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The 'polysemous' codon—a codon with multiple amino acid assignment caused by dual specificity of tRNA identity

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In some *Candida* species, the universal CUG leucine codon is translated as serine. However, in most cases, the serine tRNAs responsible for this non-universal decoding (tRNA^{Ser}CAG) accept *in vitro* not only serine, but also, to some extent, leucine. Nucleotide replacement experiments indicated that m^{1}G37 is critical for leucylation activity. This finding was supported by the fact that the tRNA^{Ser}CAGs possessing the leucylation activity always have m^{1}G37, whereas that of *Candida cylindracea*, which possesses no leucylation activity, has A37. Quantification of defined aminoacetylated tRNAs in cells demonstrated that 3% of the tRNA^{Ser}CAGs possessing m^{1}G37 were, in fact, charged with leucine *in vivo*. A genetic approach using an auxotroph mutant of *C.maltosa* possessing this type of tRNA^{Ser}CAG also suggested that the *URA3* gene inactivated due to the translation of CUG as serine was rescued by a slight incorporation of leucine into the polypeptide, which demonstrated that the tRNA charged with multiple amino acids could participate in the translation. These findings provide the first evidence that two distinct amino acids are assigned by a single codon, which occurs naturally in the translation process of certain *Candida* species. We term this novel type of codon a 'polysemous codon'.

Keywords: aminoacyl-tRNA synthetase/*Candida*/genetic code/translational fidelity

Introduction

The universality of the genetic code was once considered to be one of the essential characteristics of life, which led to the conception of the 'frozen accident theory'. This theory proposes that all extant living organisms use the universal genetic code, which was born by accident and 'frozen', and that they originate from a single, closely interbreeding population (Crick, 1968). However, in recent years a number of non-universal genetic codes have been reported in various non-plant mitochondrial systems, as well as in several nuclear systems (reviewed in Osawa *et al.*, 1992; Osawa, 1995), which contradict the frozen accident theory.

Among these deviations from the universal codes, Kawaguchi *et al.* (1989) demonstrated that CUG, a universal leucine codon, is translated as serine in an asporogenic yeast, *Candida cylindracea*. We identified the serine tRNA having the anticodon CAG, which is responsible for the assignment of codon CUG as serine (termed tRNA^{Ser}CAG), and revealed its decoding mechanism by means of an *in vitro* translational assay system (Yokogawa *et al.*, 1992; Suzuki *et al.*, 1994). Furthermore, when we investigated the distribution of this non-universal genetic code in fungi, as well as *C.cylindracea*, eight other *Candida* species—*C.albicans*, *C.zeylanoides*, *C.lusitaniae*, *C.tropicalis*, *C.melbiosica*, *C.parapsilosis*, *C.guilliermondii* and *C.rugosa*—were found to utilize the codon CUG for serine instead of leucine, all having tRNA^{Ser}CAG as the mediator in the unusual decoding (Ohama *et al.*, 1993; Ueda *et al.*, 1994). Several other investigators have also shown that the codon CUG is actually translated as serine *in vivo* in *C.albicans* and *C.maltosa* (Santos and Tuite, 1995a; Sugiyama *et al.*, 1995; Zimmer and Schunck, 1995).

One of the most remarkable structural features observed in most of these tRNA^{Ser}CAGs is that the nucleotide 5′-adjacent to the anticodon (position 33) is occupied not by the conserved U residue (U33) but by a G residue (G33). It has been speculated that U33 is necessary for forming the U-turn structure of the anticodon loop in all tRNAs reported so far (Quigley and Rich, 1976; Sprinzl *et al.*, 1996). Moreover, the nucleotide at position 37, 3′-adjacent to the anticodon CAG, is 1-methyl guanosine (m^{1}G) in almost all tRNA^{Ser}CAGs except for that of *C.cylindracea* (A37), while all the serine tRNAs in fungi corresponding to the universal serine codons UCN (N: A, C, U or G) and AGY (Y: U or C) have modified adenosine at this position without exception (Sprinzl *et al.*, 1996). The question then arises as to why these tRNA^{Ser}CAGs specific for this non-universal codon possess such unique features in the sequence surrounding the anticodon.

In the past 10 years, the mechanism by which aminoacyl-tRNA synthetases recognize their cognate tRNAs has been extensively investigated both *in vitro* and *in vivo* (Schimmel, 1987; Schulman and Abelson, 1988; Yarus, 1988; Normanly and Abelson, 1989; Shimizu *et al.*, 1992; McClain, 1993; Schimmel *et al.*, 1993). This line of study began with the artificial conversion of leucine tRNA of *Escherichia coli* to serine tRNA by Abelson's group 10 years ago (Normanly *et al.*, 1986). Recently, tRNA identity elements of *Saccharomyces cerevisiae* leucine tRNA were elucidated using unmodified variants synthesized by T7 RNA polymerase (Soma *et al.*, 1996), indicating that in addition to the discriminator base, A73, the second letter of the anticodon, A35, and the nucleotide 3′-adjacent to the anticodon, m^{1}G37, are important for recognition by leucyl-tRNA synthetase (LeuRS). The majority of *Candida* tRNA^{Ser}CAGs have A35 and m^{1}G37, while the discriminator is occupied by a nucleoside other than adenosine

(mostly G73). In this respect, tRNA^{Ser}CAG seems to be a potentially chimeric tRNA molecule capable of being recognized not only by seryl- but also by leucyl-tRNA synthetases.

Previously, we showed that these tRNA^{Ser}CAGs would have originated from the serine tRNA corresponding to codon UCG (Ueda *et al.*, 1994). This suggests an evolutionary pathway in which conversion from A to m^{1}G would have taken place at position 37 just after the emergence of tRNA^{Ser}CAG had brought about a change in the universal code. Since such a mutation at position 37 might potentially result in the leucylation of tRNA^{Ser}CAG, we attempted to elucidate the charging properties of these tRNA^{Ser}CAGs both *in vitro* and *in vivo*. Based on the results of *in vitro* aminoacylation reactions using tRNA variants constructed by the microsurgery method, the direct analysis of aminoacylated tRNAs in cells and a genetic approach, we demonstrate here that these serine tRNAs are actually leucylated both *in vitro* and *in vivo*. Furthermore, m^{1}G at position 37 was found to be indispensable for the leucylation of tRNA^{Ser}CAGs. In fact, the tRNA^{Ser}CAG of *C.cylindracea*, which has A at position 37, exhibits no leucylation activity. *C.cylindracea* has a high G+C content (63%) and utilizes CUG as a major serine codon. However, the other *Candida* species have no such high G+C content and utilize the CUG as a minor serine codon (Kawaguchi *et al.*, 1989; Lloyd and Sharp, 1992; our unpublished observation). Considering the relationship between the usage of the codon CUG as serine and the leucylation properties of tRNA^{Ser}CAG, it seems that only *Candida* species with a genome in which the incidence of the CUG serine codon is very low possess serine tRNA^{Ser}CAG that can be leucylated. Furthermore, such tRNA^{Ser}CAGs charged with heterogeneous amino acids should be utilized equally in the translation process. This is the first demonstration that a single tRNA species is assigned to two different amino acids in the cell. We propose designating this type of codon having multiple amino acid assignment as a 'polysemous codon'. The correlation between the dual-assignment state and the pathway of genetic code diversification is also discussed.

Results

Candida zeylanoides tRNA^{Ser}CAG is leucylated in vitro

First the leucylation of tRNA^{Ser}CAGs from *C.zeylanoides* and *C.cylindracea* was examined using LeuRS partially purified from *C.zeylanoides*, since it is known that leucine tRNAs of yeast have one of their identity determinants at position 37 (Soma *et al.*, 1996) and tRNA^{Ser}CAGs of *C.zeylanoides* and *C.cylindracea* have different nucleotides at this position (m^{1}G and A, respectively) (Figure 1A). Both tRNAs showed almost full serylation activity (~1200–1500 pmol/A_{260} unit), as shown in Figure 1B. The tRNA^{Ser}CAG of *C.zeylanoides* was evidently leucylated (the kinetic parameters are given in the uppermost row of Table I), though the charging activity was lower than that for serylation. This low acceptance of leucine of tRNA^{Ser}CAG may be due to the partial purification of LeuRS and high K_m value of LeuRS towards tRNA^{Ser}CAG. On the contrary, tRNA^{Ser}CAG of *C.cylindracea* was not leucylated at all, as was the case when another species

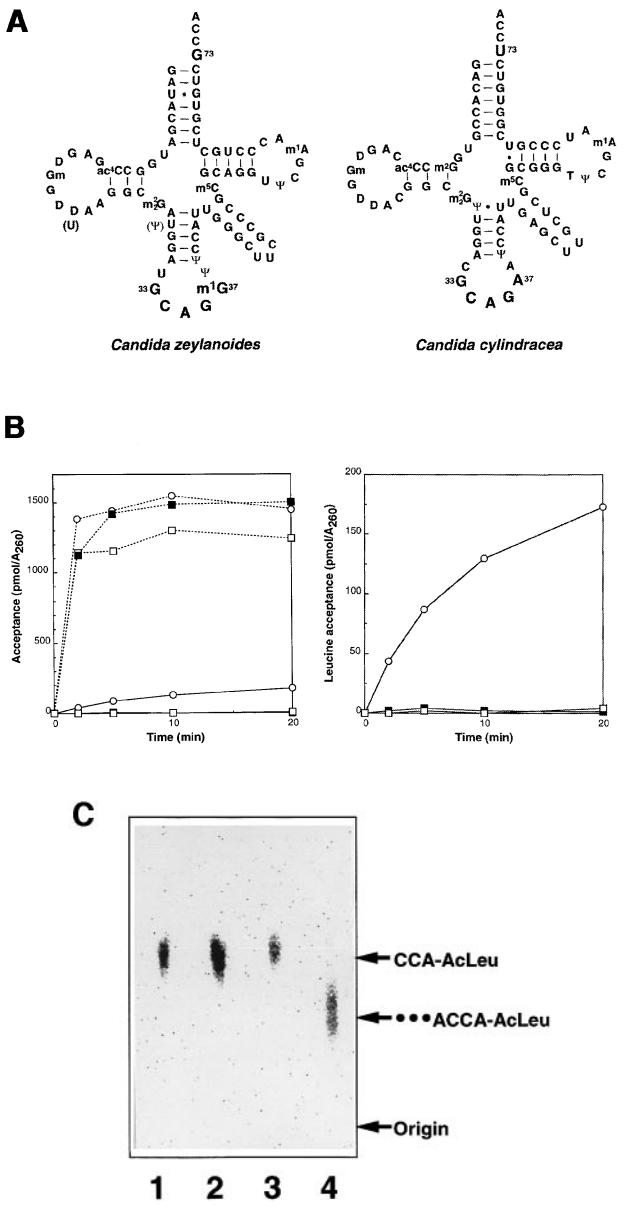

Fig. 1. Aminoacylation of *Candida* tRNA^{Ser}CAGs with serine and leucine. (**A**) Cloverleaf structures of tRNA^{Ser}CAGs from *C.zeylanoides* and *C.cylindracea* (Yokogawa *et al.*, 1992; Ohama *et al.*, 1993). The numbering system and abbreviations for modified nucleotides conform to Sprinzl *et al.* (1996) and Crain and McCloskey (1996), respectively. (**B**) Time-dependent aminoacylation with SerRS or LeuRS from *C.zeylanoides* cells. Aminoacylation reactions were carried out with 0.7 μM tRNAs and with same amounts of enzyme activities calculated using cognate tRNAs. Serylation and leucylation are shown by dotted and solid lines, respectively. The right-hand frame shows the solid curves from left-hand frame plotted with an enlarged ordinate. The aminoacylation of *C.zeylanoides* tRNA^{Ser}CAG (○) and of *C.cylindracea* tRNA^{Ser}CAG (□) are compared; *C.cylindracea* tRNA^{Ser}GCU (■), having no leucylation activity, is shown as a control. (**C**) TLC analysis of acetylleucyl-tRNA fragments derived from leucylated tRNA^{Ser}CAGs. After leucylation with [^{14}C]leucine, leucyl-tRNAs were acetylated with acetic anhydride. Acetyl-[^{14}C]leucyl-tRNA^{Ser}CAG of *C.zeylanoides* digested with RNase T1 (lane 1), and acetyl-[^{14}C]leucyl-tRNA^{Leu}s digested with RNase U2 (lane 3) or RNase T1 (lane 4) were developed on a TLC plate. Lane 2 is the pattern developed using a mixed sample from lanes 1 and 3. Samples containing radioactivity of 200 c.p.m. were spotted onto a cellulose TLC plate and developed by saturated ammonium sulfate/1 M NaOAc (pH 5.5)/isopropanol/dH_2O(20/9/1/20). The radioactivities were visualized by an imaging analyzer (BAS-1000, Fuji Photo Systems).

Table I. Kinetic parameters for mutants of tRNA^{Ser}CAG from *C.zeylanoides* with leucyl-tRNA synthetase of *C.zeylanoides*

Strain	K_m (μM)[a]	V_{max} (pmol/min)	V_{max}/K_m (relative)
Native	5.0	3.3	1.0
z-G33C	1.3	3.1	3.8
z-G33U	1.4	1.2	1.3
z-G33G	5.6	2.2	0.59
z-G33A	6.7	2.5	0.56

[a]The apparent K_m values are given since the LeuRS used was a partially purified fraction.

of serine tRNA specific for codon AGY (Y: U or C) (tRNA^{Ser}GCU) was employed as a control substrate (Figure 1B, right-hand graph). The K_m value of *C.zeylanoides* LeuRS towards tRNA^{Ser}CAG (5.0 μM) is only one order of magnitude larger than that of the serylation of this tRNA (0.22 μM) as well as that of leucylation toward the cognate leucine tRNAs of *S.cerevisae* (0.34 μM; Soma *et al.*, 1996).

In order to verify that the leucylation activity observed for the tRNA^{Ser}CAG of *C.zeylanoides* actually came from the tRNA^{Ser}CAG itself, and not from a trace amount of leucine tRNA contaminating the tRNA sample, the leucylated 3′-terminal RNA fragment derived from leucyl-tRNA^{Ser}CAG was analyzed in the following manner. ^{14}C-leucylated tRNA^{Ser}CAG from *C.zeylanoides* was first acetylated with acetic anhydride to prevent deacylation, and then digested with RNase T1. The resulting 3′-terminal fragment with ^{14}C-labeled acetylleucine was analyzed by cellulose TLC. The results are shown in Figure 1C. If leucylated tRNA^{Ser}CAG were digested with RNase T1, ^{14}C-labeled acetylleucyl-CCA should be released as a labeled fragment (Figure 1C, lane 3), because G is located at position 73 of the tRNA^{Ser}CAG (Figure 1A, left-hand structure). Any contaminated leucine tRNAs, if they exist, will give some ^{14}C-labeled fragments larger than the tetramer (Figure 1C, lane 4), because all the leucine tRNAs of yeasts so far analyzed (Sprinzl *et al.*, 1996) including those of *C.zeylanoides* (T.Suzuki, unpublished result) are known to have A73 at their 3′-ends, which are resistant to RNase T1. The mobility of the acetylleucyl-oligonucleotide derived from tRNA^{Ser}CAG from *C.zeylanoides* (Figure 1C, lane 1) was identical to that of acelylleucyl-CCA prepared from the RNase U2 digests of leucyl-tRNA^{Leu}s from *C.zeylanoides* (lane 3).

This observation clearly demonstrates that leucine is definitely attached to the tRNA possessing G73; the tRNA therefore must be tRNA^{Ser}CAG and not tRNA^{Leu}. Thus, it is concluded that the tRNA which incorporated leucine *in vitro* is in fact tRNA^{Ser}CAG. This deduction is supported by the results of an additional experiment: incorporation of [^{14}C]leucine into the tRNA^{Ser}CAG sample with LeuRS was reduced by the addition of SerRS and non-labeled serine to the reaction mixture (data not shown), which clearly indicates that the same tRNA molecule is competitively aminoacylated by these two enzymes.

To conclude that tRNA^{Ser}CAG is aminoacylated with leucine, we carried out a further experiment. The tRNA^{Ser}CAG was charged with serine and serylated tRNA^{Ser}CAG was separated from non-aminoacylated tRNA^{Ser}CAG by gel-electrophoresis under acidic conditions. After deacylation, the leucylation activity of the tRNA^{Ser}CAG was unequivocally detected. This experiment clearly indicates that a tRNA^{Ser}CAG molecule with serylation activity simultaneously possesses leucine-accepting activity.

Leucyl-tRNA synthetase from *C.zeylanoides* also leucylated tRNA^{Ser}CAGs from *C.albicans*, *C.lusitaniae* and *C.tropicalis* (data not shown), but the tRNA^{Ser}CAG of *C.cylindracea* was not leucylated at all. This charging property was not due to the heterologous combination of the synthetase and tRNA, since similar results were observed with LeuRSs from both *C.cylindracea* and *S.cerevisiae* (data not shown).

m^{1}G37 is responsible for recognition by leucyl-tRNA synthetase

Among the tRNA^{Ser}CAGs of several *Candida* species, that of *C.cylindracea* is unique because it alone possesses no leucylation capacity. A sequence comparison of these tRNAs (Figure 1A) prompts us to speculate that the nucleotide at position 37 is strongly associated with leucylation, because all tRNA^{Ser}CAGs possessing leucylation activity have m^{1}G in common, while only the tRNA^{Ser}CAG of *C.cylindracea*, which possesses no leucylation activity, has A at this position.

To examine the validity of this speculation, a series of tRNA^{Ser}CAG variants was constructed by the *in vitro* transcription method using T7 RNA polymerase, as well as by the microsurgery method, and the leucylation activity of each variant was measured. When the tRNA^{Ser}CAG of *C.zeylanoides* synthesized by *in vitro* transcription was employed as a substrate, no leucylation activity was detected, not even for the tRNA transcript having G37 (Figure 3A). On the other hand, as shown in Figure 3A, serylation activity exceeded 1000 pmol/A_{260}unit. These results strongly suggested that some nucleoside modification is necessary in tRNA^{Ser}CAG for recognition by LeuRS. We thus attempted to replace the m^{1}G37 of *C.zeylanoides* tRNA^{Ser}CAG with G (the variant is symbolized as m^{1}G37G) or A (m^{1}G37A), by the microsurgery method (Figure 2A and B; for details, see Materials and methods) to examine the contribution of m^{1}G37 to leucylation and the contribution of A37 of *C.cylindracea* tRNA^{Ser}CAG to the prevention of leucylation.

When aminoacylation of m^{1}G37A and m^{1}G37G was examined (Figure 3A), the results indicated that both substitutions lead to complete loss of leucylation (Figure 3A, right-hand graph), although no apparent influence was observed on serylation (Figure 3A, left-hand graph). These findings strongly indicate that the methyl group of m^{1}G37 plays a crucial role in enhancing the leucylation activity of tRNA^{Ser}CAG.

The slight reduction in leucylation activity observed in the control variant z-G33G (Figure 2A) compared with native tRNA (Figure 3A, right-hand graph) was found to have resulted from the partial deacetylation of 4-acetyl cytidine (ac^{4}C) due to acid treatment of the 5′-half fragment of tRNA^{Ser}CAG (see Materials and methods). This is considered further in the Discussion.

G33 acts as a modulator of leucylation

In addition to m^{1}G37, another unique feature of the serine tRNA^{Ser}CAGs in these *Candida* species is the presence

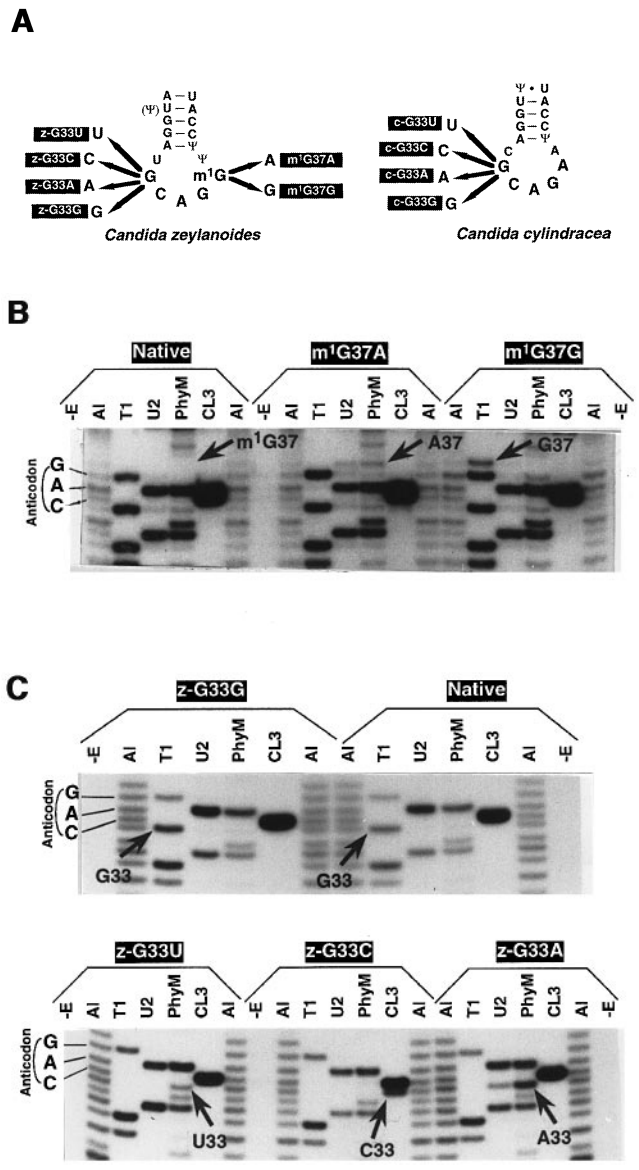

Fig. 2. Construction of tRNA variants with mutation at position 33 or 37 by the microsurgery method. (**A**) Sequences of the anticodon region of variants of tRNA^{Ser}CAGs from *C.zeylanoides* and *C.cylindracea*. The mutated nucelotides are shown by white letters on a black background. (**B**) Gel electrophoretic patterns showing RNA sequences around the anticodon for two variants mutated at position 37 (m^{1}G37A and m^{1}G37G) by Donis-Keller's method (Donis-Keller, 1980). –E, Al, T1, U2, PhyM and CL3 indicate no treatment and alkaline digestion, RNase T1 (specific for G), RNase U2 (for A > G), RNase PhyM (for A and U) and RNase CL3 (for C) treatments, respectively. Bands corresponding to A or G appeared at position 37 in m^{1}G37A or m^{1}G37G, respectively, while none was observed in the native tRNA (shown by arrows). (**C**) Gel electrophoretic patterns showing RNA sequences around the anticodon for variants mutated at position 33 by Donis-Keller's method. The nucleotide at position 33 in each variant was confirmed to have been replaced as expected (shown by arrows).

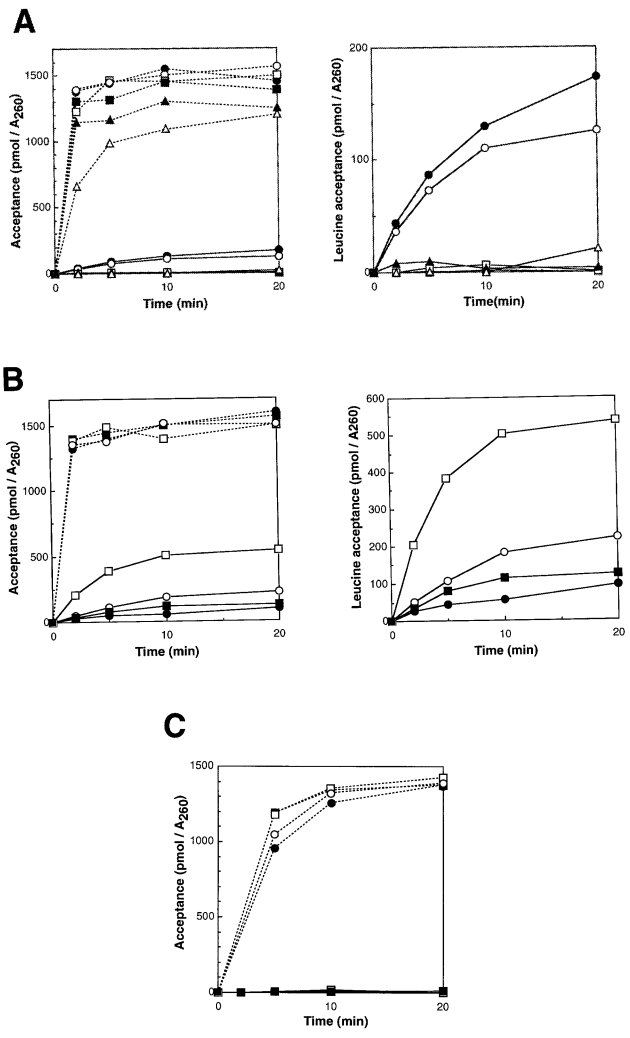

Fig. 3. Aminoacylation of tRNA variants with serine and leucine. (**A**) Effect of replacement of m^{1}G37 on leucylation. Variants: *C.zeylanoides* tRNA^{Ser}CAG (●), *C.cylindracea* tRNA^{Ser}CAG (▲), m^{1}G37A (□), m^{1}G37G (■), z-G33G (○) and the transcript of *C.zeylanoides* tRNA^{Ser}CAG (△). Solid and dotted lines indicate leucylation and serylation, respectively [and also in (B) and (C)]. The right-hand frame shows the solid curves from the left-hand frame plotted with an enlarged ordinate [and also in (C)]. (**B**) Effect of the replacement of G33 of *C.zeylanoides* tRNA^{Ser}CAG on leucylation. Variants: z-G33C (□), z-G33U (○), z-G33G (■) and z-G33A (●). (**C**) No leucylation was observed in any variant from *C.cylindracea* tRNA^{Ser}CAG with a mutation at position 33. Variants: c-G33C (□), c-G33U (○), c-G33G (■) and c-G33A (●).

of G at position 33, where a pyrimidine (mostly U) is completely conserved in usual tRNAs (Sprinzl *et al.*, 1996). Since we considered it is possible that this notable feature may be in some way related to the unusual aminoacylation characteristics described above and/or to the translation of non-universal genetic code, we examined the effect of residue 33 on the aminoacylation and translation activities of mutated tRNA^{Ser}CAG by introducing a point mutation at this position in the tRNAs of *C.zeylanoides* and *C.cylindracea* using the microsurgery method (see Figures 2A and C, and 7B and C).

The effect of mutation at position 33 in these two tRNAs was found to be quite different. In the case of the *C.cylindracea* tRNA, none of the mutations at position 33 caused leucylation of the tRNA, as was observed with the native tRNA^{Ser}CAG, and there was no reduction in serylation activity (Figure 3C). In contrast, the replacement of G33 by pyrimidines in *C.zeylanoides* tRNA^{Ser}CAG considerably enhanced the leucylation activity (Figure 3B, right-hand graph), while no significant difference was observed in the serylation activity (Figure 3B, left-hand graph). The kinetic parameters of leucylation for the

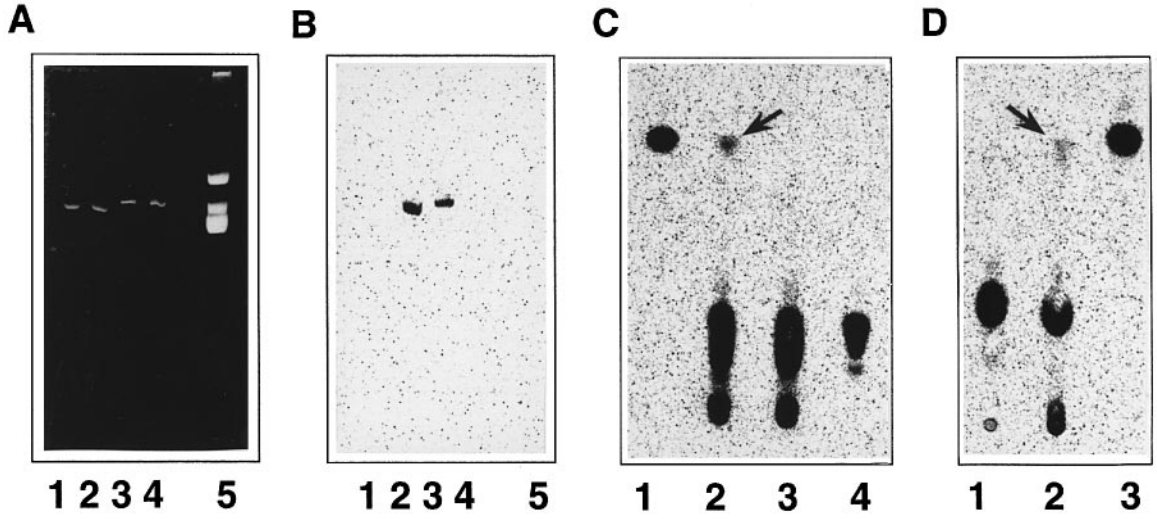

Fig. 4. Identification on TLC plates of amino acids attached to tRNA^{Ser}CAGs obtained from cells of *C.cylindracea* and *C.zeylanoides*. (**A**) [^{14}C]acetylaminoacyl-tRNA^{Ser}CAGs purified on 10% PAGE stained by toluidine blue. Lanes: uncharged tRNA^{Ser}CAG (lane 1) and [^{14}C]acetylaminoacyl-tRNA^{Ser}CAG (lane 2) from *C.cylindracea*, and uncharged tRNA^{Ser}CAG (lane 4) and [^{14}C]acetylaminoacyl-tRNA^{Ser}CAG (lane 3) from *C.zeylanoides*. Unfractionated tRNA from *C.zeylanoides* is also shown (lane 5). (**B**) Autoradiograph of (A). [^{14}C]acetylaminoacyl-tRNAs were visualized by an imaging analyzer. Lanes correspond to these in (A). (**C**) Identification of acetylamino acids attached to tRNA^{Ser}CAGs analyzed by TLC. Lanes 2 and 3 show the spots corresponding to acetylated amino acids released from *C.zeylanoides* tRNA^{Ser}CAG (lane 2) and *C.cylindracea* tRNA^{Ser}CAG (lane 3) with alkaline treatment. Lanes 1 and 4 show the spots corresponding to acetylleucine and acetylserine as markers, respectively. (**D**) Analysis of acetylamino acids attached to tRNA fragments on a TLC plate. Lane 2 shows the spot corresponding to the acetylamino acids derived from the RNase T1 fragment of *C.zeylanoides* tRNA^{Ser}CAG. Lanes 1 and 3 indicate the spots corresponding to acetylleucine and acetylserine, respectively. Ten micrograms of [^{14}C]acetylaminoacyl-tRNA^{Ser}CAG from *C.zeylanoides* was digested with RNase T1 and developed on cellulose TLC plates under the same conditions as (C). CCA fragments with [^{14}C]acetylamino acids were scraped from the plate from which the fragments were eluted with H20 and desalted by Sep-pak C18 under the conditions described in the literature (Wang *et al.*, 1990). [^{14}C]acetylamino acids discharged from the fragments were developed on TLC and visualized by an imaging analyzer (BAS-1000, Fuji Photo Systems).

variants of *C.zeylanoides* tRNA are shown in Table I. It is notable that the K_m values of the two pyrimidine mutants, z-G33U (1.4 μM) and z-G33C (1.3 μM), are clearly lower than those of the two purine mutants, z-G33A (6.7 μM) and z-G33G (5.6 μM). The V_{max} value of z-G33U (1.2 pmol/min) is 39% of that of z-G33C (3.1 pmol/min), which could explain why z-G33U shows lower leucylation activity than z-G33C despite having nearly the same K_m value (Figure 3B, right-hand graph). Judging from the sequence analysis (data not shown), the slight reduction in the leucylation of z-G33G (5.6 μM) compared with that of the native tRNA^{Ser}CAG (5.0 μM) is probably due to the partial deacetylation of ac^{4}C at position 12, as mentioned above. This was confirmed by the observation of a slight reduction in leucylation activity also in acid-treated native tRNA^{Ser}CAG (data not shown). It is thus concluded that replacement of a pyrimidine by a purine at position 33 has a repressive effect on leucylation of the tRNA^{Ser}CAG of *C.zeylanoides*.

The translation efficiencies of the variants with a mutation at position 33 were also examined in a cell-free translation system of *C.cylindracea* (Yokogawa *et al.*, 1992; Suzuki *et al.*, 1994), to evaluate the effect of G33. A change from G to U at position 33 apparently enhanced the translation activity 2.5-fold, although their decoding properties did not change at all (data not shown). We thus consider that G33 serves as a modulator of leucylation of tRNA^{Ser}CAG, despite a slight disadvantage in translation activity.

Evidence for leucylation of C.zeylanoides tRNA^{Ser}CAG in vivo

At this point, we had established that the tRNA^{Ser}CAG of *C.zeylanoides* is actually able to accept leucine *in vitro*. However, considering the facts that SerRS and LeuRS coexist in cells and, judging from their K_m values, that the affinity of tRNA^{Ser}CAG toward SerRS is one order of magnitude higher than that toward LeuRS, we needed to ascertain whether the tRNA^{Ser}CAG of *C.zeylanoides* is in fact leucylated *in vivo*. For this purpose, we adopted a newly developed method for quantifying an individual aminoacyl-tRNA in cells (Suzuki *et al.*, 1996).

Aminoacyl-tRNAs separately prepared from cells of *C.zeylanoides* and *C.cylindracea* were immediately subjected to acetylation using [1-^{14}C]acetic anhydride to label the amino acids as well as to stabilize the aminoacylated tRNAs. From each of the acetylated aminoacyl-tRNA mixtures, tRNA^{Ser}CAGs from *C.zeylanoides* and *C.cylindracea* were fished out by a solid-phase-attached DNA probe as described previously (Tsurui *et al.*, 1994; Wakita *et al.*, 1994). A single band for each of the aminoacyl-tRNAs was detected by staining (Figure 4A) with which the radioactivity coincided in each case (Figure 4B).

Acetylated amino acids attached to these tRNAs were deacylated by alkaline treatment and analyzed by TLC. As shown in Figure 4C, acetylserine was observed as a major amino acid derivative in both tRNA^{Ser}CAGs, but acetylleucine was detected only in the *C.zeylanoides* tRNA^{Ser}CAG; the acetylserine and acetylleucine spots were identified as described previously (Suzuki *et al.*,

1996). The radioactivities remaining on the origins probably came from the direct acetylation of some nucleotides in the tRNAs, as discussed previously (Suzuki *et al.*, 1996). From comparison with the radioactivity of acetylserine, it was calculated that ~3% of the tRNA^{Ser}CAG was attached with acetylleucine. These results were reproducible.

Digestion of purified acetyl-aminoacyl tRNA^{Ser}CAG with RNase T1 also gave only a ^{14}C-labeled CCA fragment, as shown in Figure 1C. When the acetylated amino acid released from the fragment purified from the corresponding spot on TLC was analyzed by TLC, the ratio of acetylleucine to acetylserine was also found to be 3% (Figure 4D), indicating that acetylleucine is covalently attached to the tRNA^{Ser}CAG fragment with G73. It thus became clear that the tRNA^{Ser}CAG of *C.zeylanoides* was in fact charged with leucine by 3% of the amount of serylation of the same tRNA^{Ser}CAG in *C.zeylanoides* cells.

Incorporation of leucine is dependent on the CUG codon in C.maltosa

Aminoacylation has generally been considered to be the final stage determining translational accuracy (reviewed in Parker, 1989; Kurland, 1992; Farabaugh, 1993). However, in the case of tRNA^{Gln} charged with glutamate in the chloroplast, Glu-tRNA^{Gln} is rejected by an elongation factor so that the chloroplast translation machinery does not employ the mischarged aminoacyl-tRNA (Stanzel *et al.*, 1994). It is likely that this is an exceptional case due to the lack of glutamyl-tRNA synthetase in the chloroplast.

In order to prove that leucylated tRNA^{Ser}CAGs actually participate in the translation process in *Candida* cells without such a rejection mechanism, we utilized a *URA3* gene expression system derived from *S.cerevisiae* in *C.maltosa*, which was developed by Sugiyama *et al.* (1995). *Candida maltosa* utilizes the codon CUG as serine and possesses the relevant tRNA^{Ser}CAG gene (Sugiyama *et al.*, 1995; Zimmer and Schunck, 1995). Since the tRNA^{Ser}CAG gene has G at position 37, G37 should be modified to m^{1}G in tRNA, and tRNA^{Ser}CAG may hence become chargeable with leucine in addition to serine in *C.maltosa* cells.

In the *URA3* gene of *S.cerevisiae*, only one CTG codon appears, at the 45th position, and this is translated as leucine according to the universal genetic code, which is essential for the activity of orotidine 5′-monophosphate decarboxylase (ODCase) (Rose *et al.*, 1984; Sugiyama *et al.*, 1995). In the present study, this URA3 gene, with the CTG codon replaced by various leucine or serine codons, was utilized as a marker gene (Figure 5A). First, a plasmid in which the *S.cerevisiae* URA3 gene was inserted downstream of a *C.maltosa*-specific promoter (C-p) was constructed and designated as pCSU–CTG (Sugiyama, 1995). As controls, mutant plasmids of pCSU–CTG, in which the codon CTG was replaced by either the serine codon TCT or the leucine codon CTC, were constructed and named pCSU–TCT and pCSU–CTC, respectively. In addition, a plasmid (pCCU) consisting of the *URA3* gene of *C.maltosa* having a CTT leucine codon at the corresponding site, combined with the *C.maltosa*-specific promoter, was also used as a positive control. These variant plasmids were introduced into a URA3-defective *C.maltosa* strain CHU1 (*his5*, *ade1*, *ura3*::*C-ADE1/ura3*::*C-ADE1*) (Ohkuma *et al.*, 1993), the growth of which was monitored on minimal medium SD plates in the presence and absence of uracil.

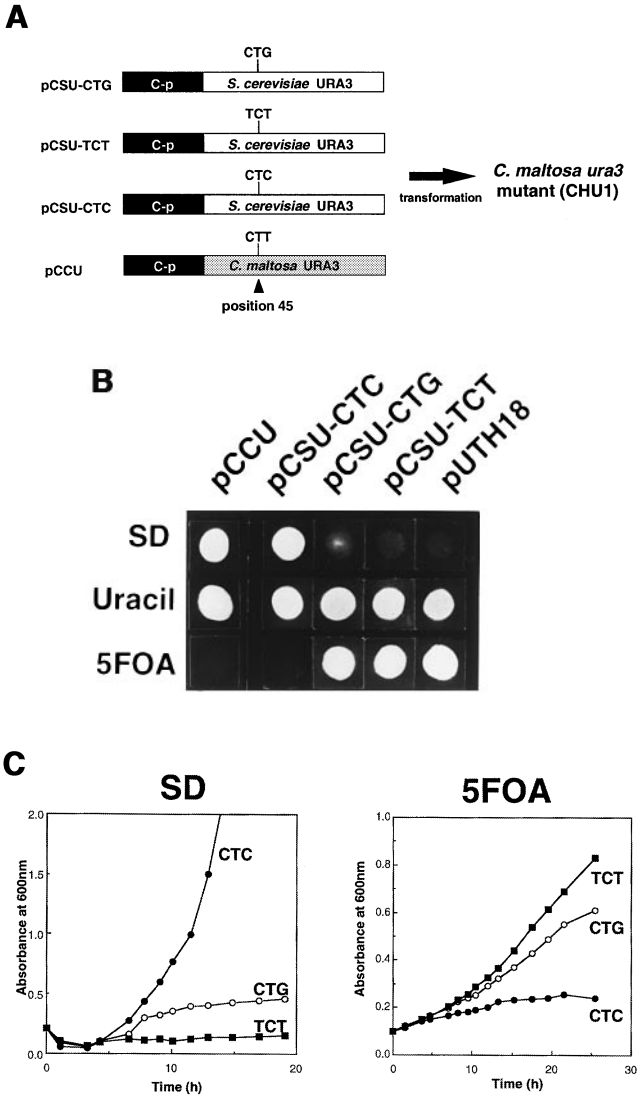

Fig. 5. Complementation of *C.maltosa URA3* mutation by *S.cerevisiae URA3* variants. (**A**) Construction of *URA3* genes transformed into *C.maltosa URA3* mutant. The 45th codon of the *S.cerevisiae URA3* gene was replaced by codon TCT or CTC, as shown. C-p indicates the promoter of the *URA3* gene from *C.maltosa*. (**B**) Growth on agar plate of *C.maltosa* cells harboring the constructed plasmids. Growth on the SD plate supplied with uracil and on the plate containing 5FOA was observed after overnight incubation, and that on the SD plate was after incubation for three nights. (**C**) Growth curves of *C.maltosa* cells harboring the constructed plasmids in SD- (left) and 5FOA- (right) liquid media. The curves indicate the growth of cells harboring pCSU–CTC (●), pCSU–CTG (○) or pCSU–TCT (■).

When uracil was supplied to the SD plate for the positive control experiments, all the transformants grew normally (Figure 5B, middle row). However, in the absence of uracil, cells harboring pCCU and pCSU–CTC showed normal growth, whereas no growth was observed in those harboring pCSU–TCT and pUTH18 that contained no *URA3* gene insertion. Cells harboring pCSU–CTG showed weak but significant growth (Figure 5B, uppermost row). These results demonstrate that if the codon at position 45 is translated as leucine, active ODCase will be produced and the cells will be able to grow, but translation of the

codon with serine will produce inactive ODCase and the cells will be unable to grow. The result with cells harboring pCSU–CTG clearly demonstrates that the *URA3* mutation on the *C.maltosa* chromosome was in some way complemented by the introduced pCSU–CTG plasmid, suggesting that the CTG codon was read at least partially as leucine in *C.maltosa* cells possessing $tRNA^{Ser}CAG$.

In order to quantify the growth rate of the cells harboring pCSU–CTG, the viability of the cells was examined in liquid medium without uracil. As shown in Figure 5C, whereas translation of the CTG codon as serine completely blocked cell growth in the case of pCSU–TCT, and full complementation was observed in the case of pCSU–CTC in which the CTC codon was read as leucine, intermediate cell growth was observed in the case of pCSU–CTG, indicating that ODCase was expressed in an active form, albeit at a low level, when there was a slight incorporation of leucine at the CTG codon. The slow growth of the cells harboring pCSU–CTG was not due to the spontaneous reversion of the CTG codon to another leucine codon or due to any other mutation, because the cells harvested from the colony on the SD-plate show the same growth phenotype. These results are unlikely to reflect the different expression levels of the *URA3* gene variants because the *URA3* mRNA level is not altered by mutations at position 45 (Ohkuma, 1993). Furthermore, the possibility that the *URA3* gene with CTG at position 45 is translated more efficiently than the gene with TCT at the same site due to codon preference (Ikemura, 1982) is excluded by the fact that the TCT codon is the most preferred of all the serine codons, including the CUG codon, in *C.maltosa* (Sugiyama *et al.*, 1995).

ODCase activity resulting from the translation of the *URA3* gene was examined in the presence of a pyrimidine analog, 5-fluoroorotic acid (5FOA), an inhibitor in pyrimidine biosynthesis. Incorporation of 5FOA with ODCase results in the formation of 5-fluorouridylate, which is harmful to cell propagation (Boeke *et al.*, 1984). Thus, *URA3*-defective strains grow normally on a medium containing 5FOA, whereas cells possessing the active *URA3* gene are unable to grow on this medium. Cells harboring the respective plasmids were cultivated in the presence of 5FOA in addition to uracil.

As shown in the bottom row of Figure 5B, cells harboring pCSU–CTG exhibited similar growth on the agar plate to those with pCSU-TCT and pUTH18, although the transformants with pCSU–CTC and pCCU were unable to grow. These results indicate that the CTG codon at position 45 was mainly translated as serine in *C.maltosa*, so as to produce the inactive ODCase. However, when the liquid medium was supplied with 5FOA, a slight reduction in the growth rate was observed in the case of pCSU–CTG, compared with pCSU–TCT (Figure 5C), while very slow growth was observed in the case of pCSU–CTC used as a control. In order to detect a low level of ODCase activity arising from a slight incorporation of leucine at the CUG codon in the 45th position, we adjusted the ratio of 5FOA and uracil as shown in Materials and methods. This growth rate reduction clearly suggests that the slow growth observed in the SD medium was due to low expression of active ODCase. Thus it is concluded that the CUG codon is partially translated as leucine in *C.maltosa* cells.

We believe that $tRNA^{Ser}CAG$ is the only molecule responsible for the leucine insertion corresponding to codon CUG in *C.maltosa* cells, based on the following obervations. We have purified and sequenced a number of leucine and serine tRNAs from *Candida* species, in which codon CUG is translated as serine, and failed in finding tRNA with the anticodon sequence potentially complementary to codon CUG other than $tRNA^{Ser}CAG$ (Yokogawa *et al.*, 1992; Ohama *et al.*, 1993; Suzuki *et al.*, 1994; Ueda *et al.*, 1994; our unpublished observation). Futhermore, tRNA genes for serine and leucine from these *Candida* species were sequenced following the amplification by cloning and/or PCR methods, and we found that only $tRNA^{Ser}CAG$ is able to translate codon CUG (Yokogawa *et al.*, 1992; Ohama *et al.*, 1993; Suzuki *et al.*, 1994; Ueda *et al.*, 1994; our unpublished observation). Thus, it could be concluded that only the $tRNA^{Ser}CAG$ species inserts leucine into polypeptide corresponding to codon CUG.

Discussion

The observations presented here clearly demonstrate that, in certain living organisms, a single codon can be simultaneously assigned to two distinct amino acids. Most codons in the genetic code degenerate, but our findings show that some amino acids are also able to degenerate with respect to a particular codon. Such codon ambiguity is governed by a tRNA acceptable to two amino acids simultaneously, as described above. We propose to designate a codon corresponding to multiple amino acids a 'polysemous codon'.

A high degree of accuracy in tRNA aminoacylation has been considered crucial for preserving fidelity in protein synthesis. It has been established that aminoacyl-tRNA synthetase is able to discriminate precisely its cognate amino acid from other structurally related amino acids at the adenylation reaction step, and its cognate tRNAs from non-cognate ones (reviewed in Parker, 1989; Kurland, 1992). The misacylation error in this process has been estimated to range between 10^{-4} and 10^{-5} (Lin *et al.*, 1984; Okamoto *et al.*, 1984). Discrimination of cognate tRNA from non-cognate tRNAs is mediated by positive and negative identity determinants localized on the tRNA molecule (Yarus, 1988; Normanly and Abelson, 1989). The only exception reported so far is that $tRNA^{Gln}$ is aminoacylated with glutamate in Gram-positive bacteria and in some organelles (Lapointe *et al.*, 1986; Schön *et al.*, 1988). However, this differs from misaminoacylation in that this process is indispensable to compensate for the lack of glutamyl-tRNA synthetase in these organisms. In general, high fidelity in the aminoacylation process is considered to be indispensable for translating genes into functionally active proteins with a high degree of accuracy.

The discovery of a polysemous codon in a *Candida* species contradicts the established notion of aminoacylation with high fidelity. We have shown that a single tRNA is acceptable to two different amino acids, and that it can therefore transfer two different amino acids corresponding to a particular codon. The expression experiment using the ODCase-encoding *URA3* gene containing codon CUG at the site essential for its activity (see also Sugiyama *et al.*, 1995) suggested that leucine

could be incorporated into the gene product corresponding to codon CUG in *C.maltosa*, as judged from the complementation tests with the *URA3* mutation. Although the amount of leucine incorporated per CUG codon was not quantitatively determined, it is clear that the incorporation was mediated by the leucyl-tRNA^{Ser}CAG. We thus concluded that codon CUG was simultaneously assigned to serine and leucine in the normal translation process in *C.maltosa*. A quantitative analysis of the amino acids attached to the tRNA indicated that 3% of tRNA^{Ser}CAG is leucylated in *C.zeylanoides* cells. Such a high level of leucylation is far beyond conventional misacylation, whose rate is estimated to be less than 10^{-4}. Unless a proofreading mechanism exists on the ribosome, incorporation of leucine at CUG codon sites may reflect the relative ratio of tRNA^{Ser}CAG leucylation, which is two orders of magnitude higher than that of conventional mistranslation.

To date, artificial manipulations of molecules participating in the translation process, such as the overproduction of aminoacyl-tRNA synthetase (Swanson *et al.*, 1988), mutations of tRNAs etc. and/or control of growth conditions, such as deprivation of amino acids in the medium (Edelmann and Gallant, 1977; O'Farrell, 1978; Parker and Precup, 1986), have been found to increase the error rate in translation (reviewed in Parker, 1989). However, our observation is based on experiments using wild-type cells grown in a rich medium suitable for high viability. In these respects, the polysemous codon is a phenomenon completely different from these artificial translational errors. It is known that many examples exist for alternative decoding of universal codons—initiation codons other than AUG (Gold, 1988; Kozak, 1983), leaky stop codons caused by nonsense suppresser or native tRNAs (Murgola, 1985), the UGA codon used for incorporation of selenocysteine (Leinfelder *et al.*, 1988) and so on. However, because of strong dependence on the context effects or possible secondary structures of mRNAs, these recoding events are those which are programed in the mRNAs (Gesteland *et al.*, 1992). We have sequenced several genes in *Candida* genomes, but we could not find any secondary structure around the codon CUG in these genes. Considering that the polysemous codon is mediated by a single tRNA, it is unlikely that a polysemous codon occurs under the influences of the neighboring regions in mRNAs. Alternative decoding of a polysemous codon CUG is possible, assuming that LeuRS is overexpressed under a certain physiological condition. Depending on the increased amount of the LeuRS in cells, incorporation of leucine corresponding to codon CUG may occur frequently, which causes the production of polypeptides with new functions. This possibility should be examined in further experiments.

The idea of a polysemous codon also differs from the 'near-cognate' concept proposed by Schultz and Yarus (1994). They claimed that ambiguous decoding may occur as a consequence of an irregular codon–anticodon interaction induced by the 27–43 base pair at the anticodon stem of the tRNA, resulting in a genetic code change transition state. The polysemous codon found in our study is caused by the tRNA aminoacylation process of tRNA with codon–anticodon interaction proceeding precisely in the conventional manner (Suzuki *et al.*, 1994). Furthermore, since the hypothesis of Schultz and Yarus is based on experiments using an artificial mutation, and it does not reflect experimental observation in an extant living organism.

On the basis of peptide sequences, several research groups have reported that codon CUG corresponds only to serine in *C.maltosa* (Sugiyama *et al.*, 1995) and *C.albicans* (Santos and Tuite, 1995a; White *et al.*, 1995). No leucine-inserted peptide was detected in these studies. However, we consider that any peptide with a leucine which was inserted for the codon CUG might have been missed during purification or was undetectable in the peptide sequencing, because the amount of leucine-inserted peptide (~3%) would have been too low to be positively identified in sequencing experiments.

We have shown that tRNA^{Ser}CAG in *Candida* species is a chimera of tRNA^{Ser}CAG and tRNALeuCAG in so far as it is the substrate for both SerRS and LeuRS. The K_m value for LeuRS is 5.0 μM, which is only one order of magnitude larger than that for SerRS (0.22 μM). In an *in vitro* aminoacylation experiment >30% of tRNA^{Ser}CAG subjected to the reaction could be converted to leucyl-tRNA^{Ser}CAG using an increased amount of LeuRS and a longer incubation time (data not shown). We observed that while the presence of SerRS and non-radioactive serine reduced leucylation, complete loss of leucylation could not be achieved (data not shown), indicating that the affinity of LeuRS toward tRNA^{Ser}CAG is relatively high.

In proliferating cells of *C.zeylanoides*, the leucyl-tRNA^{Ser}CAG in the cells was estimated to be 3% of the seryl-tRNA^{Ser}CAG, which is much lower than that obtained in the *in vitro* experiments. We consider that such a reduction in leucylation is due to the competition for the tRNA^{Ser}CAG between SerRS and LeuRS in the cells. Despite this competition, the distinct detection of leucylated tRNA^{Ser}CAG *in vivo* supports the existence of an ambiguous aminoacylation reaction toward the single tRNA^{Ser}CAG species.

The polysemous codon results from the coexistence of tRNA identity determinants for serine and leucine in a single tRNA molecule. Construction of tRNA^{Ser}CAG variants by the microsurgery method led to the finding that a single methyl moiety of m^1G at position 37 is involved in the leucylation process. In contrast, the tRNA^{Ser}CAG of *C.cylindracea*, which has A at the same position, is deprived of such leucine-accepting activity. Himeno and his co-workers noted that three nucleotides of leucine tRNAs were strongly recognized by *S.cerevisiae* LeuRS using unmodified variants transcribed by T7 RNA polymerase (Soma *et al.*, 1996). Although the discriminator base, A73, is the strongest recognition site among them, A35 and G37 in the anticodon loop also play roles as determinants in tRNA. They were able to compare the activities of variants mutated at position 37 with A or G using the variants with A at the discriminator position which effectively elevates leucylation activity. In our work, we utilized serine tRNA with a modified nucleoside and with G at the discriminator position as a substrate for LeuRS, because the T7 transcript of tRNA^{Ser}CAG showed no activity for leucylation. Our experiments using microsurgery methods indicated that m^1G is of great importance in leucylation, despite the fact that the presence of G at the discriminator position is unsuitable for the recognition of LeuRS. Some modified nucleotides in tRNA are known

to be involved in recoginition of some synthetases (Muramatsu *et al.*, 1988). Pütz *et al.* (1994) showed that m^1G at position 37 of yeast $tRNA^{Asp}$ is one of the negative determinants for arginyl-tRNA synthetase.

We have also demonstrated that the nucleotide at position 33, where only $tRNA^{Ser}CAG$ uniquely possesses G, modulates the leucine-accepting activity. G33 may prevent $tRNA^{Ser}CAG$ from excessive leucylation, which might be deleterious to the cells. Leuker and Ernst (1994) failed to express *S.cerevisiae* $tRNA^{Leu}$ genes having anticodons UAG or CAG on a low-copy (two or three copies) plasmid in *C.albicans*, indicating that such a high level of leucine residue incorporation into the CUG codon causes a lethal situation. In contrast, Santos and Tuite (1995b) reported that the *C.albicans* $tRNA^{Ser}CAG$ gene existing in a single copy plasmid could be expressed in *S.cerevisiae* cells, but that the viability of the cells decreased substantially. This finding suggests the polysemous state may be tolerated only when the ambiguous translation is under a strict constraint. We consider that G33 functions as a negative modulator in the leucylation of $tRNA^{Ser}CAG$, thereby controlling the relative seryl- to leucyl-$tRNA^{Ser}CAG$ ratio.

Several lines of experiment have suggested that U33 is involved in the tRNA function on ribosomes, such as in rigid codon–anticodon interaction, proper GTP hydrolysis of the ternary complex and the efficient translation of termination codons (Bare *et al.*, 1983; Dix *et al.*, 1986). Indeed, the replacement of G33 by U in *C.cylindracea* $tRNA^{Ser}CAG$ increased the efficiency of *in vitro* translation by 2- to 3-fold (data not shown). The negative effect of G33 on translation may indicate involvement in some mechanism for decoding the polysemous codon. This possibility needs to be clarified by further study. Nevertheless, we have shown here that one of the roles of G33 is the suppression of leucylation, and we consider that the nucleotide at position 33 is not directly involved in recognition by LeuRS. On the basis of our observation that no leucylation was detectable in the *C.cylindracea* $tRNA^{Ser}CAG$ variants in which G33 was replaced by a pyrimidine base (c-G33U and c-G33C), we speculate that G33 influences the location and/or conformation of m^1G37, accompanied by the alteration of the anticodon loop structure, decreasing the affinity of LeuRS toward $tRNA^{Ser}CAG$.

It has been generally considered that reconstructed tRNA does not lose its activity during the several reaction steps needed in the microsurgery method, such as cleavage of the tRNA strand and ligation of tRNA fragments (Ohyama *et al.*, 1985). However, a slight reduction of leucylation activity was observed in the control variant, z-G33G, compared with that of the native tRNA (Figure 3A, right-hand graph), which turned out to result from the partial deacetylation of 4-acetyl cytidine (ac4C) due to acid treatment of the 5′-half fragment (see Materials and methods). Nevertheless, it is reasonable to deduce the effect of base replacement on the aminoacylation activity by comparing the activities of these reconstructed tRNAs, because the same 5′-half fragments were used for all the manipulated tRNA molecules of *C.zeylanoides*.

A plausible mechanism by which LeuRS could recognize cognate leucine and serine tRNAs specific for codon CUG is illustrated in Figure 6. LeuRS contacts and

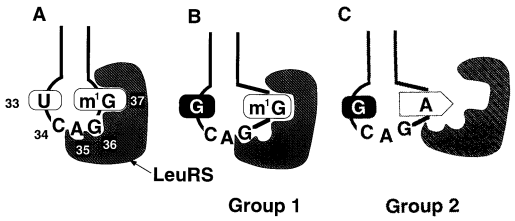

Fig. 6. Schematic diagrams showing a possible evolutionary process for the recognition of $tRNA^{Ser}CAG$s by LeuRS focusing on the effects of nucleotides at positions 33 and 37 on leucylation. (**A**) Model for the anticodon loop of normal $tRNA^{Leu}CAG$ recognized by leucyl-tRNA synthetase. (**B**) Role of m^1G 37 and G33 in recognition of the anticodon by LeuRS. The presence of m^1G at position 37 enhances the affinity between the anticodon and LeuRS, whereas G33 serves to lower it. This model represents the situation for the $tRNA^{Ser}CAG$s from *C.zeylanoides* and *C.maltosa*. (**C**) Model showing complete loss of the affinity between the anticodon and LeuRS for *C.cylindracea* $tRNA^{Ser}CAG$ due to the presence of A37.

recognizes its cognate leucine tRNA from the 3′-side of the anticodon loop, which is afforded by the uridine-turn structure due to U33 (Figure 6A). The methyl moiety of m^1G37 is directly recognized by LeuRS. In the case of *C.zeylanoides*, the anticodon loop distorted by G33 decreases the affinity toward LeuRS, judging from the observation that G33 increased the K_m value for leucylation approximately 4- to 5-fold in comparison with that with prymidine bases at the position (Figure 6B). In *C.cylindracea*, m^1G is replaced by A, which means that the tRNA has lost the two major determinants for LeuRS, m^1G and the discriminator base (Figure 6C). Consequently, LeuRS is unable to recognize $tRNA^{Ser}CAG$ at all, and G33 concomitantly loses its function as a modulator. LeuRS is, of course, unable to recognize other serine isoacceptor tRNAs corresponding to universal codons, because they have modified A at position 37.

How did this interaction between LeuRS and $tRNA^{Ser}CAG$ evolve? *Candida* species utilizing CUG as serine can be classified into two distinct groups: group 1 contains the species that have $tRNA^{Ser}CAG$ with leucylation activity, and includes *C.zeylanoides*, *C.maltosa* and others (see Figure 6B); group 2, which is represented solely by *C.cylindracea*, contains species that have $tRNA^{Ser}CAG$ without leucylation activity (Figure 6C). A plausible evolutionary process is that group 1 would have arisen prior to group 2 after the genetic code change, which is speculated on the basis of the following observations. First, the homology between $tRNA^{Ser}CAG$s in group 1 and its isoacceptor tRNAs for codon UCG is higher than that between the $tRNA^{Ser}CAG$ from *C.cylindracea* and its isoacceptor (Ueda *et al.*, 1994). Second, *C.cylindracea* (group 2) possesses high copy numbers of the $tRNA^{Ser}CAG$ genes (~20 copies) on the diploid genome (Suzuki *et al.*, 1994), while low copy numbers (two or four copies) are observed for group 1 $tRNA^{Ser}CAG$ genes (Santos *et al.*, 1993; Sugiyama *et al.*, 1995; T.Suzuki, personal observations). Third, the codon CUG is utilized as a major serine codon on several genes in *C.cylindracea*, such as lipase (Kawaguchi *et al.*, 1989) and chitin synthase (unpublished results), while CUG appears infrequently on the genomes of other species belonging to group 1 (Lloyd and Sharp, 1992; Sugiyama *et al.*, 1995; T.Suzuki, personal observations). During the course of the change in the genetic code, the genome should pass through a state

where the codon is under restrictive usage. Species with a high frequency of usage of the serine codon CUG are likely to be posterior to those with a lower frequency of usage. Furthermore, group 1 has a comparatively A+T-rich genome which would be a disadvantage for the usage of codon CUG, whereas the opposite situation occurs in group 2 (Ohama *et al.*, 1993). Fourth, the phylogenetic tree of these species and relatives constructed by using several genes also supports this evolutionary pathway (manuscript in preparation). Group 1, with a polysemous codon, thus seems to be closer to the point of genetic code change than group 2.

On the basis of their sequence similarity, Pesole *et al.* and ourselves have proposed that $tRNA^{Ser}CAG$ could have originated from the serine tRNA isoacceptor responsible for codon UCG (Ueda *et al.*, 1994; Pesole *et al.*, 1995). Thus, the nucleotide at position 37 seems likely to have mutated in the direction modified A→m^1G-(group 1)→unmodified A (group 2). This hypothesis raises two questions: (i) why did the mutation changing modified A to m^1G, which give rise to a polysemous codon disadvantageous for the fidelity of protein synthesis, occur; and (ii) why was there a subsequent reversion in group 2? These questions can only be answered by clarifying the evolutionary significance of the polysemous codon.

Alternative splicing generates a multiple protein sequence from a single gene at the mRNA level. In contrast, a polysemous codon potentially results in diversification of the amino acid sequence of the protein derived from a single gene in the translation process. We have shown that the polysemous state of a codon is tolerated when the codon appears infrequently, as observed in group 1 species. Furthermore, our analyses of the nucleotide sequences of several genes of groups 1 and 2 suggest that codon CUG tends to appear in non-housekeeping genes with high frequency (data not shown). Whereas a housekeeping gene requires a high degree of fidelity in the translation process to maintain its function, which is essential for the fundamental mechanism of the cell, non-housekeeping genes could allow the ambiguity of protein synthesis caused by a polysemous codon. We speculate that such ambiguity could have given rise to proteins with multiple amino acid sequences in non-house-keeping genes, which may have conferred multifunctionality on the proteins. Since the *C.cylindracea* strain was developed industrially for the production of lipase, such multifunctionality, caused by a polysemous codon, would not have been essential to cell growth, but might have been advantageous for the natural evolution of the cell. The advantage of a polysemous codon in evolution is of particular interest to us, and is a question which we are now addressing.

Materials and methods

Materials

[^{14}C]acetic anhydride (185 MBq/mmol) was purchased from American Radiolabeled Chemicals. Uniformly labeled [^{14}C]serine (5.99 MBq/mmol) and leucine (11.5 MBq/mmol) were from Amersham. 5-fluoroorotic acid monohydrate (5FOA) was from PCR inc. 3′-Biotinylated DNA probes were synthesized by Sci. Media, Japan. Synthetic RNA oligomers and a chimeric oligonucleotide composed of DNA and 2′-*O*-methyl RNA were synthesized by Genset Co. Ltd. Most of the enzymes used for the microsurgery were from Takara Shuzo (Tokyo, Japan). Other chemicals were obtained from Wako Chemical Industries.

Strains and media

Candida cylindracea (ATCC14830) was obtained from the American Type Culture Collection. *Candida zeylanoides* (JCM1627) was from the Japan Collection of Microorganisms (JCM, RIKEN, Wako, Saitama, Japan). *Candida maltosa* strain CHU1 (*his5*, *ade1*, *ura3*::*C-ADE1*/*ura3*::*C-ADE1*) was kindly provided by Drs M.Takagi and R.Ohtomo of the Department of Agricultural Chemistry, The University of Tokyo. Complete medium YPD (1% yeast extract, 2% Bacto-peptone and 2% dextrose) and minimal medium SD [0.67% yeast nitrogen base without amino acids (Difco) and 2% dextrose] supplied with 24 mg/ml uracil were used for the cultivation of yeast cells.

SD-plates with or without uracil were prepared by adding agar at a final concentration of 2% to each medium. 5FOA medium (1.4% yeast nitrogen base without amino acids, 4% dextrose, 20 mg/l uracil and 4 mg/ml 5-fluoroorotic acid) was adjusted to evaluate the different viabilities of transformants with pCSU–TCT and pCSU–CTG in the presence of 5FOA. 5FOA plates consisted of 0.7% yeast nitrogen base without amino acids (Difco), 2% dextrose, 50 mg/l uracil, 1 mg/ml 5FOA and 2% agar, prepared according to the literature (Boeke *et al.*, 1984).

Plasmid construction and transformation

In order to introduce mutation at the 45th codon in the reading frame of the *S.cerevisiae URA3* gene, pUC-CSU (Sugiyama *et al.*, 1995) was first cut by *Eco*T14I so as to remove a fragment consisting of 80 bps, including the 45th CTG codon. A totally synthesized DNA fragment with a mutation of CTG to TCT at the 45th position was inserted into the vector in place of the removed fragment to construct pUC–CSU–TCT. The *Hin*dIII fragment of pUC–CSU–TCT consisting of the *C.maltosa* promoter and the *S.cerevisiae URA3* coding region where the 45th position was mutated from CTG to TCT was inserted into the *Sma*I site of pUTH18 (Ohkuma *et al.*, 1993) to construct pCSU–TCT. Plasmid pUTH18 containing an autonomously replicating sequence of *C.maltosa* (Takagi *et al.*, 1986) and C-HIS5 (Hikiji *et al.*, 1989) were used as expression vectors developed from pUC18 by Ohkuma *et al.* (1993).

Other plasmids—pCSU–CTG, denoted as pCSU in the literature (Sugiyama *et al.*, 1995), consisting of the *C.maltosa* promoter and *S.cerevisiae URA3* coding region having one CTG codon at the 45th position, pCSU–CTC (Sugiyama *et al.*, 1995) consisting of the *C.maltosa* promoter and *S.cerevisiae URA3* coding region where the 45th position was mutated from CTG to CTC, and pCCU (Sugiyama *et al.*, 1995) having the native *URA3* of *C.maltosa*—were kindly provided by Drs M.Takagi and R.Ohtomo of the Department of Agricultural Chemistry, University of Tokyo. Transformation of *C.maltosa* was carried out by electroporation as follows. An overnight culture of *C.maltosa* CHU1 in YPD medium was harvested by centrifugation at 0°C. The cells were washed with 5 ml ice-cold 1 M sorbitol solution and collected by centrifugation. The pellet was resuspended with 200 µl chilled 1 M sorbitol. A 50 µl aliquot of the cells mixed with an appropriate amount of the plasmid DNA was pulsed by an electroporator (Electroporator II, Invitrogen Corp.) under the conditions described in the manufacturer's instruction manual. The electrified cells were spread on a SD-plate containing uracil and incubated at 30°C.

In vitro aminoacylation assay

Seryl- or leucyl-tRNA synthetases were partially purified from *C.zeylanoides* cells as described previously (Suzuki *et al.*, 1994), both of the activities having been completely separated by column chromatographies. The aminoacylation reaction was carried out at 37°C in 100 µl of reaction mixture containing 100 mM Tris–HCl (pH 7.5), 10 mM KCl, 50 mM NaCl, 15 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, 15 µM [^{14}C]serine (5.99 GBq/mmol) or 15 µM [^{14}C]leucine (11.5 GBq/mmol), 0.1–2.0 µM purified tRNA, and appropriate units of partially purified SerRS or LeuRS from *C.zeylanoides*. The reaction was started by addition of the enzyme to the pre-incubated reaction mixture. After appropriate incubation periods, 25 µl of the reaction mixture was spotted onto a filtration paper (Wattman 3MM), which was washed with 5% TCA to remove the free [^{14}C]amino acids. The radioactivity remaining on the filter was then measured by a liquid scintillation counter.

Large-scale purification of $tRNA^{Ser}CAG$s from C.zeylanoides and C.cylindracea

Candida cylindracea cells (3.1 kg) were treated with phenol, from which 150 000 A_{260} units of unfractionated tRNA were extracted. Eighty thousand A_{260} units of tRNA mixture were obtained by DEAE–cellulose chromatography with stepwise elution, which was then applied onto a DEAE–Sephadex A-50 column (6×100 cm). Elution was performed with a linear gradient of NaCl from 0.375 to 0.525 M in a buffer

consisting of 20 mM Tris–HCl (pH 7.5) and 8 mM $MgCl_2$. The fraction rich in $tRNA^{Ser}$ was applied onto a RPC-5 column (1×80 cm) and eluted with a linear gradient of NaCl from 0.4 to 1 M NaCl in a buffer consisting of 10 mM Tris–HCl (pH 7.5) and 10 mM $Mg(OAc)_2$. As a result of these chromatographies, 300 A_{260} units of purified $tRNA^{Ser}CAG$ were finally obtained.

One hundred and fifty thousand A_{260} units of tRNA from *C.zeylanoides* cells (3.7 kg) were fractionated on DEAE–Sepharose fast-flow column (3.5×130 cm) with a linear gradient of NaCl from 0.25 to 0.4 M in a buffer consisting of 20 mM Tris–HCl (pH 7.5) and 8 mM $MgCl_2$. About 300 A_{260} units of *C.zeylanoides* $tRNA^{Ser}CAG$ were finally obtained by further column chromatography with Sepharose 4B in a reverse gradient of ammonium sulfate from 1.7 to 0 M with a buffer consisting of 10 mM NaOAc (pH 4.5), 10 mM $MgCl_2$, 6 mM β-mercaptoethanol and 1 mM EDTA.

Construction of tRNA variants with mutation at position 33

The microsurgery procedures were basically carried out according to the literature (Ohyama *et al*., 1985, 1986). Limited digestion of 4 mg purified $tRNA^{Ser}CAG$ from *C.zeylanoides* with RNase T1 was performed at 0°C for 30 min in a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 100 mM $MgCl_2$, 0.5 mg/ml of the tRNA and 25 000 units/ml RNase T1 (Sigma). After phenol extraction, the resulting fragments were treated with 0.1 N HCl at 0°C for 12 h in order to cleave the 2′, 3′ cyclic phosphate of the 3′-end of the fragments formed in the limited digestion, and then the 5′- and 3′-half fragments were separated by 10% PAGE containing 7 M urea (10×10 cm). Four hundred and thirty micrograms of the 5′-half and 520 µg of the 3′-half fragments were recovered from the gel. The purified 5′-half fragment was dephosphorylated with bacterial alkaline phosphatase (Takara Shuzo), and G33 at the 3′-end of the 5′-half fragment was removed by oxidation with sodium periodate as described in the literature (Keith and Gilham, 1974). After dephosphorylation of the 3′-end of this fragment, the truncated 5′-half fragment was ligated with each of four synthetic tetramers—pUCAGp, pCCAGp, pACAGp or pGCAGp—with T4 RNA ligase (Takara Shuzo) at 10°C for 16 h in 200 µl of a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 3.5 mM DTT, 15 µg/ml BSA, 5% PEG6000, 480 µM ATP, 4.0 nmol of the 5′-half fragment, 20 nmol of the tetramer and 200 units T4 RNA ligase. Ligation efficiency was >80%. Ligated fragments were separated from unligated ones by MonoQ column chromatography with a linear gradient of NaCl from 0.38 to 0.5 M in a buffer consisting of 20 mM Tris–HCl (pH 7.5) and 1 mM EDTA. The fragments were precipitated with isopropanol and dephosphorylated. The ligated 5′-half and the phosphorylated 3′-half were incubated at 65°C for 10 min and annealed at room temperature. The ligation was performed in 300 µl of a reaction mixture consisting of 58 mM Tris–HCl (pH 7.5), 17.5 mM $MgCl_2$, 3.5 mM DTT, 10 µg/ml BSA, 50 µM ATP, 50 µg annealed tRNA and 200 units T4 RNA ligase. The 5′-end of the ligated tRNA was phosphorylated and the 3′-end was dephosphorylated simultaneously with T4 polynucleotide kinase (Toyobo) at pH 6.9, and then the 3′-end was repaired at 37°C for 1 h with CCA enzyme partially purified from *C.zeylanoides* in 400 µl of a reaction mixture consisting of 50 mM Tris–HCl (pH 8.7), 10 mM $MgCl_2$, 100 mM KCl, 1.75 mM ATP, 0.5 mM CTP, 2.5 mM DTT and an appropriate amount of the CCA enzyme. The reconstituted tRNAs were purified by 12% PAGE containing 7 M urea (0.5×20×40 cm). About 50 µg of each of the four variants mutated at G33 from *C.zeylanoides* $tRNA^{Ser}CAG$ were finally obtained; these were denoted as z-G33U, z-G33C, z-G33A and z-G33G, respectively. Transfer RNA variants mutated at position 33 from *C.cylindracea* $tRNA^{Ser}CAG$ were constructed. Limited digestion of 10 mg purified $tRNA^{Ser}CAG$ from *C.cylindracea* using RNase A was performed to obtain the 5′-half and 3′-half fragments. The dephosphorylated 5′-half fragment was ligated with each of four kinds of dimer—pUCp, pCCp, pGCp or pACp—for mutation at position 33. Each of the ligated 5′-half fragments was annealed with the phosphorylated 3′-half fragment and ligated at the anticodon loop. Then the 3′-end of each variant was repaired with T4 polynucleotide kinase and the CCA enzyme as described above. About 65 µg of each of the purified variants of *C.cylindracea* $tRNA^{Ser}CAG$ mutated at position 33 was obtained; these were denoted as c-G33U, c-G33C, c-G33A and c-G33G, respectively.

Construction of tRNA variants mutated at position 37

To obtain the mutation of m^1G37 of *C.zeylanoides* $tRNA^{Ser}CAG$, we selected the restrictive digestion technique with RNase H using a chimera oligonucleotide splint composed of DNA and 2′-*O*-methyl RNA as developed by Inoue *et al.* (1987). We designed a splint oligonucleotide according to the literature (Hayase *et al*., 1990) which was designated as CZE-37 (5′GmCmCmCmAmAmUmGmGmAmAmdCdCdTdG–CmAmUmCmCmAmUm3′), possessing a cleavage site between positions 37 and 38 of *C.zeylanoides* $tRNA^{Ser}CAG$. Two hundred micrograms of purified $tRNA^{Ser}CAG$ from *C.zeylanoides* was incubated at 65°C for 10 min with 14.4 nmol CZE-37 in a buffer consisting of 40 mM Tris–HCl (pH 7.7), 0.5 mM NaCl, 0.1 mM DTT, 0.0003% BSA and 0.4% glycerol (500 µl), and then annealed at room temperature. Magnesium chloride was added to the mixture up to a final concentration of 4 mM and the reaction was carried out at 30°C for 2 h by the addition of 600 units of RNase H (Takara Shuzo). About 60 µg of the cleaved 3′-half fragment was obtained by purification using 10% PAGE containing 7 M urea. Either of two synthetic oligo-RNAs, pCAGAp or pCAGGp, was ligated with the same 5′-half fragment digested by RNase T1 as the variants mutated at position 33 under the conditions described above. The ligated and dephosphorylated 5′-half fragments were annealed and ligated with the 3′-half fragment digested by RNase H. About 50 µg of each of the two variants from *C.zeylanoides* mutated at position 37—m^1G37A and m^1G37G—was obtained by the phosphorylation of the 5′-end and purification by 12% PAGE containing 7 M urea.

Identification of amino acids attached to $tRNA^{Ser}CAGs$ in the cells

Identification of aminoacyl-$tRNA^{Ser}CAG$ from *Candida* cells was carried out by a new method developed recently by us (Suzuki *et al.*, 1996). The experimental conditions were the same as those reported. To fish out the aminoacyl-tRNAs, we designed two 3′-biotinylated DNA probes: 5′AGCAAGCTCAATGGATTCTGCGTCC3′ for *C.cylindracea* $tRNA^{Ser}CAG$ and 5′GAAGCCCAATGGAACCTGCATCC3′ for *C.zeylanoides* $tRNA^{Ser}CAG$. These probes were immobilized with streptavidin agarose (Gibco BRL) as reported previously (Wakita *et al.*, 1994).

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