Non-universal decoding of the leucine codon CUG in several *Candida* species

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**Abstract**

It has been reported that CUG, a universal leucine codon, is read as serine in an asporogenetic yeast, *Candida cylindracea*. The distribution of this non-universal genetic code in various yeast species was studied using an *in vitro* translation assay system with a synthetic messenger RNA containing CUG codons in-frame. It was found that CUG is used as a serine codon in six out of the fourteen species examined, while it is used for leucine in the remaining eight. The tRNA species responsible for the translation of codon CUG as serine was detected in all the six species in which CUG is translated as serine. The grouping according to the CUG codon assignments in these yeast species shows a good correlation with physiological classification by the chain lengths of the isoprenoid moiety of ubiquinone and the cell-wall sugar contained in the yeasts. The six *Candida* species examined in which CUG is used as serine belong to one distinct group in Hemiascomycetes.

**Introduction**

Since 1979, when a non-universal genetic code was first found in human mitochondria (1), genetic code variations have been reported not only for mitochondria but also for nuclear genes (2). The non-universal codons in the nuclear genes had all been related to termination codons. CUG was believed to be a universal leucine codon in Ascomycetes, such as filamentous fungi (Euascomycetes) and yeasts (Hemiascomycetes), as in other organisms. In 1989, however, Kawaguchi *et al.* (3) found that CUG codes for serine instead of leucine in an asporogenetic yeast, *Candida cylindracea* (4). Amongst the nuclear genetic codes, this is a unique instance in which the assignment of an amino acid codon deviates from the universal genetic code. The present study was undertaken to determine the distribution of the non-universal serine codon CUG in various yeasts.

Translation of a synthetic mRNA having CUG codons in-frame in cell-free extracts (the S30 fractions) from fourteen yeast species indicates that in six species, including *C. cylindracea*, codon CUG is translated as serine, while in the other eight species CUG is read as leucine. Serine tRNA with the anticodon sequence CAG, which is complementary to codon CUG, was found in all the six species in which CUG is used as a serine codon. From physiological classification by ubiquinone and cell-wall sugar in these yeasts, it was deduced that these six *Candida* species belong to a distinct group in Hemiascomycetes.

**Materials and Methods**

**Yeast species**

Yeast strains used were obtained from the Japan Collection of Microorganisms (JCM, RIKEN, Hirosawa, Wako, Saitama 351-01, Japan) and from the American Type Culture Collection (ATCC) (Table 1). *Candida albicans* (strain C9) was from Drs M.Homma and K.Tanaka of Nagoya University, School of Medicine.

**Cell culture**

Each species was cultured aerobically in a rotary air-shaker (180–200 rpm) at 25°C, in a medium containing, per liter, 7 g yeast extract (Difco), 3 g malt extract (Difco), 20 g peptone (Difco) and 20 g dextrose. Cells were harvested between the early log phase and mid-log phase.

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**D14890, D12941, D14891, D14940**
Preparation of cell-free extract (S30 fraction)

The preparation of the S30 fraction was essentially the same as that for *Saccharomyces cerevisiae* (5–8), with the modifications described below. Cells (~ 2 g) were suspended in 5 ml of extraction buffer (8.5% mannitol, 30 mM Hepes-KOH(pH7.4), 3 mM Mg(OAc)2, 100 mM KOAc, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride). The suspension was transferred to a 125 ml screw-cap glass bottle with 8 g of acid-washed glass beads (0.5 mm-diameter), and shaken vigorously up and down by hand in a vertical motion for 10–15 minutes with occasional cooling on ice for 20 seconds. The extract was pooled, and the glass beads were washed with 3 ml of the extraction buffer. The combined extract (~ 8 ml) was centrifuged and used as a template for transcription.

Hind III sites of plasmid vector Bluescript KS (Strategene) downstream from the *Hind* III promoter. The resulting plasmid was linearized with *Kpn* I, and the double-stranded DNA fragment was inserted between the *Kpn* I and *Xba* I sites (Fig. 1), were synthesized by a Gene-Assembler Plus DNA Synthesizer (Pharmacia). Restriction enzyme sites for *Kpn* I and for *Hind* III were included at each 5'-end of the single-stranded fragments for cloning.

Preparation of T3 RNA polymerase-promoted transcript

DNA was synthesized, with the construction of the 5'-upstream region of the initiation codon having a sequence similar to that of native mRNAs of *S. cerevisiae* (9), so that the derived mRNA could be translated efficiently *in vitro*. Two single-stranded DNA fragments, strand-1 (64 nucleotides) and strand-2 (60 nucleotides), having 13 complementary bases at the 3'-ends, were synthesized with DNA polymerase and the lacking complementary strands were then synthesized with DNA polymerase. The resulting plasmid was linearized with *Hind* III and used as a template for transcription.

In vitro translation assay using synthetic mRNA

Cell-free translation was carried out in 50 μl of reaction mixture, containing 25 mM Hepes-KOH(pH7.4), 150 mM KOAc, 2.5 mM Mg(OAc)2, 0.5 mM ATP, 0.1 mM GTP, 2 μg creatine of the supernatant from the top was passed through a Sephadex G-25 (medium) column (22×1.5 cm) in the absence of mannitol to remove low-molecular weight components. After measurement of A260, the main fraction (~ 4 ml) was concentrated by Centricon C-10 (10,000 MW cut-off, Amicon) so as to obtain the S30 fraction of 5−10 μg of protein per one μl. The S30 fraction so obtained was divided into 200 μl aliquots, rapidly frozen with liquid nitrogen, and stored at −70°C until use. The S30 fraction was active for at least a month.

### Table 1. Some properties of Ascomycetes

<table>
<thead>
<tr>
<th>Species</th>
<th>Genomes G+C %</th>
<th>Ubiquinone type</th>
<th>Gelactose in cell wall</th>
<th>Amino acid assignment of codon CUG</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em> (ATCC 24830)</td>
<td>40</td>
<td>O8</td>
<td>-</td>
<td>leucine</td>
<td>1, 2</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>45</td>
<td>O7</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Phaffa membraneiicae</td>
<td>40</td>
<td>O7</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candida rugosa</td>
<td>50</td>
<td>O7</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candida hansenii</td>
<td>44</td>
<td>O9</td>
<td>-</td>
<td>serine</td>
<td></td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>33</td>
<td>O9</td>
<td>-</td>
<td>serine</td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>38</td>
<td>O9</td>
<td>-</td>
<td>serine</td>
<td></td>
</tr>
<tr>
<td>Candida zeylanica (JCM 1593)</td>
<td>50</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candida rugosa</td>
<td>50</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candida parapsilosis (JCM 1785)</td>
<td>50</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candida tropicalis (JCM 1627)</td>
<td>50</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candida albicans (C9)</td>
<td>38</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Candidaandida (ATCC 1111)</td>
<td>33</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
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</tr>
<tr>
<td>Candida parapsilosis (JCM 1785)</td>
<td>50</td>
<td>O9</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
<tr>
<td>Cryptococcus humicolous (JCM 1457)</td>
<td>63</td>
<td>O10</td>
<td>-</td>
<td>leucine</td>
<td></td>
</tr>
</tbody>
</table>

1 | Used in this study.
2 | ATCC: American Type Culture Collection.
3 | From Barnett et al. (17); Nakase, T. (unpublished) for asterisk.
4 | Barnett et al. (17); Gorin and Spencer (18); Nakase, T. (unpublished) for asterisk.
5 | a: this study; b: from Kawagushi et al. (3) and Yokogawa et al. (4); c: from Kalb et al. (30); D: from Germann et al. (31).
phosphokinase (Sigma), 25 mM creatine phosphate (Sigma), 2 mM glucose-6-phosphate (Sigma), 2 mM DTT, 1.0 mM CaCl<sub>2</sub>, and the S30 fraction (125 μg protein).

The endogenous mRNA in the S30 fraction was removed in two steps; first by precipitation at 20°C for 10 min. to perform run-off translation of polysome, and then by digestion with micrococcal nuclease (0.4 u/μl) (Pharmacia) for 20 min. at 20°C, followed by the addition of EGTA (1.6 mM) to stop the nuclease digestion.

Ten μM each of non-labelled methionine, non-labelled leucine (or [3H]leucine, 1285.2 TBq/mmol) and non-labelled serine (or [3H]serine, 1398.6 TBq/mmol), and 100 μg of synthesized mRNA were added and incubated at 20°C for 2 hr.

Twelve μl aliquots were taken out, and after treatment with 1 N NaOH and 10% (w/v) trichloroacetic acid (TCA), the TCA-insoluble radioactivity was measured by a liquid scintillation counter.

**RESULTS**

*In vitro* translation assay for the amino acid assignment of codon CUG

To determine whether codon CUG is translated as serine or leucine in various yeast species, an *in vitro* translation system was constructed by the S30 fractions from these species and a synthetic mRNA having CUG codons in-frame which had been transcribed from a synthetic DNA by T3 RNA polymerase (Fig. 1). To test the reliability of the *in vitro* translation assay, the S30 fraction from Saccharomyces cerevisiae (CUG = leucine) and that from Candida cylindracea (CUG = serine) were incubated with the synthetic mRNA with [3H]leucine or [3H]serine. With the
Figure 4. *In vitro* translation of synthetic mRNA with the S30 fractions from seven *Candida* species. For legends, see Fig. 2.

Figure 5. Nucleotide sequences of serine tRNA with anticodon CAG, from six *Candida* species: *C. parapsilosis, C. zeylanoides, C. albicans, C. cylindracea* (4), *C. rugosa* and *C. melibiosica*.

S30 fraction from *S. cerevisiae* a time-dependent incorporation of [³H]leucine occurred, while almost no incorporation of [³H]serine was observed (Fig. 2a). With the S30 fraction from *C. cylindracea*, the situation was reversed, i.e., [³H]serine was incorporated, and [³H]leucine was not (Fig. 2b). These profiles were observed with magnesium concentrations between 2.5 mM and 4.0 mM, the maximum incorporation being with 2.5 mM. When no mRNA was added to the assay system, no appreciable incorporation of [³H]leucine or [³H]serine was detected, so that a possible contribution of endogenous mRNA in the S30 fraction could be ruled out. Thus, with this present *in vitro* system it is possible to determine whether codon CUG is for leucine or serine in various yeast species.

A predominant incorporation of [³H]serine was observed with the S30 fractions from *Candida parapsilosis, Candida zeylanoides, Candida albicans, Candida rugosa* and *Candida*
Serine tRNA with anticodon sequence CAG complementary to codon CUG

Serine isoacceptor tRNAs were isolated from *C. parapsilosis*, *C. zeylanoides*, *C. albicans* (14, 15), *C. cylindracea* (4), *C. rugosa* and *C. melibiosica*. The genes were amplified by PCR using primers indicated by solid lines and sequenced. Splicing sites are shown by arrows.

Figure 6. Nucleotide sequences of tRNA<sup>33°CAG</sup> genes from six *Candida* species: *C. parapsilosis*, *C. zeylanoides*, *C. albicans* (14, 15), *C. cylindracea* (4), *C. rugosa* and *C. melibiosica*. The genes were amplified by PCR using primers indicated by solid lines and sequenced. Splicing sites are shown by arrows.

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The amino acid-charging activities of these tRNAs were examined by seryl-tRNA and leucyl-tRNA synthetases partially purified from *S. cerevisiae*. All the tRNAs having anticodon CAG accepted serine to a considerable extent (more than 1,000 pmoles/A<sub>260</sub>), without appreciable acceptance of leucine (less than 5 pmoles/A<sub>260</sub>). tRNA<sup>33°CAG</sup> from *C. cylindracea* was a good substrate for seryl-tRNA synthetase, in spite of having U at the discriminator position where G is located in the other five tRNAs and eukaryotic serine tRNAs reported so far. As described above, it is clear that these tRNAs from *Candida* species have enough tRNA identity elements as serine tRNAs, though we have not determined enzymatic parameters of these tRNAs for seryl-tRNA synthetases. Thus it can be concluded that the tRNA with anticodon CAG from these *Candida* species is a serine tRNA corresponding to codon CUG.

Distribution of non-universal CUG codons in Hemi-ascomycetes

By comparing the amino acid assignments with the chain length of the isoprenoid moiety of ubiquitine (17) and the composition of cell-wall sugar (18), the yeast species could be classified into three groups. The first group is one in which CUG codes for leucine, the ubiquinone type is either Q-9 or Q-10, and the cell wall contains galactose (+Gal) (18). —Group I; *Zygoascus helenicus*, *C. magnolidae*, *C. azyma*, *Yarrowia lipolytica*, *Candida diversa*, *Candida rugopelliculosa* and *Trichosporon cutaneum* (Basidiomycetes) in addition to *S. cerevisiae* (Fig. 4).

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DISCUSSION

We have established an *in vitro* translation assay system for amino acid assignments of codons in Hemiascomycetes. The genetic codes in certain organisms are usually determined by comparing the amino acid sequence of a protein and the nucleotide sequence of its gene DNA. However, the purification of a protein and determination of its amino acid sequence are often laborious tasks. There is also the possibility of RNA editing (19). Thus, assuming that these tRNA genes are of the same phylogenetically closer to Group I than the others (unpublished observations). All tRNAs except that from C. albicans cell-free extracts using α-, β-globin and Bromo mosaic virus coat protein mRNAs, and yet translation of codon CUG by tRNASer-CAG has not been reported. The nucleotide sequence of tRNASer-CAG in the six Candida species (Group II) is very similar; the sequence homologies of the tRNA from C. parapsilosis, C. albicans and C. zeylanoides are more than 90%, whereas those of C. melibiosica, C. cylindracea and C. rugosa are about 70–80% (see Fig. 5). These similarities correlate to the phylogenetic relationship predicted from their 5S rRNA sequences (our unpublished observation). All tRNAs except that from C. cylindracea have G at position 73 (discriminator nucleotide), which is one of the characteristics of serine tRNAs, whereas they show a feature of leucine tRNA that m'G is located at the 3' adjacent position of the anticodon. However, it is notable that G23 is present in all the species. This unique feature and the phylogenetic relationship of these tRNAs, deduced from their sequence similarities would imply that tRNASer-CAG is derived from a single ancestor.

We have proposed that tRNASer-CAG is derived from an intron-containing serine tRNA based on the nucleotide sequence of tRNASer-CAG of C. cylindracea. Here the sequences of tRNASer-CAG genes of the Candida species in which CUG is translated as serine are determined. Among these six tRNA genes, the tRNA genes of two species, C. cylindracea and C. melibiosica, are interrupted by introns, while the other four tRNA genes lack intron. According to the similarities of 5S rRNA sequences it is suggested that C. cylindracea and C. melibiosica, which have the intron-containing tRNA genes, are phylogenetically closer to Group I than the others (unpublished data). Thus, assuming that these tRNA genes are of the same origin, it is likely that intron have disappeared during the evolution of the tRNASer-CAG.

According to the codon capture theory (2, 21), production of an unassigned codon is the first step for neutral genetic code change. Unassigned codons may be produced by directional mutation pressure (AT- or GC-pressure), which primarily determines the synonymous codon choice, especially in extremely high or low G+C bacteria (2). There is also a strong correlation between the relative amount of isoacceptor tRNA for an amino acid and the usage of the corresponding synonymous codons (22, 23). In its extreme form, a codon becomes unassigned by removal of the codon and the corresponding tRNA, as exemplified by CGG in *Mycoplasma capricolum* (G+C: 25%) (24) or by AGA and AUA in *Micrococcus luteus* (G+C: 74%) (22).

One remarkable feature of yeasts resides in the wide range of their genomic G+C content (e.g., 27.1% for *Hanseniaspora valbyensis* and 63.4% for *Rhodotorula fujisansensis*). The GC contents of the genomes of these yeasts (Table 1) are highly diversified, even in closely related species. For example, C. albicans is very closely related to C. zeylanoides based on the sequence homology of 5S rRNA (94%); unpublished), and yet the G+C contents are 36% and 56%, respectively. This suggests that the direction of the mutation pressure has rapidly changed within a very short time, even in closely related species in yeasts. The range of genomic G+C-content in Group II used in this study is 34% to 63%, while that in Group I is 43% to 60%. It is thus possible that CUG became unassigned during the emergence of Group II yeasts under strong AT-pressure by converting to another A+T-rich synonymous codon, accompanied by a loss of the corresponding tRNA. A relaxation of AT-pressure, i.e., an increase in GC-pressure, could have brought the reappearance of CUG as serine upon emergence of a tRNA translating the codon as serine.

Naturally, such an unassigned codon capture by the serine tRNA is acceptable at any dispensable site in a gene. For example, a dispensable UUG leucine can mutate to CUG serine, etc. However, in one of the lipase genes of C. cylindracea, CUG is used as a serine codon in the catalytic center (25, 26), showing that a serine codon, even at an important site, can change to CUG by a nearly neutral process. The change of the universal serine codon UCN (N: U, C, A, or G) or AGY (Y: U or C) to CUG cannot occur by a single mutation — it must pass through an intermediate codon such as UUR (R: G or A) or CCN (Pro). The gene having such an intermediate codon becomes functionally inferior or inactive if it occurs at an important site. However, most of the eukaryotic genes exist multiply in the genome and therefore deleterious mutation in one of the genes would not affect the viability of the cell. The ‘inferior’ gene would become a pseudogene at one time, but at another time it would be revived by a second mutation at the site originally occupied by a serine codon such as UCG [e.g., UCG (Ser) — UUG (Leu) — CUG (Ser)]. The latter process would not be impossible if the creation of tRNA has preceded the change of UUG (Leu) to CUG (Ser). Indeed, there would be no other way of near-neutral generation of CUG serine codons at important sites.

Naturally, the higher the GC-pressure, the higher the usage of codon CUG, regardless of its assignment, serine or leucine (3, 27). For example, CUG is a rare leucine codon in *S. cerevisiae* (G+C: 40%) (28) and is also a rare codon (for serine) in *C. albicans* (G+C: 34%) (29), while it is a predominant serine codon in *C. cylindracea* (G+C: 63%) in which several copies of the genes for tRNASer-CAG have been detected (unpublished).

Propagation of codon CUG for serine in Group II yeasts is also possible through duplication of the genes containing CUG after its reassignment.
We infer that all of the serine CUG codons in Group II yeasts arose through individual mutations of various other codons, including universal serine codons, by a nearly neutral process as described above. Indispensable serine sites in a protein gene, only a single copy of which exists in an organism, would not have been directly involved in this type of reassignment, because a mutation, if it occurred at these sites, would be removed by negative selection. Therefore, such code changes could occur only when multiples of the same gene exist.

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