



Review/Revue

The genetic code of the fungal CTG clade

Code génétique du clade fongique « CTG »

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ABSTRACT

Genetic code alterations discovered over the last 40 years in bacteria and eukaryotes invalidate the hypothesis that the code is universal and frozen. Mitochondria of various yeast species translate the UGA stop codon as tryptophan (Trp) and leucine (Leu) CUN codons (N = any nucleotide) as threonine (Thr) and fungal CTG clade species reassigned Leu CUG codons to serine and translate them ambiguously in their cytoplasm. This unique sense-to-sense genetic code alteration is mediated by a Ser-tRNA containing a Leu 5'-CAG-3' anticodon (ser-tRNA_{CAG}), which is recognized and charged with Ser (~97%) by the seryl-tRNA synthetase (SerRS) and with Leu (~3%) by the leucyl-tRNA synthetase (LeuRS). This unusual tRNA appeared 272 ± 25 million years ago and had a profound impact on the evolution of the CTG clade species. Here, we review the most recent results and concepts arising from the study of this codon reassignment and we highlight how its study is changing our views of the evolution of the genetic code.

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R É S U M É

Les altérations du code génétique, découvertes durant les 40 dernières années chez les bactéries et les eukaryotes, invalident l'hypothèse selon laquelle le code est universel et gelé. Chez les levures, les mitochondries d'espèces variées traduisent le codon stop UGA en tryptophane (Trp) et les codons leucine (Leu) CUN (N = l'un des 4 nucléotides) en thréonine (Thr). Les espèces fongiques du clade « CTG » ont réassigné le codon Leu CUG en serine et les traduisent sans ambiguïté dans leurs cytoplasmes. Cette altération unique sens-sens du code génétique est due à un Ser-tRNA contenant un anticodon Leu 5'-CAG-3' (ser-tRNA_{CAG}) qui est reconnu et chargé en Ser (~97%) par la seryl-tRNA synthétase (SerRS) et en Leu (~3%) par le leucyl-tRNA synthétase (LeuRS). Ce tRNA inhabituel est apparu il y a 272 ± 25 millions d'années et a eu un impact profond sur l'évolution des espèces du clade « CTG ». Ici, nous présentons une revue des résultats et concepts les plus récents issus de l'étude de cette réassignation de codon et nous soulignons comment cette étude est en train de changer nos idées sur l'évolution du code génétique.

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1. Introduction

The reassignment of the CUG codon from Leu to Ser occurs in at least 75 *Candida* species and in *Pichia*

stipitis, *Debaryomyces hansenii* and *Lodderomyces elongisporus* [1,2], which form the so-called CTG clade [3]. *C. zeylanoides*, *C. dubliniensis*, *C. tropicalis*, *C. guilliermondii*, *C. albicans* and many others translate CUGs ambiguously (Ser + Leu) and their proteins contain ~97% of Ser and ~3% of Leu at CUG positions, while *C. cylindracea* decodes CUGs as Ser only [4,5]. The change of identity of the CUG codon is

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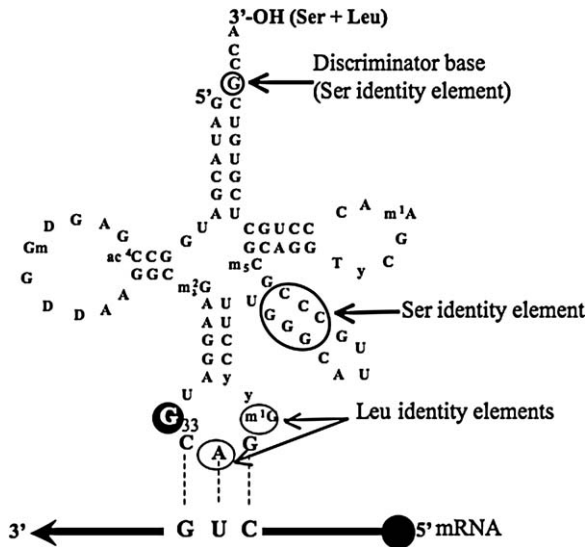


Fig. 1. *C. albicans* tRNA_{CAG}^{Ser}. The *C. albicans* tRNA_{CAG}^{Ser} is a hybrid tRNA with identity elements for both leucyl and seryl tRNA synthetases (LeuRS and SerRS). Its anticodon arm is characteristic of leucine tRNAs and contains two leucylation identity elements, namely A₃₅ and m¹G₃₇, whereas the acceptor stem and the variable arm are characteristic of serine tRNAs. The discriminator base (G₇₃) and the G3C3 run of the extra-arm are characteristic of serine tRNAs [25].

mediated by a novel tRNA_{CAG}^{Ser} (Fig. 1), whose peculiar double recognition by SerRS and LeuRS leads to synthesis of two different aminoacyl-tRNAs, namely a Ser-tRNA_{CAG}^{Ser} and a Leu-tRNA_{CAG}^{Ser}, which compete for CUGs at the ribosome A-site during mRNA translation [6]. The mischarged leu-tRNA_{CAG}^{Ser} is neither edited by the LeuRS nor discriminated by the translation elongation factor 1 (eEF1A) and, consequently, Leu and Ser are incorporated into the proteome at CUG positions *in vivo* [7]. Apart from this, the tRNA_{CAG}^{Ser} contains a unique guanosine at position 33 (G₃₃), which is located adjacent to the 5' base of the 5'-CAG-3' anticodon (Fig. 1), a position occupied by a critical uridine (U₃₃) required for the U-turn of the anticodon-loop [8]. G₃₃ induces a long-range distortion of the top of the anticodon stem of the tRNA_{CAG}^{Ser} and lowers its leucylation and decoding efficiencies [9,10]. Finally, the discriminator base (N₇₃) of the tRNA_{CAG}^{Ser} is guanosine (G₇₃) which is an identity element of Ser tRNAs (Fig. 1). This raises the intriguing question of how the LeuRS recognizes this tRNA because a single A₇₃ to G₇₃ mutation in *S. cerevisiae* tRNA^{Leu} converts its identity from Leu to Ser [11]. In other words, the *C. albicans* LeuRS should not recognize the tRNA_{CAG}^{Ser} as the tRNA identity elements are conserved between these two yeasts. But the tRNA_{CAG}^{Ser} contains A₃₅ and m¹G₃₇ in its anticodon-loop, which are directly recognized by the LeuRS (Fig. 2) [11], suggesting that these two identity elements are sufficient for proper recognition of the tRNA_{CAG}^{Ser} by the LeuRS. This question needs to be clarified at the structural and enzymatic levels using X-ray crystallography of the LeuRS-tRNA_{CAG}^{Ser} complex and aminoacylation assays.

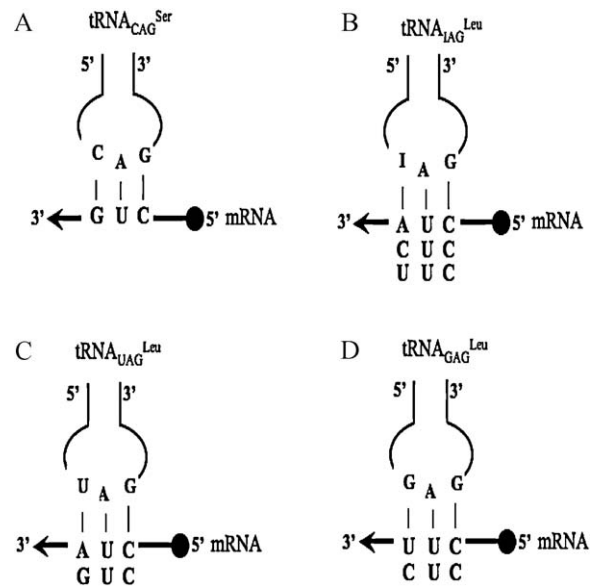


Fig. 2. Decoding of the CUN codon family in *C. albicans* and *S. cerevisiae*. A, B. (In *C. albicans* CUG codons are decoded by a mutant serine tRNA_{CAG}^{Ser} while the CUU, CUC and CUA codons are decoded by a tRNA_{IAG}^{Leu}). C, D. In *S. cerevisiae*, a tRNA_{UAG}^{Leu} decodes CUA and CUG codons and a tRNA_{GAG}^{Leu} decodes the CUU and CUC codons.

2. The reassignment of CUG codons in the fungal CTG clade

The reassignment of CUGs in the fungal CTG clade strongly supports the “*Ambiguous Intermediate Theory*” of the genetic code, which postulates that codons are reassigned through ambiguous decoding [12–14]. Interestingly, such codon ambiguity erased 98% of the CUGs of the CTG clade ancestor through mutation to the frequently used Leu UUA and UUG codons. The “new” CUG codons present in extant species of the CTG clade evolved recently from codons coding for Ser or amino acids with similar chemical properties thus showing that the CUGs of the CTG clade species are phylogenetically unrelated to the CUGs of the other fungal species [3,15]. Comparative genomics and molecular phylogeny studies showed that the tRNA_{CAG}^{Ser} appeared 272 ± 25 million years ago, prior to the divergence between the *Saccharomyces* and *Candida* genera (170 ± 27 million years ago), via insertion of an adenosine in the anticodon of a serine tRNA_{CAG}^{Ser} gene [15]. However, the evolutionary pathway of CUG reassignment has not yet been reconstructed and one does not fully understand how CUGs changed their identity. In particular, selective advantages produced by CUG ambiguity, which would have been critical for its initial selection and posterior CUG reassignment, are poorly understood. In any case, the appearance of the mutant tRNA_{CAG}^{Ser} created a unique cellular situation where CUGs were decoded by two distinct tRNAs, namely the new mutant ser-tRNA_{CAG}^{Ser} and the standard fungal leu-tRNA_{CAG}^{Leu}. In the CTG clade ancestor, these two tRNAs competed for approximately 100 My for CUG codons and introduced significant ambiguity at CUG

positions [15,16]. The mutant tRNA_{CAG}^{Ser} was lost in the lineage that originated *Saccharomyces* spp., maintaining CUG identity for Leu, and was selected in the lineage that originated the CTG clade, thus reassigning CUG identity from Leu to Ser [15].

3. Decoding of CUN codons in *Candida* spp.

The reassignment of the CUG codon from Leu to Ser affected the decoding properties of Leu CUN codons [17]. In CTG clade species, CUGs are decoded by the tRNA_{CAG}^{Ser}, while CUA, CUU and CUC codons are decoded by a single tRNA with a 5'-IAG-3' anticodon (tRNA_{IAG}^{Leu}) (Fig. 2). A single tRNA_{IAG}^{Leu} decodes three different codons as inosine (I) at the first anticodon position base pairs with A, C or U at the third codon position through extended wobble, but the strength of codon-anticodon interactions is variable. The CUU codon is cognate for this tRNA and interacts strongly with it. A similar situation occurs for the 5'-IAG-3' interaction with the CUC codon however the 5'-IAG-3'-CUA interaction is weak and repressed CUA usage [15]. Conversely, in *S. cerevisiae* the CUN codon family is decoded by two tRNAs: a tRNA_{UAG}^{Leu} decodes CUA and CUG codons and a tRNA_{GAG}^{Leu} decodes CUC and CUU codons. In *S. pombe* and in many other fungi the CUN codon family is decoded by three different tRNAs: a tRNA_{GAG}^{Leu} decodes CUC and CUU codons, a tRNA_{UAG}^{Leu} decodes CUA and a tRNA_{CAG}^{Leu} decodes CUG codons. Therefore, the appearance of the novel tRNA_{CAG}^{Ser} influenced CUN decoding and the evolution of Leu tRNAs in the CTG clade species [3,15].

Natural mistranslation of CUGs (~3% Leu and ~97% Ser) in extant fungal species of the CTG clade suggests that it may play a role in the biology of these fungi because it elevates the translational error rate by ~3000-fold (basal error is 10⁻⁵) [6,18]. Such a high error rate is highly detrimental in non-adapted species and should have been eliminated by natural selection. Recent studies provide further indirect evidence for a role of CUG ambiguity as engineered *C. albicans* strains tolerate up to 28% of Leu

misincorporation at CUG codons (28,000-fold increase over typical translational error). This does not have visible effects on growth rate, but rather impacts on genome stability and phenotypic diversity [4,19]. Indeed, hyper-mistranslator strains of *C. albicans* display a diverse array of forms, up regulate lipase and proteinase secretion and flocculate [19]. These cells also generate population heterogeneity, produce aerial hyphae and have high frequency of white-opaque switching and often form long filaments [19]. Interestingly, morphological variation, yeast-hypha transition, proteinase and lipase secretion and adhesins, are important *Candida* spp. virulence traits [20], suggesting that CUG ambiguity may be relevant for adaptation.

4. The proteomic relevance of CUG reassignment and ambiguity

The global impact of CUG ambiguity is better comprehended if one analyses the genome distribution of CUGs. The *C. albicans* genome encodes 26,148 CUGs distributed over 66% of its genes at a frequency of 1 to 38 CUGs per gene. The majority of the genes contain between 1 to 5 CUGs (57.7%) (Fig. 3A). Considering the insertion of Ser and Leu at each of these CUG positions the number of protein molecules that can be produced from *C. albicans* genes is given by the expression 2ⁿ, where n = number of CUGs per gene (Fig. 4 for the case n = 3). A genome-wide analysis of CUGs showed that *C. albicans* has the capacity to synthesize 283 billion different protein molecules from its 6438 genes (Fig. 3B) [4]. In other words, mistranslation of each *C. albicans* gene produces an array of protein molecules containing Leu or Ser at CUG positions, creating heterogeneous populations of protein molecules differing in Leu or Ser at CUG positions [4]. The biological implications of this phenomenon are profound as each *C. albicans* cell contains a unique combination of protein molecules (the proteome is statistical) and, therefore, the probability of finding two identical cells in a population is extremely small, even if cells are grown under the same conditions and express the

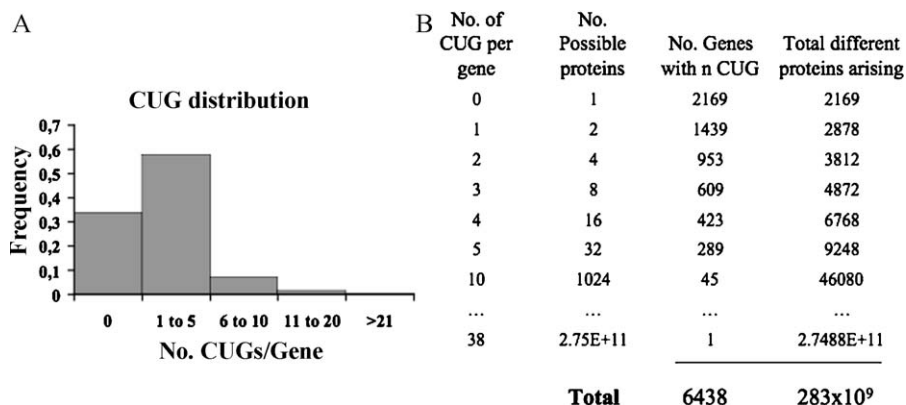


Fig. 3. Distribution of CUG codons in *C. albicans* genes. A. One third of the *C. albicans* genes do not contain CUG codons. The majority (57.7%) contains between 1 to 5 CUGs and 7.1% have between 6 and 10. Only a small fraction of genes have more than 10 CUG codons. B. Ambiguous CUG decoding expands the *C. albicans* proteome exponentially. The total theoretical number of combinatorial proteins encoded by the *C. albicans* genome is 283 billion, considering insertion of Ser (100%) or Leu (100%) at CUGs [6].

Ambiguity level		3% Leu 97% Ser	5% Leu 95% Ser	28% Leu 72% Ser
Type of molecules	3 Ser	91.4%	85.7%	37.3%
	2 Ser / 1 Leu	2.7%	4.5%	14.5%
		2.7%	4.5%	14.5%
		2.7%	4.5%	14.5%
	1 Ser / 2 Leu	0.085%	0.24%	5.6%
		0.085%	0.24%	5.6%
		0.085%	0.24%	5.6%
	3 Leu	0.00026%	0.013%	2.2%

Fig. 4. Production of combinatorial proteins in *C. albicans*. The table shows the type of protein molecules that can be produced from a gene containing 3 CUG codons. The probability of synthesis of the protein isoforms depends on the level of leucine incorporation; 3% at 30 °C, 5% at pH 4.0 and 28% in recombinant cells. Leu and Ser insertion at each CUG occurs randomly and is only dependent on the level of ambiguity.

same genes. If one takes into consideration the number of molecules *per cell* for each protein, a picture of extreme proteome complexity emerges. In yeast low and high abundance proteins are represented by 50 and 10^6 molecules per cell, respectively [21]. Assuming that: (1) all *C. albicans* genes are expressed; (2) 10% of the proteins with lowest CAI values are represented by 5000 molecules/cell; (3) 10% of the proteins with the highest CAI value are represented by 50,000 molecules/cell; and (4) the remaining 80% of genes are represented by 20,000 molecules/cell [11], then the approximate number of different protein molecules that result from 3% of CUG ambiguity is 6.7×10^6 . This value increases up to 10.7×10^6 in cells mistranslating at 5% and in the case of engineered *C. albicans* strains that mistranslate CUGs at 28% the total number of different protein molecules that can be produced is 42.8×10^6 . In other words, the *C. albicans* proteome is plastic, highly complex, and its size is unrelated to its gene pool by several orders of magnitude. Whether novel functions are associated to mistranslated proteins or whether they simply represent a nuisance to the cell remains to be elucidated.

5. CUGs are enriched in specific gene classes

C. albicans CUG usage is repressed in highly expressed genes (high CAI value) and is more relaxed in genes whose expression is low (low CAI value). Indeed, 83% of the highly expressed genes do not have CUGs while 81% of the genes expressed at low level have at least 1 CUG, indicating that CUGs are rarely used (0.43% usage). If one takes into consideration the Specific Codon Usage (SCU), which in this case measures the relative frequency of CUGs normalized to Ser abundance [22], then a clearer picture of CUG usage emerges. The SCU_{CUG} of genes containing one Ser-CUG residue and no other Ser is 1.0 while that of genes containing two Ser-CUGs and 18 additional Ser residues encoded by other Ser codons is 0.1. This allows one to obtain a global picture of

Table 1
Functional categories (GO terms) of genes with highest SCU_{CUG} .

Gene functional categories	SCU_{CUG}
Extrinsic membrane protein (ISS)	0.094
Golgi to endosome transport (ISS)	0.090
Spliceosome complex (ISS)	0.078
DNA repair (IEA)	0.070
Centromere (ISS)	0.069
Regulation of redox homeostasis (ISS)	0.068
Nuclear membrane (ISS)	0.067
AP-N adaptor complex (ISS)	0.064
Golgi to vacuole transport (ISS)	0.063
DNA replication factor complex	0.062
Chromatin silencing (ISS)	0.062
SAGA complex (ISS)	0.061
CCR4-NOT complex (ISS)	0.060
mRNA splicing (IEA)	0.059
Spindle pole (ISS)	0.057
Chromatin (ISS)	0.057
Golgi to plasma membrane transport (ISS)	0.057
Mitosis (IEA)	0.056
Cytokinesis (IEA)	0.056
Protein targeting (ISS)	0.055

Candida albicans genes were grouped by their Gene Ontology (GO) terms and the average CUG usage (SCU_{CUG}) of each category was calculated. CUGs are enriched in specific GO terms. The impact of CUG mistranslation is maximal in these genes.

CUG usage in *C. albicans* and identify functional classes of genes enriched in CUGs by carrying out a global survey of SCU_{CUG} values in Gene Ontology (GO) lists (Tables 1 and 2).

Plasma membrane protein genes show 2-fold increase in CUG usage (Table 1) and similar positive CUG usage biases were found in genes encoding nuclear membrane proteins and in genes encoding proteins of the SAGA-complex which is a large multi-protein complex with histone acetyltransferase activity involved in transcriptional regulation (ex: *Gcn5p*; [23]). Conversely, spindle pole protein genes involved in the organization of the

Table 2
Functional categories (GO terms) of genes with lowest SCU_{CUG} .

Gene functional categories	SCU_{CUG}
Ribosome (ISS)	0.0057
Cytosolic ribosome (sensu Eukarya) (ISS)	0.0061
ATP synthesis coupled proton transport (ISS)	0.0062
Respiratory chain complex	0.0109
Carbohydrate metabolism (ISS)	0.0154
Chromatin assembly/disassembly (ISS)	0.0161
Hydrogen-transporting ATPase	0.0186
Extracellular (ISS)	0.0189
Protein biosynthesis (ISS)	0.0190
Ergosterol biosynthesis (ISS)	0.0193
DNA-directed RNA polymerase	0.0203
Proteasome	0.0209
RNase complex (ISS)	0.0242
Glycogen metabolism (ISS)	0.0249
Ubiquitin-dependent protein catabolism (ISS)	0.0250
Peroxisome	0.0253
Endocytosis (IEA)	0.0254
Eukaryotic translation initiation factor complex (ISS)	0.0261
Heme biosynthesis (ISS)	0.0264
35S primary transcript processing (ISS)	0.0268

CUG usage is repressed in genes of specific GO terms categories. The impact of CUG ambiguity in these genes is minimal.

cytoskeleton and CCR4-NOT complex genes involved in transcriptional regulation, mRNA degradation and post-transcriptional modifications [24], show negative CUG usage bias, indicating that CUGs are under negative selective pressure in these genes (Table 2). CUG codons are also repressed in ribosomal protein genes, however these proteins are highly expressed and CUG repression is likely related to expression level rather than protein function.

6. Conclusions

Fungal species of the CTG clade use a mutant Ser tRNA_{CAG}^{Ser} to decode thousands of CUGs as Ser. This tRNA appeared prior to the split between *Saccharomyces* and *Candida* genera and competed for over 100 My with the wild type tRNA_{CAG}^{Leu} for CUGs during mRNA decoding [15,16]. The *Saccharomyces* spp. ancestor lost the mutant Ser tRNA_{CAG}^{Ser} and maintained the standard identity of the CUG codon (Leu), while the ancestor of the CTG clade lost the cognate tRNA_{CAG}^{Leu}, retained the mutant tRNA_{CAG}^{Ser}, and changed the identity of the CUG codon from Leu to Ser. This reassignment resulted in massive mutational change of CUGs to the UUG or UUA Leu codons [15]. Therefore, the CUGs present in extant species of the CTG clade are “new” CUGs that were captured by the tRNA_{CAG}^{Ser} upon mutation of codons from amino acid families belonging to Ser or amino acids with similar chemical properties [15]. Finally, the double identity of the CUG codon creates statistical proteins and proteomes whose biological implications are still poorly understood. Whether *Candida* spp. regulate and take advantage of CUG ambiguity remain important questions for future studies.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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