequence as has already been reported [33].

$tRNA^{Glu}$ was purified to a single species (1,500 A_{260} units) from the crude *E.coli* tRNA mixture by a combination of DEAE-Sephadex A 50 column chromatographies at different pHs, 7.5 and 4.0, according to the literature [34]. It was actually $tRNA_2^{Glu}$ by sequencing, and it is hereafter called simply $tRNA^{Glu}$.

The molecular extinction coefficients $[\epsilon]_{260}$ of these tRNAs were determined to be 6,800 for $tRNA^{Lys}$ and 6,500 for $tRNA^{Glu}$ in TNM buffer consisting of 0.01 M Tris−HCl, pH 7.5, 0.2 M NaCl and 0.01 M $MgCl_2$, by the reported method using alkaline degradiation [35].

The aminoacylation assay of tRNAs was carried out at 37°C in a reaction mixture (50μl) containing 100 mM Tris−HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 10 mM KCl, 6.5 μM uniformly labeled amino acids (11.5 GBq/mmol of [^{14}C]-Lys or 9.25 GBq/mmol of [^{14}C]-Glu), and an appropriate amount of S100 fraction (40 μg/ml) obtained from *E.coli* A19 cells harvested at the early log phase.

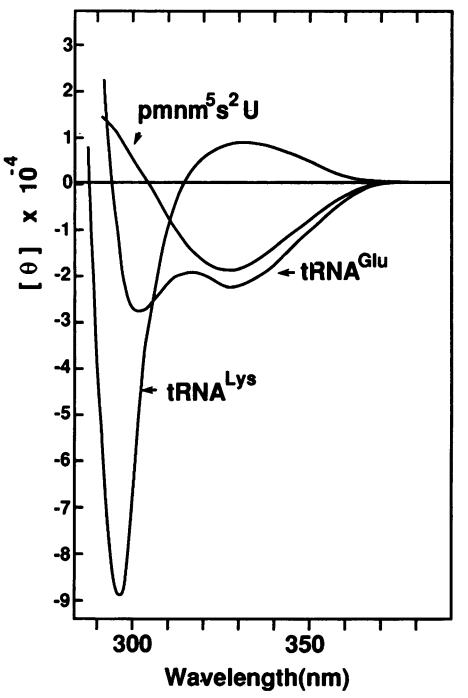

Figure 2. CD spectra of mnm^5s^2U-5′-phosphate ($pmnm^5s^2U$), and $tRNA^{Lys}$ and $tRNA^{Glu}$ in the near UV region (290−390 nm).

CD and UV measurements. CD spectra were obtained with a JASCO (Japan Spectroscopic Co. Ltd) J-500A spectropolarimeter as already reported [19, 20]. The temperature was set at 20°C

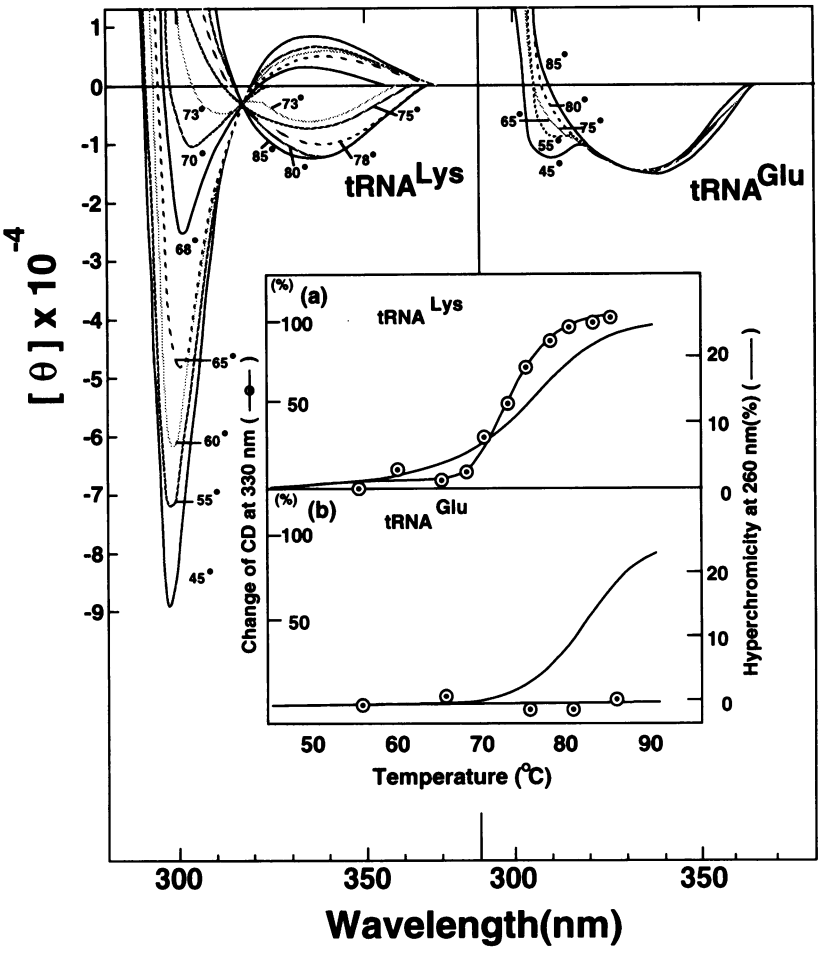

Figure 3. CD spectra of $tRNA^{Lys}$ and $tRNA^{Glu}$ in the near UV region at various temperatures. The temperature was raised at 1°C/min and CD spectra were measured at the defined fixed temperatures. Insert: Melting profiles of $tRNA^{Lys}$ and $tRNA^{Glu}$ monitored by CD at 335 nm (-⊙-) or by UV at 260 nm (—).

unless otherwise indicated. Cells of 5 mm and 1 cm path lengths were used for measurement.

UV spectra were obtained with a Hitachi UV 228 double-beam spectrophotometer. Melting profiles were obtained with a Corning-Gilford Response II spectrophotometer.

All the measurements were made using the TNM buffer described above.

H_2O_2 treatment. H_2O_2 treatment was carried out essentially as reported previously [19]; 5 A_{260}/ml tRNAs (~9 μM equivalent to mnm^5s^2U monomer) were mixed with the TNM buffer in a volume of 1 ml, into which 1 μl of 35% H_2O_2 was added. The reaction was followed by CD spectra at 20°C. It is known that H_2O_2 attacks the sulfur atom of mnm^5s^2U, which is in a condition accessible to the solvent, resulting in an H_2O_2-altered nucleoside having no CD band above 300 nm [19, 36]. Thus, by measuring the time-dependent CD spectral change at the wavelength where the band strength shows the maximum value (330 nm for mnm^5s^2U), the reactivity of mnm^5s^2U towards H_2O_2 can be followed quantitatively [19, 22].

Preparation of the anticodon fragment containing mnm^5s^2U. 50 A_{260} units of $tRNA^{Lys}$ was digested with RNase T_1 (5 units/1 A_{260} unit of tRNA) in 50 mM Tris−HCl (pH 7.5) and 10 mM EDTA at 37°C for 24 h. The reaction was stopped by phenol extraction. The fragment containing mnm^5s^2U was purified by DEAE Sephadex A-25 column chromatography with a linear gradient of NaCl (0.14 M−1.5 M) in 20 mM Tris buffer (pH 7.5) (total 600 ml) containing 7 M urea [18, 37]. $5A_{260}$ units of the anticodon fragment containing mnm^5s^2U, A-C-U-mnm^5s^2U-U-U-t^6A-A-U-C-A-A-U-U-G, were obtained, and its sequence was confirmed by sequencing according to Donis-Keller's method [38], base analysis, and 5′-end analysis.

Time-dependent digestion of $tRNA^{Lys}$ with RNase T_1. To ascertain the CD spectral change of $tRNA^{Lys}$ due to RNase T_1 digestion, $tRNA^{Lys}$ (9.5 A_{260} units/ml) in 50 mM Tris−HCl (pH 7.5) and 5 mM $MgCl_2$ was digested with RNase T_1 (17 Units per 1 A_{260} unit of tRNA) at 30°C. Under these conditions, tRNA can maintain its native higher-order structure, and the rate of digestion is slow enough to be followed by CD spectral change. The CD spectra were measured at various times, and simultaneously 5 μl of the sample solution was withdrawn and applied onto 20% polyacrylamide gel containing 7 M urea to estimate the extent of digestion. The bands were visualized by staining the gel with toluidine blue.

Limited RNase digestion. $tRNA^{Lys}$ and $tRNA^{Glu}$ were labelled with ^{32}P at their 5′- or 3′-end and purified by electrophoresis

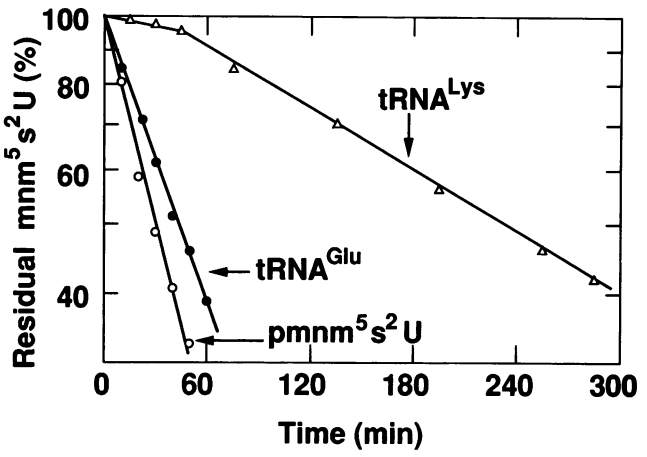

Figure 4. Time courses of the reactions with H_2O_2 of pmnm^{5}s^{2}U (o), tRNA^{Lys} (Δ) and tRNA$^{Glu}_2$ (●). The reactions were followed by CD spectra at 330 nm.

on a 20% polyacrylamide containing 7 M urea, as already reported [39, 40]. The [^{32}P]labelled tRNA (10^6 cpm) was mixed with 1 A_{260} unit of cold tRNA^{Met}, and an aliquot (usually 3×10^5 cpm/0.3 A_{260} unit tRNA in 30 μl) was digested at 37°C for an appropriate number of minutes with RNase T_2 (0.05 units/1 A_{260} unit tRNA), nuclease S_1 (7 units/1 A_{260} unit tRNA) and RNase V_1 (1 unit/1 A_{260} unit tRNA), separately, in the same buffer conditions of 50 mM Tris−HCl (pH 7.5) and 5 mM $MgCl_2$.

Limited digestion with RNase T_1 was carried out basically according to the literature [38]. The above-mentioned labeled tRNA (30 μl) was reacted with RNase T_1 (2 units/1 A_{260} unit tRNA) at 55°C for 20 min in a buffer consisting of 30 mM Na-citrate (pH 4.5), 1 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol and 9 M urea.

Limited alkaline hydrolysis was performed at 90°C for 5 min in the reaction mixture (10 μl) of [^{32}P] labeled tRNA with the carrier tRNA mentioned above, 50 mM Na_2CO_3 (pH 9.0) and 1 mM EDTA.

The strength of the sensitivity of each residue in the tRNA towards nucleases was estimated from the band strengths on the autoradiograms, which were quantified with a Corning-Gilford Response II spectrophotometer equipped with a densitometer.

RESULTS

Comparison of CD spectra between tRNA^{Lys} and tRNA^{Glu}: Figure 1 shows the cloverleaf structures of tRNA^{Lys} and tRNA^{Glu} of *E.coli*. The two tRNAs are similar with respect to the presence of a hypermodified nucleoside, 5-methylamino-methyl-2-thiouridine (mnm^{5}s^{2}U), (the chemical structure is also shown in Figure 1) at the first position of their anticodons. However, their CD spectra in the near UV region (290−340 nm) are quite different from each other, as shown in Figure 2. Whereas both tRNA^{Glu} and the 5′-phosphate of mnm^{5}s^{2}U (pnmn^{5}s^{2}U) show negative peaks centered at 330 nm, tRNA^{Lys} shows a positive peak at the same wavelength. It is well known that the trough at 330 nm is derived from the thiocarbonyl group attached at position 2 of the uridine ring of the mnm^{5}s^{2}U residue, in so far as mnm^{5}s^{2}U exists either as a monomer or as a part of the single-stranded RNA [18, 19, 41].

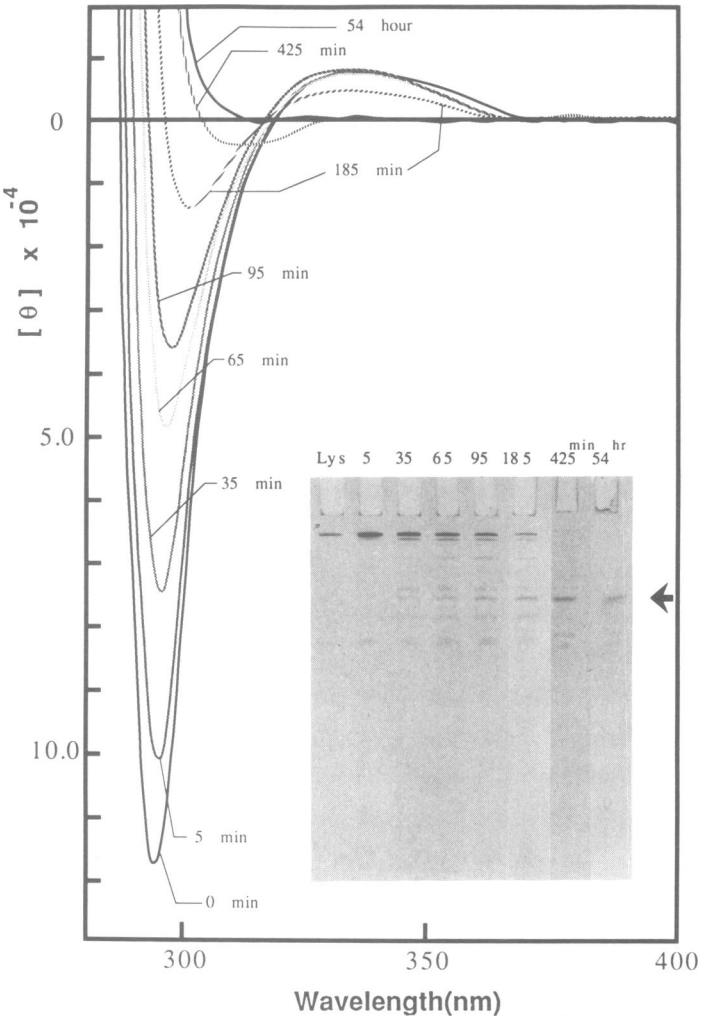

Figure 5. Time courses of the reaction of tRNA^{Lys} with RNase T_1, as followed by CD spectra. Insert: Polyacrylamide gel electrophoresis of aliquots of the RNase-treated tRNA solution after various reaction periods. The arrow shows the band corresponding to the 15 mer containing the anticodon.

The positive peak at 330 nm observed for tRNA^{Lys} is very similar to that at 310 nm shown by *Thermus thermophilus* tRNAs having a 5-methyl-2-thiouridine (m^{5}s^{2}U) residue at position 54 in the T loop [18]. The positive band is known to be derived from this m^{5}s^{2}U residue, which is base-paired with a 1-methyladenosine (m^{1}A) residue at position 58 in the tertiary structure of tRNAs [19]. In these thermophile tRNAs, the positive band at 310 nm changes to negative when the temperature is raised, the midpoint of the CD spectral change being the same as the melting temperature (Tm) of the tRNA measured by UV light at 260 nm [18, 19].

If the mnm^{5}s^{2}U residue of tRNA^{Lys} were also involved in some bonding(s) with other residues in the tertiary structure of the tRNA, the positive CD band at 330 nm should be changed to negative by raising the temperature, and this did, in fact, prove to be the case, as shown in Figure 3. Whereas the negative CD band at 330 nm observed for tRNA^{Glu} did not change at all, even when the temperature was elevated to 85°C, the positive CD peak at 330 nm observed for tRNA^{Lys} gradually decreased as the temperature increased, and eventually changed to negative above 70°C. The midpoint of the CD spectral change is 72.5°C,

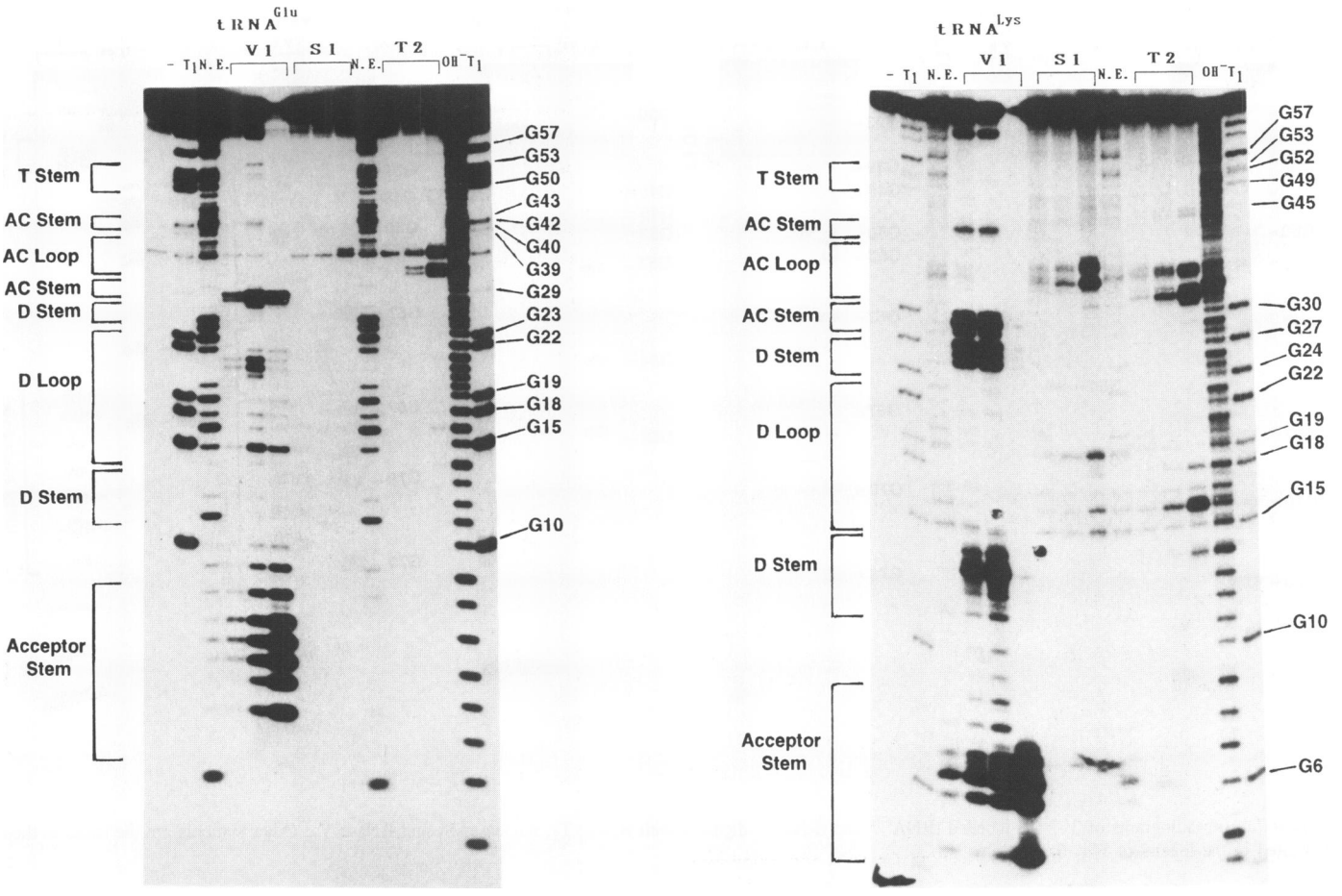

Figure 6. Autoradiograms of 5′-[^{32}P]-labelled tRNA^{Glu} (left) and tRNA^{Lys} (right) digested with RNase V_1, nuclease S_1 (both producing nucleotides with 5′-phosphate) and RNase T2 (producing nucleotides with 3′-phosphate). −, T_1, N.E. and OH^-, respectively mean minus enzyme, RNase T_1, *Neurospora crassa* endonuclease (producing nucleotides with 5′-phosphate) and alkaline treatment (producing nucleotides with 3′-phosphate). The three lanes of V_1, S_1 and T_2 are for incubation periods of (from left to right) 0.5 min, 2 min and 10 min.

which is 2.5°C lower than the Tm of the tRNA monitored by UV light at 260 nm (see Figure 3 insert). These results demonstrate that the mnm^{5}s^{2}U residue of tRNA^{Glu} exists originally in the single-stranded region in the tRNA [23, 24], and thus no conformational change occurs even at high temperature. On the other hand, the mnm^{5}s^{2}U residue of tRNA^{Lys}, probably involved in bonding(s) in the tertiary structure of tRNA, starts to change its conformation a little earlier than the onset of the conformational change of the whole tRNA molecule due to temperature elevation, and becomes a part of the single-stranded region at high temperature.

Reactivity of mnm^{5}s^{2}U residue with H_2O_2. To confirm this possibility, the reactivity of the mnm^{5}s^{2}U residue towards hydrogen peroxide (H_2O_2) was examined. It is already known that a residue of any s^{2}U derivative in a tRNA is, like its monomer, reactive with H_2O_2 when included in the single-stranded region, whereas it is much less reactive when involved in bonding(s) in the tertiary structure of the tRNA [19].

The result is shown in Figure 4. The mnm^{5}s^{2}U residue in tRNA^{Glu} reacted with H_2O_2 rather rapidly, with a rate constant of 15.5×10^{-3} min^{-1}, which is about 2/3 of the rate constant for the pmnm^{5}s^{2}U monomer. On the other hand, the mnm^{5}s^{2}U residue in tRNA^{Lys} reacted quite slowly in a two-step manner. For the first 45 min the reaction proceeded linearly at a rate constant, $k_{initial}$, of 0.7×10^{-3} min^{-1}, after which the reaction rate increased about 7-fold to a $k_{final} = 4.8\times10^{-3}$ min^{-1}. Even this rate constant is about 1/3, and the $k_{initial}$ is only 1/22, of that for tRNA^{Glu}. This result provides good evidence that the mnm^{5}s^{2}U residue in tRNA^{Lys} is, like the m^{5}s^{2}U residue in *T.thermophilus* tRNAs, in some way involved in bonding(s) in its tertiary structure.

Other lines of experimental evidence suggesting the unusual conformation of mnm^{5}s^{2}U residue in tRNA^{Lys}. The CD band at 330 nm of tRNA^{Lys} is changeable with the concentration of Mg^{2+}. Mg^{2+}-free tRNA, which was obtained by extensive dialysis first against trans-1,2-diaminocyclohexane-tetraacetic acid (CDTA) and then against distilled water [42], showed a negative trough at 330 nm, like the mnm^{5}s^{2}U monomer and tRNA^{Glu}. The intensity of the negative band gradually decreased as the concentration of Mg^{2+} increased, and eventually a new positive band appeared at 330 nm. In this process, the Mg^{2+} concentration corresponding to the midpoint of the CD band change was 0.5 mM (data not shown).

The positive CD band of tRNA^{Lys} is also changeable by RNase T_1 treatment. As shown in Figure 5, the positive CD band gradually decreased when tRNA^{Lys} was treated with

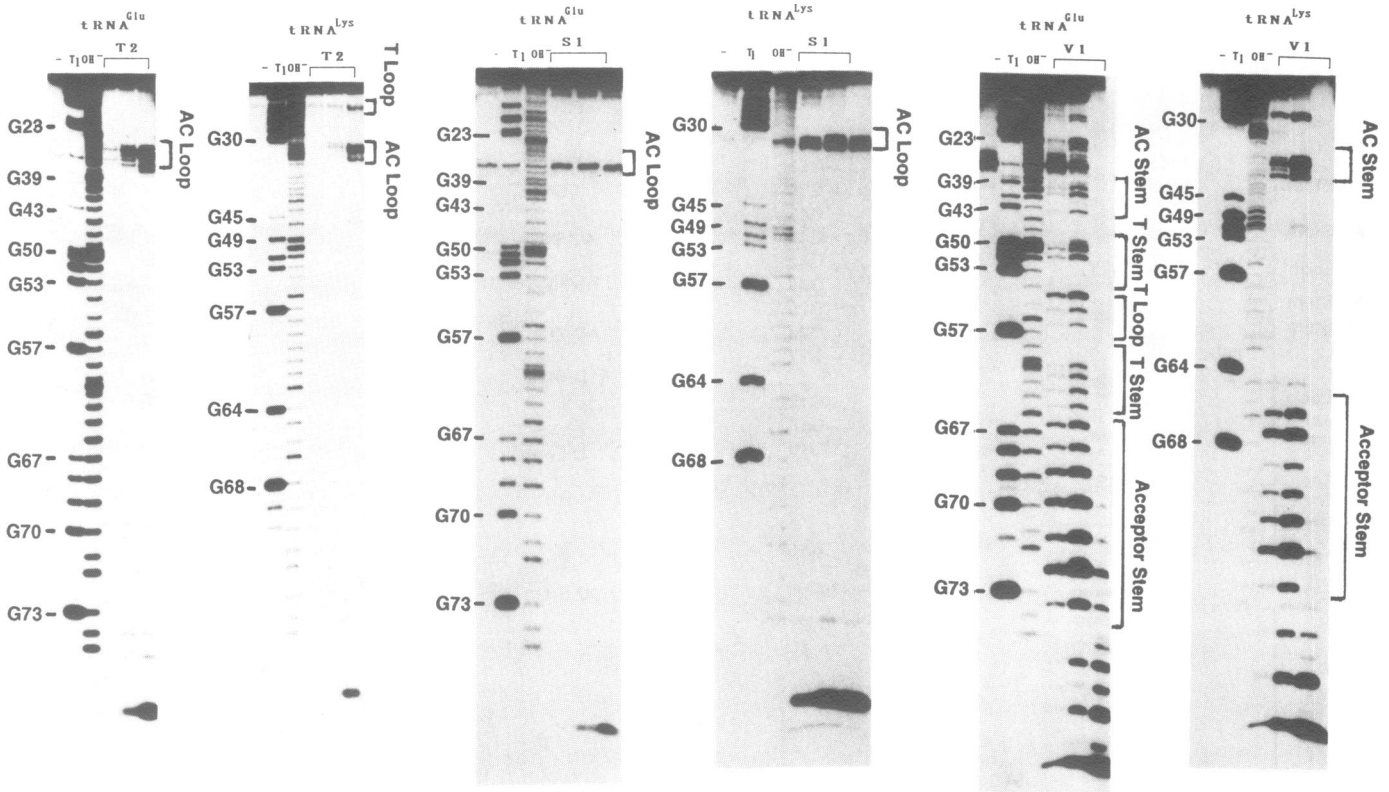

Figure 7. Autoradiograms of 3′-[^{32}P]-labelled tRNA^{Glu} and tRNA^{Lys} digested with RNase T_2, nucleases S_1 and RNase V_1. Other symbols are the same as those indicated in the legend of Fig. 6.

RNase T_1 in the presence of Mg^{2+}. After several hours the band disappeared and it seemed that digestion has proceeded completely judging from the band patterns on the electrophoretogram, shown in the insert of Figure 5. Although no negative band at 330 nm was observed in this case, the anticodon loop fragment including the mnm^{5}s^{2}U residue (15 mer, 5′-ACUmnm^{5}s^{2}UUUt^{6}AACAAUUG-3′) isolated from the complete RNase T_1 digests of tRNA^{Lys} showed a negative band at 330 nm (data not shown).

All these results clearly demonstrate that the native conformation of tRNA^{Lys} is required for a positive CD band at 330 nm.

Conformational difference between tRNA^{Lys} and tRNA^{Glu}. To obtain more detailed evidence for the conformational difference between tRNA^{Lys} and tRNA^{Glu}, attempts were made to examine the sensitivities of these tRNAs against single- and double-strand-specific nucleases. To make the reaction conditions the same, the reaction was carried out at 37°C in the buffer consisting of 50 mM Tris−HCl (pH 7.5) and 5 mM $MgCl_2$, where tRNA is considered to take the native conformation. In all these experiments, it can be said that only the primary cut was detected in the 5′- or 3′-labeled tRNAs, because the strength of any strong band did not decrease as the incubation periods were prolonged as long as 10 min (Figures 6 and 7).

RNase T_2 is an enzyme which has no base-specificity in RNA and preferentially attacks the single-stranded regions of tRNA. When tRNA^{Glu} was treated with T_2, the anticodon was the most sensitive toward the enzyme in the order of U35 > mnm^{5}s^{2}U34 = C32 = U33 = C36. The cleavage site U35 means that the cleavage occured at the 3′ side of U35. The CCA end, as well as A16 in the D loop and A46 in the variable loop, was sensitive toward T_2, and shown in Figures 6 and 7. For tRNA^{Lys}, C32 was the most reactive with RNase T_2 in the order of C32 > U33 = U35 > U36 = A31, but interestingly, mnm^{5}s^{2}U34 was not reactive. The CCA end-sequence and U7 and C48 were also sensitive. It is surprising that some residues in the D loop, such as G15, D16, D17 and G18, were also reactive with T_2 (Figures 6 and 7).

To clarify the conformational difference between tRNA^{Glu} and tRNA^{Lys}, single-strand-specific S_1 nuclease and double strand-specific RNase V_1 were then used. When tRNA^{Glu} was treated with S_1, the sensitivity of the anticodon loop was restricted to three residues in the order of U35 > C36 > m^{2}A37, as shown in Figures 6 and 7. The CCA end was also sensitive, but less reactive than U35 and C36. No other parts in the tRNA except A46 were sensitive towards S_1 in this condition. The results for tRNA^{Lys} seem quite different from those for tRNA^{Glu}, as shown in Figures 6 and 7. C32 was the most sensitive in the anticodon region, the order of sensitivity being C32 > U35 > U36 > t^{6}A37 > U33. It was striking that almost all residues of the D loop region (from A14 to D20) were sensitive to S_1, comparable to the CCA end.

All the stem regions of tRNA^{Glu} were more or less sensitive to the double-strand-specific nuclease V_1. In addition, U8, C9, A14, C20, C20A, A26, T54, ψ55 and C56 were also sensitive, so that these residues seem to be involved in tertiary interactions in tRNA^{Glu}, as observed for yeast tRNA^{Phe} [7]. tRNA^{Lys} showed

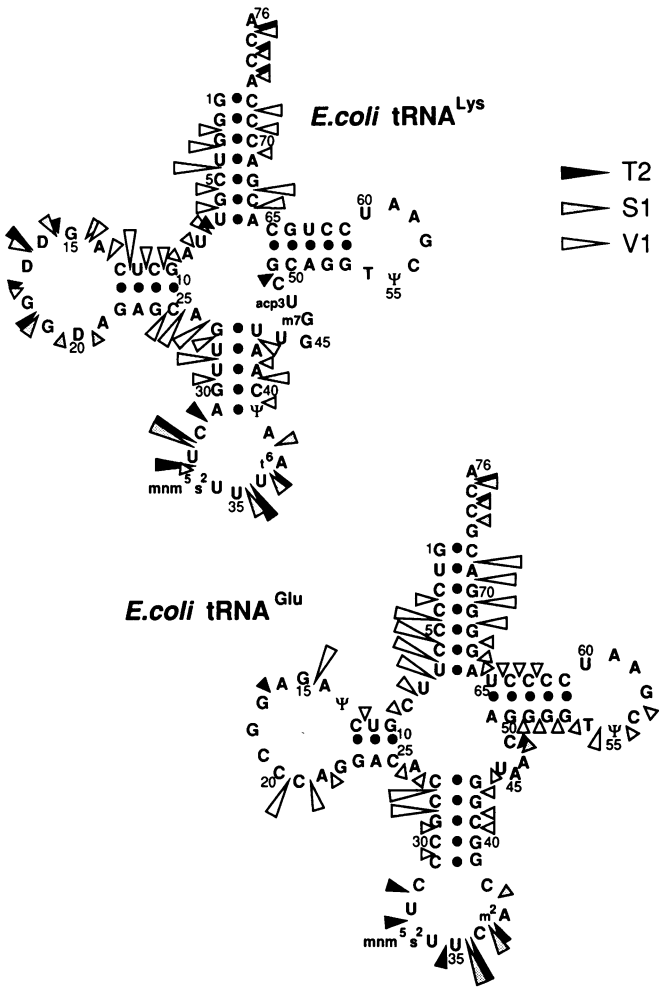

Figure 8. Cloverleaf structures of $tRNA^{Lys}$ and $tRNA^{Glu}$ in which the sites sensitive towards RNase T2 (black), nuclease S_1 (gray) and RNase V1 (white) are indicated by arrows. The length of the arrow indicates the strength of the sensitivity, which was estimated from the band strengths on the autoradiograms in Figs. 6 and 7; normalization was done in the anticodon regions.

a quite different pattern; although the anticodon, D and Acceptor stems were sensitive towards V_1 attack, the T stem was never reactive with V_1 and also the TψC sequence was insensitive towards V_1 in these conditions. A summary of the sensitivity towards various nucleases is shown in Figure 8.

These results suggest that the tertiary structure of $tRNA^{Lys}$ is in some way different from those of usual tRNAs, including $tRNA^{Glu}$ [24].

DISCUSSION

In this work, we found several lines of experimental evidence that demonstrate the unusual tertiary structure of *E.coli* $tRNA^{Lys}$, especially with respect to its anticodon loop. The CD spectra in the near UV region strongly suggest that the conformation of mnm^5s^2U of $tRNA^{Lys}$ is quite different from that of $tRNA^{Glu}$, although the anticodon loop sequences of the two tRNAs do not differ greatly — C-U-mnm^5s^2U-U-U-t^6A-A for $tRNA^{Lys}$ and C-U-mnm^5s^2U-U-C-m^2A-C for $tRNA^{Glu}$ (only the underlined parts are different; see Figure 1).

It is well known that the derivatives of s^2U preferentially take a rigid *C3'-endo-anti* conformation, even at the monomer level [36], and always give a negative CD band in the near UV region [41]. Therefore, it is almost impossible for the mnm^5s^2U34 residue to have a *syn* conformation in $tRNA^{Lys}$ by transconformation from *anti* to *syn* (if this were the case, it should give a negative CD band above 300 nm), and it is reasonable to infer that the mnm^5s^2U54 is involved in some bondings(s) with other base(s) as it maintains the *anti* conformation in $tRNA^{Lys}$, like the mnm^5s^2U54 in the *T.thermophilus* tRNAs, as described below.

The behavior of the CD band at 330 nm for $tRNA^{Lys}$ against temperature is quite similar to that at 310 nm of the tRNA of *T.thermophilus*, which we have reported previously [18, 19]. In that case, the positive CD band at 310 nm is due to a modified nucleoside m^5s^2U54 located in the T loop of *T.thermophilus* tRNA. In the native form of the tRNA, the m^5s^2U54 residue is buried inside the tertiary structure of the tRNA, being base-paired with m^1A58 and involved in the RNA A-type helix formed by the anticodon stem and T stem [7, 19].

If this also hold for $tRNA^{Lys}$, mnm^5s^2U should form a base pair with a certain base, the most likely candidate in this case being A38. Such a situation could be achieved, if a rearrangement of the anticodon loop could occur so as to mimic the T loop conformation, namely, mnm^5s^2U could move to the first position of the anticodon loop (position 32) and pair with A38 at the new position 36, which would require the rearrangement of the anticodon stem of $tRNA^{Lys}$ from the original five base-pairs of G27−U43, U28−A42, U29−A41, G30−C40 and A31−ψ39 to five new base-pairs — U29−G45, G30−U44, A31−U43, C32−A42 and U33−A41. However, judging from the digestion patterns of $tRNA^{Lys}$ with nucleases (Figs. 6−8) this possibility seems to be very unlikely, because A31, C32 and U33, which should be included in the new stem region, are sensitive to the single strand-specific RNase T2 and nuclease S_1, whereas A39 and C40, which should be included in the new loop region, are sensitive to the double strand-specific RNase V_1.

The RNase sensitivity experiments seems to be reasonable, because the results were fairly reproducible and the patterns for $tRNA^{Glu}$ digested with nuclease S_1 were not very different from those of earlier work done by other researchers, irrespective of the different conditions used (our digestion conditions were 0.14 units nuclease/μg tRNA in 50 mM Tris−HCl pH 7.5, 5 mM $MgCl_2$ at 37°C). Wurst *et al.* [43] reported that U33, mnm^5s^2U34, U35, C36 and C20 are the most reactive sites of $tRNA^{Glu}$ for the nuclease S_1 at pH 4.5 at 37°C, with 0.06 unit nuclease/μg tRNA. Wrede *et al.* [24] determined the reactive sites of $tRNA^{Glu}$ towards nuclease S_1 in the order of U35 > C36 > mnm^5s^2U34 > U33 > C20 > G73 = C74 = C75 at 58°C in the presence of 7 M urea at pH 5.0 with 0.1 unit enzyme/μg tRNA. They also reported similar sites (U35 > C36 > mnm^5s^2U34 > U33 > C20) under different conditions (5 mM $MgCl_2$ at pH 4.5 at 37°C).

One of the main differences between our findings and those of Wurst *et al.* [43] and Wrede *et al.* [23, 24] lies in the fact that U33, mnm^5s^2U34 and C20 were not reactive in our results but were reactive in both of theirs, while the opposite was true for m^2A and A46. However, the finding that both the D loop (except for C20) and T loop were insensitive towards nuclease S_1 is identical in all the cases. These differences may be attributable to the conditions (especially the pH and the presence or absence of urea) used for the nuclease digestion.

If these observations indicate that the nuclease digestion experiments in this work have been properly carried out, it is clear that the sites sensitive towards nuclease S_1, RNases T_2 and V_1 differ considerably between $tRNA^{Glu}$ and $tRNA^{Lys}$. The main differences are seen in the D loop and at the mnm^5s^2U residue in the anticodon loop. Whereas the D loop of $tRNA^{Lys}$ is very sensitive toward single-strand-specific nucleases, that of $tRNA^{Glu}$ is insensitive toward such nucleases but rather sensitive towards double-strand-specific RNase V_1, This suggests that the tertiary interaction between the D loop and the T loop is normal in $tRNA^{Glu}$, but abnormal or very weak in $tRNA^{Lys}$. As for the anticodon loop, the sensitivity of its residues as a whole towards single strand-specific nucleases is very similar in $tRNA^{Lys}$ and $tRNA^{Glu}$, whereas the sensitivity of the mnm^5s^2u residue is opposite; sensitive in $tRNA^{Glu}$, but not in $tRNA^{Lys}$. This feature is consistent with the CD spectra observations, as well as the reactivity of the mnm^5s^2U residue towards H_2O_2. One possible explanation may be that although the anticodon loop of $tRNA^{Lys}$ itself is not rearranged, as discussed above, the mnm^5s^2U is base-paired with another base, the most likely candidate being t^6A, because it exists in $tRNA^{Lys}$, but not in $tRNA^{Glu}$ and it possesses a long side-chain including imino groups probably available for hydrogen-bonding with the sulfur atom of the mnm^5s^2U base [19]. It is also reasonable that the mnm^5s^2U residue, even if it is base-paired, changes its conformation prior to the conformational change of the whole tRNA as a result of temperature elevation (Fig. 3, insert), since it is known that heat-induced conformational change of the anticodon loop precedes that of the whole tRNA molecule [44]. We are now analyzing the tertiary structure of the anticodon loop of $tRNA^{Lys}$ using NMR spectroscopy.

Assuming our contention that $tRNA^{Lys}$ possesses an unusual anticodon structure is correct, what is the purpose of its existence? The most probable answer is that it is somehow related to the 'frameshift' in the *E.coli* translation process, which has recently been found in various cases.

It is known that in *E.coli* DNA polymerase, τ and γ subunits are encoded on a single open reading frame (ORF) [45], and these two subunit proteins are produced by the (−1) frameshift at a run of 'A's' on the ORF-bearing mRNA [27, 28, 45]. This translational control by frameshifting can also be observed in the *in vitro* system [46]. The importance of the affinity between the anticodon of tRNA and the codon of the mRNA has been shown by an experiment examining how the nucleotide sequence of the site on which the frameshift occurs (AAAAAAG) influences the efficiency of the frameshift [28].

In one of the transposable element families of *E.coli*, IS 1, there are 3 kinds of ORF's on the IS 1 gene, ins A, ins B and ins B′ in which the 5′ region (79 bp) overlaps with the 3′ region of ins A. The (−1) frameshift occuring within the ins B′ area results in the fusion protein 'insA−B′−insB', which exerts transposase activity [26]. The sequence responsible for the frameshift was determined to be CTAAAAAACTC (in particular, the underlined region) by the transposing activity of IS 1−lac Z fusion proteins whose gene sequences had been altered in various ways by the site-directed mutagenesis.

What is the molecular mechanism by which these frameshifts occur? In the above cases where a stretch of A's is probably translated by $tRNA^{Lys}$ whose anticodon structure is unusual, it is inferred that the affinity between the anticodon of the tRNA and the codon of the mRNA is slightly altered. As a result, a slippage can occur leading to a 2-nucleotide translocation in the $tRNA^{Lys}$ translating the original reading-frame codon AAA [47], or a certain doublet AA in the stretch of A's may be recognized by $tRNA^{Lys}$ and translated as Lysine [26], either of which would result in the frameshift. We are now seeking to clarify these speculations experimentally by using the $tRNA^{Lys}$ and mRNA containing the stretch of A's in the *in vitro* translation system of *E.coli* [46].

Another interesting phenomenon which might be related to the unusual structure of $tRNA^{Lys}$ is that *E.coli* $tRNA^{Lys}$ is exceptionally sensitive towards the T_4 anticodon endonuclease [48]. When phage T_4 infects *E.coli*, $tRNA^{Lys}$ in the host cell is once cleaved by an anticodon nuclease (ACNase) and then ligated by an RNA ligase at the 5′ position of the anticodon. It has been suggested that the ACNase activity is coupled with the protein synthesis of the T_4-infected host cell. Thus, we will also attempt to elucidate how the unusual anticodon structure of $tRNA^{Lys}$ is related to the uncommon phenomena described above.

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