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 (tRNAserCAG) accept in vitro not only serine, but also, to some extent, leucine. Nucleotide replacement experiments indicated that m1G37 is critical for leucylation activity. This finding was supported by the fact that the tRNAserCAGs possessing the leucylation activity always have m1G37, whereas that of Candida cylindracea, which possesses no leucylation activity, has A37. Quantification of defined aminoacylated tRNAs in cells demonstrated that 3% of the tRNAserCAGs possessing m1G37 were, in fact, charged with leucine in vivo. A genetic approach using an auxotroph mutant of C. maltosa possessing this type of tRNAserCAG 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 was confirmed by the incorporation of leucine into the polypeptide, suggesting 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/translation 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 it 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 tRNAserCAG), 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. melibiosica, C. parapsilosis, C. guilliermondii and C. rugosa—were found to utilize the codon CUG for serine instead of leucine, all having tRNAserCAG 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 tRNAserCAGs is that the nucleotide 5′-adjacent to the anticodon (position 33) is occupied 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. 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; Schimmel et al., 1993; 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, m1G37, are important for recognition by leucyl-tRNA synthetase (LeuRS). The majority of Candida tRNAserCAGs have A35 and m1G37, while the discriminator is occupied by a nucleoside other than adenosine.
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 (m1G and A, respectively) (Figure 1A). Both tRNAs showed almost full seryltransfer activity (~1200–1500 pmol/1500 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 seryltransfer. 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 (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 m1G 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, m1G 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 charging 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 assignments as a ‘polysemous codon’. The correlation between the dual-assignment state and the pathway of genetic code diversification is also discussed.

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. Seryltransfer 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 (C) and of C. cylindracea tRNA^{Ser}CAG (D) are compared; C. cylindracea tRNA^{Ser}CAG, 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 [14C]leucine, leucyl-tRNAs were acetylated with acetic anhydride. Acetyl-[^14C]leucyl-tRNA^{Ser}CAG of C. zeylanoides digested with RNase T1 (lane 1), and acetyl-[14C]leucyl-tRNA^{Ser}CAG 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) or isopropanol/dH2O (20/9/1/20). The radioactivities were visualized by an imaging analyzer (BAS-1000, Fuji Photo Systems).
of serine tRNA specific for codon AGY (Y: U or C) (tRNA<sub>SerCAG</sub>) was employed as a control substrate (Figure 1B, right-hand graph). The <i>K</i><sub>m</i> value of <i>C. zeylanoides</i> LeuRS towards tRNA<sub>SerCAG</sub> (5.0 μM) is only one order of magnitude larger than that of the serylating of this tRNA (0.22 μM) as well as that of leucylation toward the cognate leucine tRNAs of <i>S. cerevisiae</i> (0.34 μM; Soma et al., 1996).

In order to verify that the leucylation activity observed for the tRNA<sub>SerCAG</sub> of <i>C. zeylanoides</i> actually came from the tRNA<sub>SerCAG</sub> itself, and not from a trace amount of leucine tRNA contaminating the tRNA sample, the leucylated 3′-terminal RNA fragment derived from leucyl-tRNA<sub>SerCAG</sub> was analyzed in the following manner. 14C-leucylated tRNA<sub>SerCAG</sub> was first acetylated with acetic anhydride to prevent deacylation, and then digested with RNase T1. The resulting 3′-terminal fragment with 14C-labeled acetylleucine was analyzed by cellulose TLC. The results are shown in Figure 1C. If leucylated tRNA<sub>SerCAG</sub> were digested with RNase T1, 14C-labeled acetylleucyl-CCA should be released as a labeled fragment (Figure 1C, lane 3), because G is located at position 73 of the tRNA<sub>SerCAG</sub> (Figure 1A, left-hand structure). Any contaminated leucine tRNAs, if they exist, will give some 14C-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 <i>C. zeylanoides</i> (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<sub>SerCAG</sub> from <i>C. zeylanoides</i> (Figure 1C, lane 1) was identical to that of acetylleucyl-CCA prepared from the RNase U2 digests of leucyl-tRNA<sub>1<sup>14</sup>CAG</sub> from <i>C. zeylanoides</i> (lane 3).

This observation clearly demonstrates that leucine is definitely attached to the tRNA possessing G73; the tRNA therefore must be tRNA<sub>SerCAG</sub> and not tRNA<sub>1<sup>14</sup>CAG</sub>. Thus, it is concluded that the tRNA which incorporated leucine in vitro is in fact tRNA<sub>SerCAG</sub>. This deduction is supported by the results of an additional experiment: incorporation of [14C]leucine into the tRNA<sub>SerCAG</sub> 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 aminoaacylated by these two enzymes.

To conclude that tRNA<sub>SerCAG</sub> is aminoacylated with serine, we carried out a further experiment. The tRNA<sub>SerCAG</sub> was charged with serine and serylated tRNA<sub>SerCAG</sub> was separated from non-aminoaacylated tRNA<sub>SerCAG</sub> by gel-electrophoresis under acid conditions. After deacylation, the leucylation activity of the tRNA<sub>SerCAG</sub> was unequivocally detected. This experiment clearly indicates that a tRNA<sub>SerCAG</sub> molecule with serylating activity simultaneously possesses leucine-accepting activity.

Leucyl-tRNA synthetase from <i>C. zeylanoides</i> also leucylated tRNA<sub>SerCAG</sub> from <i>C. albicans</i>, <i>C. lusitaniae</i> and <i>C. tropicalis</i> (data not shown), but the tRNA<sub>SerCAG</sub> of <i>C. cylindracea</i> 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 <i>C. cylindracea</i> and <i>S. cerevisiae</i> (data not shown).

**m<sup>1</sup>G<sub>37</sub> is responsible for recognition by leucyl-tRNA synthetase**

Among the tRNA<sub>SerCAG</sub> of several Candida species, that of <i>C. cylindracea</i> 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<sub>SerCAG</sub>s possessing leucylation activity have m<sup>1</sup>G in common, while only the tRNA<sub>SerCAG</sub> of <i>C. cylindracea</i>, which possesses no leucylation activity, has A at this position.

To examine the validity of this speculation, a series of tRNA<sub>SerCAG</sub> 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<sub>SerCAG</sub> of <i>C. zeylanoides</i> synthesized by in vitro transcription was employed as a substrate, no leucylation activity was detected, even for the tRNA transcript having G37 (Figure 3A). On the other hand, as shown in Figure 3A, serylating activity exceeded 1000 pmol/A<sub>260</sub> unit. These results strongly suggested that some nucleoside modification is necessary in tRNA<sub>SerCAG</sub> for recognition by LeuRS. We thus attempted to replace the m<sup>1</sup>G37 of <i>C. zeylanoides</i> tRNA<sub>SerCAG</sub> with G (the variant is symbolized as m<sup>1</sup>G37G) or A (m<sup>1</sup>G37A), by the microsurgery method (Figure 2A and B; for details, see Materials and methods) to examine the contribution of m<sup>1</sup>G37 to leucylation and the contribution of A37 of <i>C. cylindracea</i> tRNA<sub>SerCAG</sub> to the prevention of leucylation.

When aminoacylation of m<sup>1</sup>G37A and m<sup>1</sup>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 serylating activity (Figure 3A, left-hand graph). These findings strongly indicate that the methyl group of m<sup>1</sup>G37 plays a crucial role in enhancing the leucylation activity of tRNA<sub>SerCAG</sub>.

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 deacylation of 4-acetylcytidine (ac<sub>4</sub>C) due to acid treatment of the 5′-half fragment of tRNA<sub>SerCAG</sub> (see Materials and methods). This is considered further in the Discussion.

**G<sub>33</sub> acts as a modulator of leucylation**

In addition to m<sup>1</sup>G37, another unique feature of the serine tRNA<sub>SerCAG</sub>s in these Candida species is the presence

### Table I. Kinetic parameters for mutants of tRNA<sub>SerCAG</sub> from <i>C. zeylanoides</i> with leucyl-tRNA synthetase of <i>C. zeylanoides</i>

| Strain   | <i>K</i><sub>m</i> (μM)<sup>a</sup> | <i>V</i><sub>max</i> (pmol/min) | <i>V</i><sub>max</sub>/<i>K</i><sub>m</i> (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                                          |

<sup>a</sup>The apparent <i>K</i><sub>m</i> values are given since the LeuRS used was a partially purified fraction.
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Fig. 3. Aminoacylation of tRNA variants with serine and leucine. (A) Effect of replacement of m1G37 on leucylation. Variants: C.zeylanooides tRNA^{Ser}CAG (●), C. cylindracea tRNA^{Ser}CAG (▲), m1G37A (▲), m1G37G (▲), z-G33G (○) and the transcript of C.zeylanooides 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.zeylanooides 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 (●).

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. zeylanooides and C. cylindracea. The mutated nucleotides 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 (m1G37A and m1G37G) by Donis-Keller’s method (Donis-Keller, 1980). (C) No leucylation was observed in any variant from C. cylindracea tRNA^{Ser}CAG with a mutation 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).

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 transla-

tion activities of mutated tRNA^{Ser}CAG by introducing a point mutation at this position in the tRNAs of C. zeylanooides 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. zeylanooides 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
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<sup>Ser</sup>CAG (5.0 μM) is probably due to the partial deacetylation of acetyl<sup>2</sup>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<sup>Ser</sup>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<sup>Ser</sup>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<sup>Ser</sup>CAG, despite a slight disadvantage in translation activity.

**Evidence for leucylation of C. zeylanoides tRNA<sup>Ser</sup>CAG in vivo**

At this point, we had established that the tRNA<sup>Ser</sup>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<sup>Ser</sup>CAG toward SerRS is one order of magnitude higher than that toward LeuRS, we needed to ascertain whether the tRNA<sup>Ser</sup>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-<sup>14</sup>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<sup>Ser</sup>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, acetylserylserine was observed as a major amino acid derivative in both tRNA<sup>Ser</sup>CAGs, but acetylleucine was detected only in the *C. zeylanoides* tRNA<sup>Ser</sup>CAG; the acetylserylserine and acetylleucine spots were identified as described previously (Suzuki et al.,
996). 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 acetylsersine, it was calculated that ~3% of the tRNASerCAG was attached with acetylleucine. These results were reproducible.

Digestion of purified acetyl-aminoacyl tRNA SerCAG with RNase T1 also gave only a 14C-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 acetylsersine was also found to be 3% (Figure 4D), indicating that acetylleucine is covalently attached to the tRNA SerCAG fragment with G73. It thus became clear that the tRNA SerCAG of C.zeylanoides was in fact charged with leucine by 3% of the amount of serylation of the same tRNA SerCAG in C.zeylanoides cells.

**Incorporation of leucine is dependent on the CUG codon in \textit{C.maltosa}**

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

In the \textit{URA3} gene of \textit{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 \textit{S.cerevisiae} URA3 gene was inserted downstream of a \textit{C.maltosa} promoter, 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 \textit{URA3} gene of \textit{C.maltosa} having a CTT leucine codon at the corresponding site, combined with the \textit{C.maltosa}-specific promoter, was also used as a positive control. These variant plasmids were introduced into a UR3-defective \textit{C.maltosa} strain CHU1 (his5, ade1, ura3::C-

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\caption{Complementation of \textit{C.maltosa} \textit{URA3} mutation by \textit{S.cerevisiae} \textit{URA3} variants. (A) Construction of \textit{URA3} genes transformed into \textit{C.maltosa} \textit{URA3} mutant. The 45th codon of the \textit{S.cerevisiae} \textit{URA3} gene was replaced by codon TCT or CTC, as shown. C-p indicates the promoter of the \textit{URA3} gene from \textit{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. (B) Growth curves of \textit{C.maltosa} cells harboring the constructed plasmids in SD- (left) and 5FOA- (right) liquid media. The curves indicate the growth of cells harboring pCSU–CTG (□), pCSU–CTG (○) or pCSU–TCT (■). \textit{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.

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–CTG showed normal growth, whereas no growth was observed in those harboring pCSU–TCT and pUTH18 that contained no \textit{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^{SerCAG}.

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 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 UGA 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 synthesis. It has been established that aminoacyl-tRNA gene are unable to grow on this medium. Cells estimated to range between 10^{-4} and 10^{-5} (Lin et al., 1994). Thus, 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; Schöng 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

**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’.

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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<sub>Ser</sub>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<sub>Ser</sub>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<sup>−4</sup>. Unless a proofreading mechanism exists on the ribosome, incorporation of leucine at CUG codon sites may reflect the relative ratio of tRNA<sub>Ser</sub>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 suppressor 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 programmed 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<sub>Ser</sub>CAG in *Candida* species is a chimera of tRNA<sub>Ser</sub>CAG and tRNA<sub>Leu</sub>CAG in so far as it is the substrate for both SerRS and LeuRS. The *K<sub>m</sub>* 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<sub>Ser</sub>CAG subjected to the reaction could be converted to leucyl-tRNA<sub>Ser</sub>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<sub>Ser</sub>CAG is relatively high.

In proliferating cells of *C. zeylanoides*, the leucyl-tRNA<sub>Ser</sub>CAG in the cells was estimated to be 3% of the seryl-tRNA<sub>Ser</sub>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<sub>Ser</sub>CAG between SerRS and LeuRS in the cells. Despite this competition, the distinct detection of leucylated tRNA<sub>Ser</sub>CAG in *Candida* species supports the existence of an ambiguous aminoacylation reaction toward the single tRNA<sub>Ser</sub>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<sub>Ser</sub>CAG variants by the microsurgery method led to the finding that a single methyl moiety of m<sup>1</sup>G at position 37 is involved in the leucylation process. In contrast, the tRNA<sub>Ser</sub>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 near the discriminator position, A<sub>35</sub> and G<sub>37</sub> in the anticodon loop, are responsible for the tRNA<sub>Ser</sub>CAG leucylation, which is two orders of magnitude higher than that for AUG (Gold, 1988; Kozak, 1983), leaky stop codons caused by nonsense suppressor 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 programmed 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.

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to be involved in recognition of some synthetases (Muramatsu et al., 1988). Fujiwara et al. (1994) showed that m1G at position 37 of yeast tRNA\textsuperscript{Asp} is one of the negative determinants for arginyl-tRNA synthetase.

We have also demonstrated that the nucleotide at position 33, where only tRNA\textsuperscript{SerCAG} uniquely possesses G, modulates the leucine-accepting activity. G33 may prevent tRNA\textsuperscript{SerCAG} from excessive leucylation, which might be deleterious to the cells. Leuker and Ernst (1994) failed to express S.\textit{cerevisiae} tRNA\textsuperscript{Ser}\textsuperscript{Leu} genes having anticodons UAG or CAG on a low-copy (two or three copies) plasmid in \textit{C.\textit{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 \textit{C.\textit{albicans}} tRNA\textsuperscript{SerCAG} gene existing in a single copy plasmid could be expressed in \textit{S.\textit{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\textsuperscript{SerCAG}, thereby controlling the relative seryl- to leucyl-tRNA\textsuperscript{SerCAG} 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 \textit{C.\textit{cylindracea}} tRNA\textsuperscript{SerCAG} increased the efficiency of \textit{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 \textit{C.\textit{cylindracea}} tRNA\textsuperscript{SerCAG} variants in which G33 was replaced by a pyrimidine base (e-G33U and c-G33C), we speculate that G33 influences the location and/or conformation of m1G37, accompanied by the alteration of the anticodon loop structure, decreasing the affinity of LeuRS toward tRNA\textsuperscript{SerCAG}.

It has been generally considered that reconstructed tRNAs 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 \textit{C.\textit{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 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 m1G37 is directly recognized by LeuRS. In the case of \textit{C.\textit{zeylanoides}}, the anticodon loop distorted by G33 decreases the affinity toward LeuRS, judging from the observation that G33 increased the \textit{K}_\textit{m} value for leucylation approximately 4- to 5-fold in comparison with that with pyrimidine bases at the position (Figure 6B). In \textit{C.\textit{cylindracea}}, m1G is replaced by A, which means that the tRNA has lost the two major determinants for LeuRS, m1G and the discriminator base (Figure 6C). Consequently, LeuRS is unable to recognize tRNA\textsuperscript{SerCAG} 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 does this interaction between LeuRS and tRNA\textsuperscript{SerCAG} evolve? \textit{Candida} species utilizing CUG as serine can be classified into two distinct groups: group 1 contains the species that have tRNA\textsuperscript{SerCAG} with leucylation activity, and includes \textit{C.\textit{zeylanoides}}, \textit{C.\textit{maltosa}} and others (see Figure 6B); group 2, which is represented solely by \textit{C.\textit{cylindracea}}, contains species that have tRNA\textsuperscript{SerCAG} 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\textsuperscript{SerCAGs} in group 1 and its isoacceptor tRNAs for codon UCG is higher than that between the tRNA\textsuperscript{SerCAG} from \textit{C.\textit{cylindracea}} and its isoacceptor (Ueda et al., 1994). Second, \textit{C.\textit{cylindracea}} (group 2) possesses high copy numbers of the tRNA\textsuperscript{SerCAG} 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\textsuperscript{SerCAG} genes (Santos et al., 1993; Sugiyma et al., 1995; T.Suzuki, personal observations). Third, the codon CUG is utilized as a major serine codon on several genes in \textit{C.\textit{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; Sugiyma 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 tRNASerCAG 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→m1G (group 1)→unmodified A (group 2). This hypothesis raises two questions: (i) why did the mutation changing modified A to m1G, 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-housekeeping 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**

[^1C]Acetic anhydride (185 MBq/mmol) was purchased from American Radiolabeled Chemicals. Uniformly labeled[^1C]serine (5.99 MBq/ mmol) and leucine (11.5 MBq/mmol) were from Amersham. 5-fluoro-orotic 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 Genet 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* (ICM1627) was from the Japan Collection of Microorganisms (JCM, ROKEN, Wako, Saitama, Japan). *Candida maltosa* strain CHU1 (his5, ade1, ura3::ADE1/ ura3::ADE1) was kindly provided by Drs M.Takagi and R.Ohtomo of the Department of Agricultural Chemistry, The University of Tokyo. Complete minimal 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, 50 μg/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/ml 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 μg/ml 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 mutational at the 45th codon in the reading frame of the *S.cerevisiae* URA3 gene, pUC-CSU (Sugiyama et al., 1995) was first cut by EcoRI 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 HindIII fragment of pUC–CSU–TCT consisting of the *C.maltosa* promoter and the *Saccharomyces cerevisiae* URA3 coding region where the 45th position was mutated from CTG to TCT was inserted into the small Smal site of pUTH8 (Okukama et al., 1993) to construct pCSU–TCT. Plasmid pTH18 containing an autonomously replicating sequence of *C.maltosa* (Takagi et al., 1986) and C-HISS (Hikiji et al., 1989) were used as expression vectors developed from pUC18 by Okukama et al. (1993).

Other plasmids—pCSU–CTG, denoted as pCSU in the literature (Sugiyama et al., 1995), consisting of the *C.maltosa* promoter and *Saccharomyces 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 *Saccharomyces cerevisiae* URA3 coding region where the 45th position was mutated from CTG to TCT, 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 of the reaction mixture containing 100 mM Tris–HCl (pH 7.5), 10 mM KCl, 50 mM NaCl, 15 mM MgCl2, 10 mM DTT, 2 mM ATP, 15 μM[^1C]serine (5.99 GBq/mmol) or 15 μM[^3]Uleucine (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 ml ice-cold 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 electroporated cells were spread on a SD-plate containing uracil and incubated at 30°C.

**In vitro aminoclay assay**

Seryl- or leucyl-tRNA synthetases were partially purified from *C.zeylanoides* as described previously (Suzuki et al., 1994), both of the activities having been completely separated by column chromatographies. The aminoclay 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 MgCl2, 10 mM DTT, 2 mM ATP, 15 μM[^1C]serine (5.99 GBq/mmol) or 15 μM[^3]Uleucine (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 [^1C]amino acids. The radioactivity remaining on the filter was then measured by a liquid scintillation counter.

**Large-scale purification of tRNA[^54]CAGs from *C.zeylanoides* and *C.cylindracea***

*Candida cylindracea* cells (3.1 kg) were treated with phenol, from which 150,000 μg of tRNA was extracted. Eighty thousand μg of tRNA mixture were obtained by DEAE–cellulose chromatography with stepwise elution, which was then applied onto a DEAE–Sephadex A-50 column (60×100 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 MgCl2. The fraction rich in tRNA 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 ASuG units of purified tRNA were finally obtained.

One hundred and fifty thousand ASuG units of tRNA from C. zeylanoides cells (3.7 g) 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 MgCl2. About 300 ASuG units of C. zeylanoides tRNA 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 MgCl2, 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 (Ohyma et al., 1985, 1986). Limited digestion of 4 mg purified tRNA 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 MgCl2, 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 after the addition of 600 units of RNase H (Takara Shuzo). About 60 μg of the cleaved 3′-half fragment was then purified 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 T1 and purified using 10% PAGE containing 7 M urea. About 25 μg of each of the two variants from C. zeylanoides mutated at position 37—mG37A and mG37G—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 variants in the cells**

Identification of aminoacyl-tRNA taken 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 digest yeast tRNA, we designed antibacterial interference with bacterial growth. The tRNA was purified using 10% PAGE containing 7 M urea. Two hundred micrograms of each of the four variants tRNA purified by 12% PAGE was annealed and ligated with the 3′-half fragment digested by RNase T1 and phosphorylated using 10% PAGE containing 7 M urea.

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**Constructions of tRNA variants mutated at position 37**

To obtain the mutation of mG37 of C. zeylanoides tRNA, we selected the repressive 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′GmCmGmCmAmAmJmGmGmAmAmCdGdTgD– CAmUmCnCmAmUm3′), possessing a cleavage site between positions 37 and 38 of C. zeylanoides tRNA. Two hundred micrograms of purified tRNA 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 DTT, 0.1 M NaCl, 0.1 M NaOAc and 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 then purified using 10% PAGE containing 7 M urea.

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