Presence and coding properties of 2'-O-methyl-5-carbamoylmethyluridine (ncm5Um) in the wobble position of the anticodon of tRNA-Leu (U*AA) from brewer's yeast

Anne-Lise Glasser[a], Chakib El Adlouni[b], Gérard Keith[b], Elzbieta Sochacka[3], Andrzej Malkiewicz[c], Manuel Santos[d], Mick F. Tuite[d] and Jean Desgrès[a]

[a]Laboratoire de Biochimie Médicale, Faculté de Médecine, Université de Bourgogne, 21034 Dijon, France, [b]Institut de Biologie Moléculaire et Cellulaire du CNRS et Université Louis Pasteur, 67084 Strasbourg, France, [c]Institute of Organic Chemistry, Technical University (Politechnika), 90–924 Lodz, Zwirki 36, Poland and [d]Biological Laboratory, The University, Canterbury, Kent CT2 7NJ, UK

The unknown modified nucleoside U* has been isolated by enzymatic and HPLC protocols from tRNA-Leu (U*AA) recently discovered in brewer's yeast. The pure U* nucleoside has been characterized by electron impact mass spectroscopy, and comparison of its chromatographic and UV-absorption properties with those of appropriate synthetic compounds. The structure of U* was established as 2'-O-methyl-5-carbamoylmethyluridine (ncm5Um). The yeast tRNA-Leu (U*AA) is the only tRNA so far sequenced which has been shown to contain ncm5Um. The location of such a modified uridine at the first position of the anticodon restricts the decoding property to A of the leucine UUA codon.

tRNA-Leu (U*AA); Modified nucleoside; Identification; Mass spectrometry; Anticodon; Yeast

1. INTRODUCTION

Among the very few brewer's yeast tRNAs whose primary structures are unknown, we recently discovered, purified, and sequenced a new tRNA-Leu (U*AA) [1]. It is the third cytoplasmic tRNA-Leu isolated from *Saccharomyces cerevisiae* so far; the others which are known are tRNA-Leu (m5CAA) [2,3], and tRNA-Leu (UAG) [4]. The major characteristic of this new tRNA-Leu (U*AA) is the presence of an unknown nucleoside in the wobble position of the anticodon. Preliminary studies [1], including two-dimensional thin-layer chromatography and UV-absorption spectrum of non-radioactive material, showed that this unknown nucleoside is a uridine derivative, designated U*.

Using the combined means of high performance liquid chromatography (HPLC), real-time UV-absorption spectroscopy, and mass spectrometric measurements, we report here on the isolation of this U* nucleoside

from purified yeast tRNA-Leu (U*AA), and on its identification as 2'-O-methyl-5-carbamoylmethyluridine (ncm5Um). The coding properties of this modified nucleoside are also reported.

2. EXPERIMENTAL

2.1. Purification of yeast tRNA-Leu (U*AA)
The yeast tRNA-Leu (U*AA) was purified by means of countercurrent distribution of total brewer's yeast tRNA (Boehringer, Mannheim, Germany), followed by polyacrylamide gel electrophoresis, as previously described [5,6].

2.2. Isolation of the unknown U* nucleoside
The U* nucleoside was isolated from purified yeast tRNA-Leu (U*AA) using the analytical procedure previously described for the isolation of other unknown nucleosides [7,8]. This procedure can be summarized as follows:
(i) nuclease P1 (Boehringer, Mannheim, Germany) digestion, followed by bacterial alkaline phosphatase (BAP) (Sigma, St. Louis, USA) treatment, of tRNA-Leu (U*AA) sample (about 50 A_{260} U in 200 µl water);
(ii) isolation by HPLC of a U*pA dinucleotide resistant to nuclease P1 action;
(iii) combined hydrolysis by snake venom phosphodiesterase (SV-PDE) (Sigma, St. Louis, USA) and BAP of the U*pA dinucleotide sample, leading to the liberation of free U* and adenosine (A) mononucleosides;
(iv) isolation and desalting of U* mononucleoside by HPLC.

Analysis, isolation, and desalting of the ribonucleosides by HPLC were performed using the experimental conditions previously described [7,9–11].

Correspondence address: J. Desgrès, Laboratoire de Biochimie Médicale, Faculté de Médecine, Université de Bourgogne, 21034 Dijon, France. Fax: (33) 80 29 35 00.

Abbreviations: ncm5Um, 2'-O-methyl-5-carbamoylmethyluridine; cm5Um, 2'-O-methyl-5-carboxymethyluridine; ncm5U, 5-carbamoylmethyluridine; BAP, bacterial alkaline phosphatase; SV-PDE, snake venom phosphodiesterase; (TMS) derivative, trimethylsilyl derivative; EI-MS, electron impact mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

2.3. Mass spectrometry

The conversion of U* nucleoside (approx. 2 μg) to the trimethylsilyl (TMS) derivative, and the electron impact mass spectroscopy (EI-MS) of silylated U* by direct-probe introduction, were carried out as previously reported [7].

2.4. Acidic treatment of U* nucleoside

1 μg of the pure unknown compound was incubated in 100 μl 1.8 N HCl at 60°C for 1 h, and further analyzed by HPLC. The resulting product was isolated, converted to the trimethylsilyl derivative, and characterized by mass spectrometry.

3. RESULTS

3.1. Isolation of pure U* nucleoside

Fig. 1 shows the HPLC nucleoside analysis of S. cerevisiae tRNA^Leu (U*AA) after exhaustive nuclease P1 digestion followed by BAP hydrolysis. The peak that eluted at 48 min retention time was designated as a nuclease P1-resistant unknown compound, N₄₈, and collected. As shown in Fig. 2a, the unknown N₄₈ yielded two mononucleosides upon SV-PDE plus BAP treatments.

One of these mononucleosides was easily identified as unmodified adenosine (A) by comparison of its HPLC retention time and UV-absorption spectrum with those of authentic adenosine.

The second nucleoside was collected, desalted, and concentrated. This compound exhibited a UV-absorption spectrum (Fig. 2b) which was very similar to that of 5-carbamoylmethyluridine (ncm⁵U), previously identified in different eukaryotic tRNAs^Pro [12,13]. However, its HPLC retention time (25.7 min) did not correspond either to ncm⁵U nucleoside, or to any of the modified uridines so far characterized. From these results and because of the behavior of U* in two-dimen-
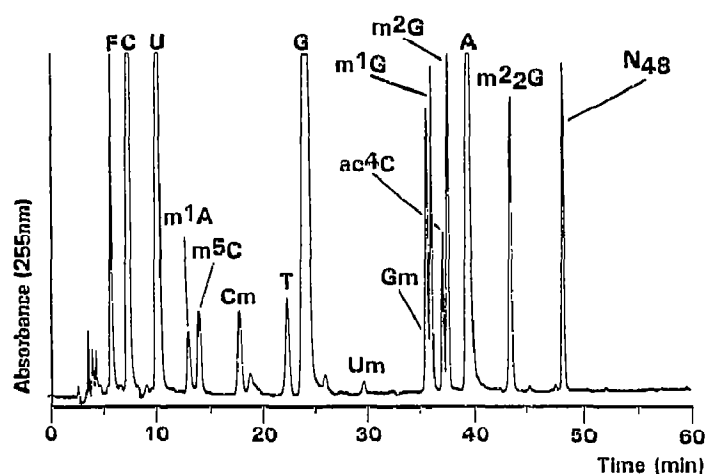

Fig. 1. HPLC chromatogram of nucleosides resulting from nuclease P1 plus bacterial alkaline phosphatase digestion of the third cytoplasmic tRNA^Leu, i.e. tRNA^Leu (U*AA), isolated from brewer's yeast [1]. The peak, N₄₈, corresponds to an unknown dinucleotide U*pA eluted at 48 min retention time.

sional thin-layer chromatography (results not shown), it was supposed that the structure of the unknown U* could be close to that of ncm⁵U. Further investigations on mass spectrometry of U* and its derivative upon acidic treatment confirmed this hypothesis.

3.2. Tentative characterization of U* as ncm⁵Um by mass spectrometry

The chemical structure of U* as 2'-O-methyl-5-carbamoylmethyluridine was postulated from the EI-MS fragmentation of U* as a TMS derivative. As reported previously for TMS derivatization of modified uridines [14], and especially of ncm⁵U from eukaryotic
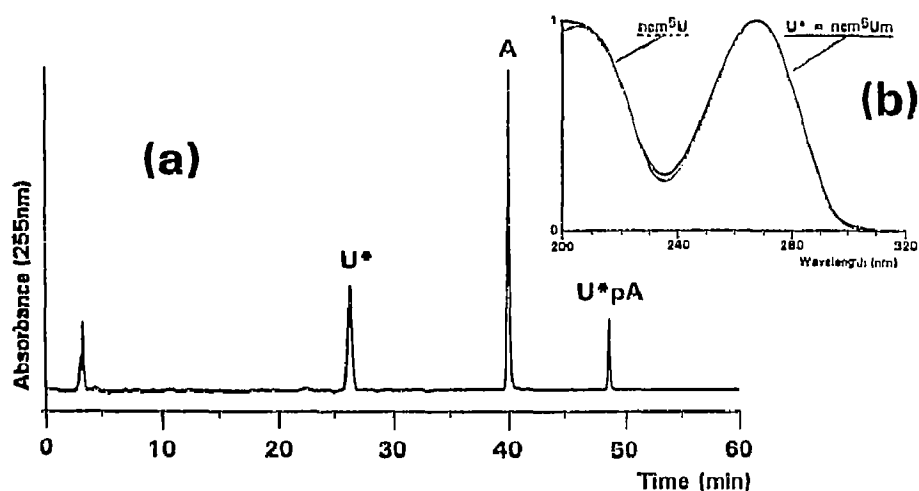

Fig. 2. (a) HPLC chromatogram of mononucleosides resulting from incomplete snake venom phosphodiesterase plus bacterial alkaline phosphatase hydrolysis of the unknown N₄₈ dinucleotide, i.e. U*pA, isolated by HPLC from brewer's yeast tRNA^Leu (U*AA). The peak designated as U* was collected, desalted, and concentrated. (b) Real-time UV-absorption spectrum of the unknown U* nucleoside from brewer's yeast tRNA^Leu (U*AA), as compared to that of authentic 5-carbamoylmethyluridine (ncm⁵U) isolated from yeast, or chicken liver, or beef liver tRNA^Pro [12].

tRNA^{Pro}'s [13], the trimethylsilylation of U* from yeast tRNA^{Leu} (U*AA) led to two TMS derivatives. From the resulting EI-mass spectrum presented in Fig. 3a, several ion series have been summarized in Table I.

The two major series of fragment ions shown in Table I result from fragmentation of the molecular ion of the (TMS)$_4$ derivative (m/z 603) designated M$_a^+$, and of the molecular ion of the (TMS)$_3$ derivative (m/z 531) designated M$_b^+$. These two molecular ions, M$_a^+$ and M$_b^+$, are in agreement with a 2'-O-methyl-5-carbamoylmethyluridine structure for the silylated U* nucleoside. Two significant fragment ions at m/z 488 (M$_a$ −115) and m/z 416 (M$_b$ −115) affirm the presence of a 5-carbamoylmethyl group on U* nucleoside, since they result, as in the fragmentation pathway previously described for the EI-MS of silylated ncm^{5}U [12,13], from the re-

moval of the fragment CO-NSi(CH$_3$)$_3$ from each molecular ion.

Another essential structure information for U* structure was gained from the ions at m/z 291, 259, 201, 187 and 159, which are characteristic of the fragmentation of a ribose moiety carrying a methoxy group on the carbon 2' [15]. The presence of the fragment ion M$_a$ −CH$_3$OH at m/z 571 and the concomitant absence of the fragment M$_a$ −(CH$_3$)$_3$SiOH at m/z 513 are also characteristic of the fragmentation pathway observed in the EI-mass spectra of the modified 2'-O-methyluridines as TMS derivatives [14]. Numerous other ion peaks at m/z 516, 386, 353, 341, 313 and 270, confirm the ncm^{5}Um structure for U* because they contain the intact base part, B$_a$ or B$_b$, plus portions of the 2'-O-methylribose moiety.
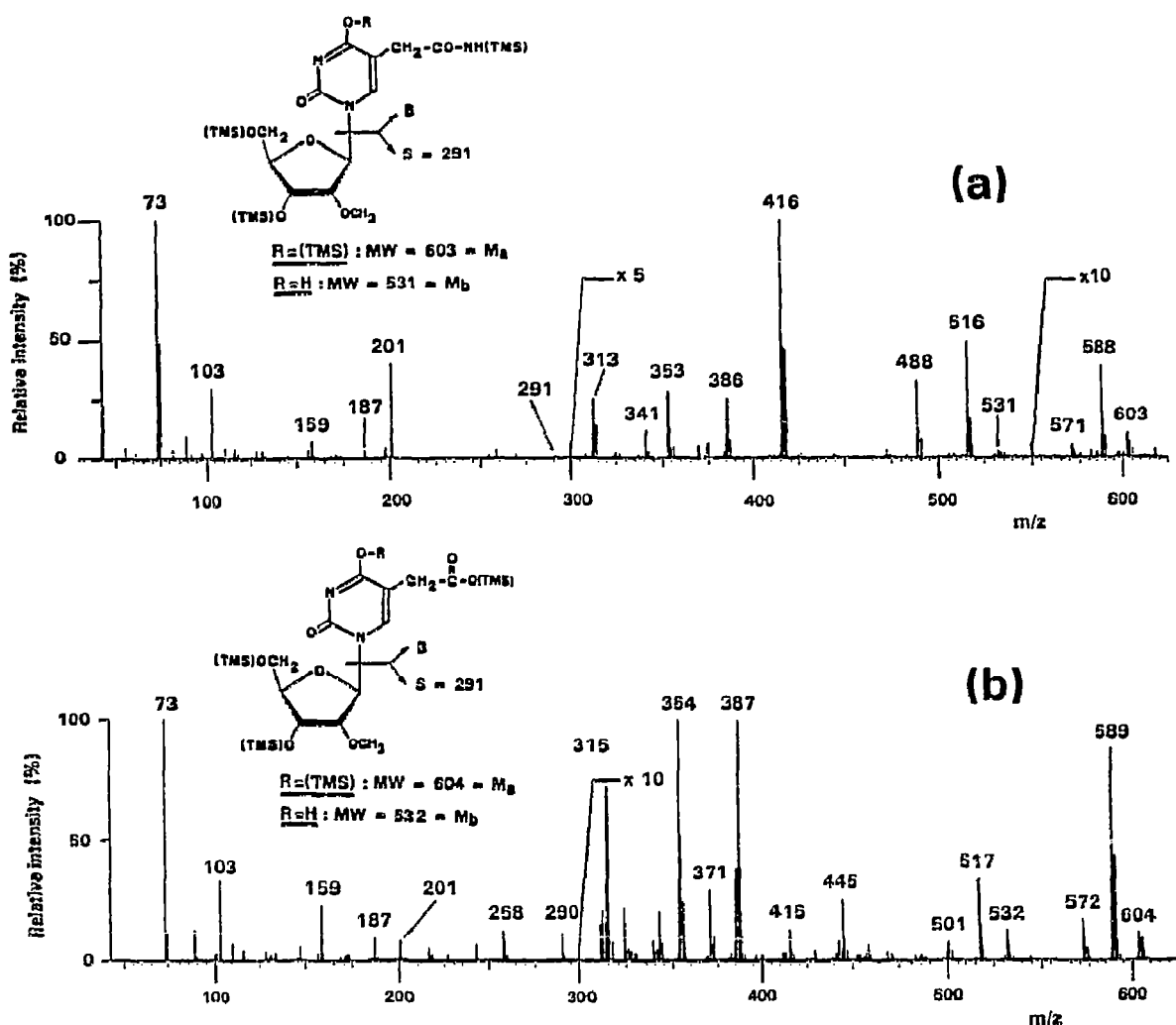

Fig. 3. Electron impact (EI) mass spectrum of the trimethylsilyl (TMS) derivative of U* nucleoside isolated from brewer's yeast tRNA^{Leu} (U*AA) (a, upper panel), as compared to that of the (TMS) derivative of the compound resulting from acidic treatment of U* (b, lower panel). The structure for this new modified uridine U* as 2'-O-methyl-5-carbamoylmethyluridine (ncm^{5}Um) was deduced from the two fragment ion series described in Table I, resulting from fragmentation of two molecular ions m/z 603 (M$_a$), and m/z 531 (M$_b$) for the (TMS)$_4$ and (TMS)$_3$ derivatives of U*, respectively. The EI-mass spectrum of the (TMS) product resulting from acidic treatment of U* (b, lower panel) confirmed this structure by leading to fragmentation pathways in agreement with a 2'-O-methyl-5-carboxymethyluridine (cm^{5}Um) structure.

Confirmation of the 5-carbamoylmethyl side chain on U* was gained from HPLC and EI-MS analysis of the compound resulting from acidic treatment of U*. When chromatographed by HPLC, the only resulting product of such a treatment of U* eluted at 16.3 min retention time, i.e. about 9.5 min earlier than U* (results not shown). This compound was collected, desalted, and submitted to EI-MS analysis as a TMS derivative. In the resulting EI-mass spectrum presented in Fig. 3b, all ion peaks with high relative intensities are in agreement with the expected fragmentation pathways of two TMS derivatives of a 2'-O-methyl-5-carboxymethyluridine (cm^{5}Um) structure: (i) molecular ion M_u^+ at m/z 604 of the (TMS)$_4$ derivative, and molecular ion M_b^+ at m/z 532 of the (TMS)$_3$ derivative; (ii) molecular ion series at m/z 589 (M_u –CH$_3$), 572 (M_u –CH$_3$OH), 517 (M_b–CH$_3$); (iii) base ion peaks containing the intact base part B_u of the (TMS)$_4$ derivative plus portions of the sugar moiety, e.g. fragment ions at m/z 501 (B_u +188), 445 (B_u +132), 415 (B_u +102), 387 (B_u +74), 371 (B_u + 58), 354 (B_u +41), 315 (B_u +2); (iv) sugar ion series at m/z 290 (S –1), 201 (S –90), 187 (S –104), resulting from the cleavages of the 2'-O-methylribose moiety. Thus, the side chain linked to the carbon 5 of U* nucleoside is really a carbamoylmethyl group, since acidic treatment of U* hydrolyses the amide function of this CH$_2$-CO-NH$_2$ group carried by ncm^{5}Um into CH$_2$-COOH acid group carried by the resulting cm^{5}Um.

Table I

Fragment-ion series from electron impact mass spectroscopy of U* nucleoside (i.e. ncm^{5}Um) isolated from the yeast leucine tRNA (U*AA), as trimethylsilyl [(TMS)$_4$, and (TMS)$_3$] derivatives

Mass (m/z)	From molecular ion (TMS)$_4$		From molecular ion (TMS)$_3$	
	Ion$^+$	Composition	Ion$^+$	Composition
603	M_u	Molecular ion (TMS)$_4$	–	–
588	M_u –15	M_u –CH$_3$	–	–
571	M_u –32	M_u –CH$_3$OH	–	–
488	M_u –115	M_u –CON(TMS)	–	–
531	–	–	M_b	Molecular ion (TMS)$_3$
416	M_u –187	M_u –CH$_3$OH –C$_3$H$_3$CONH(TMS)	M_b –115	M_b –CON(TMS)
516	B_u +204	B_u +C$_2$H$_2$O$_2$(TMS)$_2$	M_b –15	M_b –CH$_3$
386	B_u +74	B_u +H+(TMS)	–	–
353	B_u +41	B_u +C$_2$HO	–	–
341	B_u +29	B_u +CHO	–	–
313	B_u +1	B_u +H	–	–
270	–	–	B_b +30	B_b +CH$_2$O
291	S	Sugar moiety	S	Sugar moiety
259	S –32	S –CH$_3$OH	S –32	S –CH$_3$OH
201	S –90	S –(TMS)OH	S –90	S –(TMS)OH
187	S –104	S –H –CH$_2$O(TMS)	S –104	S –H –CH$_2$O(TMS)
159	S –132	C$_3$H$_3$O$_2$(TMS)$_2$	S –132	C$_3$H$_3$O$_2$(TMS)$_2$
103	–	CH$_2$O(TMS)	–	CH$_2$O(TMS)

B_u, base moiety of (TMS)$_4$ derivative; B_b, base moeity of (TMS)$_3$ derivative.

3.3. Definitive confirmation of the chemical structure for U* as ncm^{5}Um

Recently, the authentic ncm^{5}Um nucleoside was chemically synthesized, and its structure was characterized and rigorously established by protonated nuclear magnetic resonance (^{1}H NMR) and EI-MS of underivatized compound [16]. HPLC under standard chromatographic conditions and real-time UV-spectrometric measurements were applied on this reference ncm^{5}Um nucleoside. HPLC retention time and UV-absorption spectrum of the standard compound were strongly identical to those of U* nucleoside isolated from yeast tRNA^{Leu} (U*AA). In addition, the resulting products upon acidic treatments of U* and reference ncm^{5}Um exhibited identical chromatographic and UV-spectrometric properties.

These results definitively confirmed the chemical structure of U* as 2'-O-methyl-5-carbamoymethyluridine (ncm^{5}Um) (Fig. 4a), as well as the 2'-O-methyl-5-carboxymethyl-uridine (cm^{5}Um) structure for the nucleoside resulting from acidic treatment of U* (Fig. 4b).

3.4. ncm^{5}Um decodes only A in protein synthesis

In vitro protein synthesis experiments (not shown) of α-globin mRNA (containing 18 CUN and 2 UUG leucine codons), β-globin mRNA (containing 21 CUN leucine codons), and Brom mosaic virus (BMV) coat protein RNA 4 (containing all 6 leucine codons), led to radioactive protein only of the latter when [^{14}C]leucyl tRNA^{Leu} (ncm^{5}UmAA) was used. But with [^{14}C]leucyl tRNA^{Leu} (UAG), we obtained all three radioactive proteins, and with [^{14}C]leucyl tRNA^{Leu} (m^{5}CAA), we obtained only radioactive α-globin and BMV coat protein, meaning that the modifications of the pyrimidine nucleotide located at the wobble position of PyAA leucine anticodons restrict the decoding properties to either A, when it is ncm^{5}Um, or G, when it is m^{5}C.
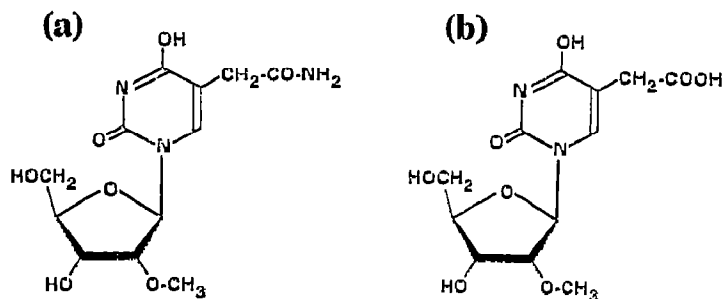

Fig. 4. Structures of (a) 2'-O-methyl-5-carbamoylmethyluridine (ncm^{5}Um), located at the first position of the anticodon in the third cytoplasmic tRNA^{Leu} (ncm^{5}UmAA) isolated from brewer's yeast, and (b) 2'-O-methyl-5-carboxymethyluridine (cm^{5}Um), obtained by acidic treatment of ncm^{5}Um.

4. DISCUSSION

Among all the tRNAs so far sequenced, the yeast tRNA^{Leu} (U*AA) is the first tRNA which has been shown to contain 2'-O-methyl-5-carbamoylmethyluridine (ncm^{5}Um). In previous studies [17], this modified nucleoside was tentatively characterized in crude unfractionated yeast tRNAs by its chromatographic, electrophoretic, and UV-absorption spectrometric properties as compared with those of semi-synthetic methyl ester and amide derivatives of 2'-O-methyl-5-carboxymethyluridine (cm^{5}Um). At this time, the author predicted that the postulated ncm^{5}Um should be found in a leucine, isoleucine, or valine isoacceptor tRNA.

Our previous [1] and present studies are complementary to these results and hypothesis since: (i) they rigorously establish the chemical structure of ncm^{5}Um, and (ii) they clearly define the yeast tRNA carrying this modified nucleoside as the tRNA^{Leu} recently isolated from brewer's yeast. This tRNA is the only one in yeast carrying ncm^{5}Um, as checked throughout all fractions of a counter-current distribution of total yeast tRNAs (not shown).

Our in vitro protein synthesis results allow a better clarification of the respective functions of the three known yeast tRNAs^{Leu} in the translation of the 6 leucine codons in yeast. Indeed, the tRNA^{Leu} (ncm^{5}UmAA) decodes the codon UUA (where ncm^{5}Um base pairs with A), the tRNA^{Leu} (m^{5}CAA) decodes the codon UUG (where m^{5}C base pairs with G), whereas the tRNA^{Leu} (UAG) decodes the 4 CUN leucine codons according to the 'wobble' hypothesis [18], and despite the in vitro results by others [19] who found that this tRNA could recognize all 6 leucine codons (CUN, UUA and UUG), but in the absence of the two other tRNAs^{Leu}.

In addition, recent studies [20], have shown that the modifications of U in position 34 of tRNAs contribute to the correct and efficient codon recognition through the regulation of the conformational 'rigidity–flexibility' of the first letter of the anticodon. Therefore, in the case of ncm^{5}Um in yeast tRNA^{Leu} (U*AA), the collaborative effects of both 2'-O-methylation and 5-substitution, could bring the required rigidity to this nucleotide to allow the exclusive recognition of A in the third position of the codon.

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