Volume 314, number 3, 381–385 FEBS 11902 December 1992

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

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Received 21 October 1992; revised version received 31 October 1992

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 (ncm^{5}Um). The yeast tRNA^{Leu} (U*AA) is the only tRNA so far sequenced which has been shown to contain ncm^{5}Um. 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} (m^{5}CAA) [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 (ncm^{5}Um). The coding properties of this modified nucleoside are also reported.

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Abbreviations: ncm^{5}Um, 2′-*O*-methyl-5-carbamoylmethyluridine; cm^{5}Um, 2′-*O*-methyl-5-carboxymethyluridine; ncm^{5}U, 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. EXPERIMENTAL

2.1. *Purification of yeast tRNA^{Leu}(U*AA)*

The yeast tRNA^{Leu} (U*AA) was purified by means of counter-current 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 (SVPDE) (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].

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, N_{48}, and collected. As shown in Fig. 2a, the unknown N_{48} 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 (ncm^5U), previously identified in different eukaryotic $tRNAs^{Pro}$ [12,13]. However, its HPLC retention time (25.7 min) did not correspond either to ncm^5U nucleoside, or to any of the modified uridines so far characterized. From these results and because of the behavior of U* in two-dimensional thin-layer chromatography (results not shown), it was supposed that the structure of the unknown U* could be close to that of ncm^5U. Further investigations on mass spectrometry of U* and its derivative upon acidic treatment confirmed this hypothesis.

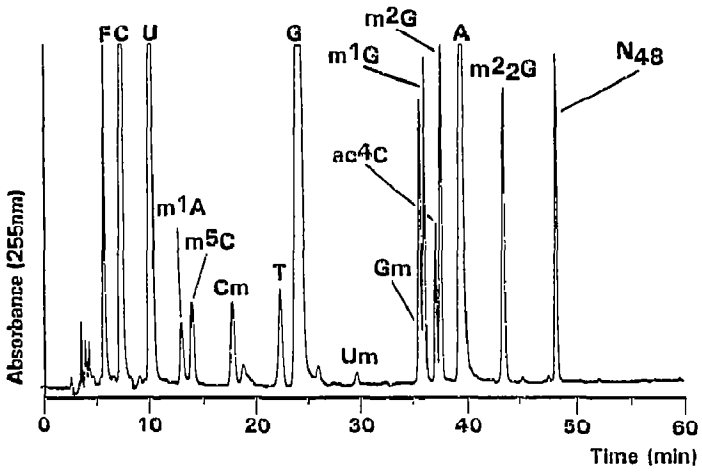

Fig. 1. HPLC chromatogram of nucleosides resulting from nuclease P1 plus bacterial alkaline phosphatase digestion of the third cytoplasmic $tRNA^{Leu}$, i.e. $tRNA^{Leu}$ (U*AA), isolated from brewer's yeast [1]. The peak, N_{48}, corresponds to an unknown dinucleotide U*pA eluted at 48 min retention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

3.2. *Tentative characterization of U* as ncm^5Um 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 ncm^5U from eukaryotic

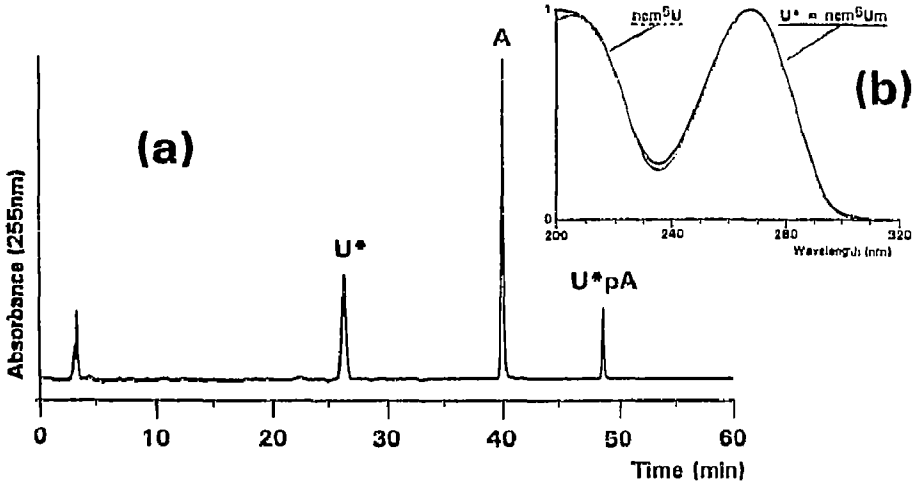

Fig. 2. (a) HPLC chromatogram of mononucleosides resulting from incomplete snake venom phosphodiesterase plus bacterial alkaline phosphatase hydrolysis of the unknown N_{48} dinucleotide, i.e. U*pA, isolated by HPLC from brewer's yeast $tRNA^{Leu}$ (U*AA). The peak designated as U* was collected, desalted, 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 (ncm^5U) 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^5U [12,13], from the removal of the fragment $CO\text{-}NSi(CH_3)_3$ 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 $-CH_3OH$ at *m/z* 571 and the concomitant absence of the fragment M_a $-(CH_3)_3SiOH$ 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^5Um structure for U* because they contain the intact base part, B_a or B_b, plus portions of the 2′-*O*-methylribose moiety.

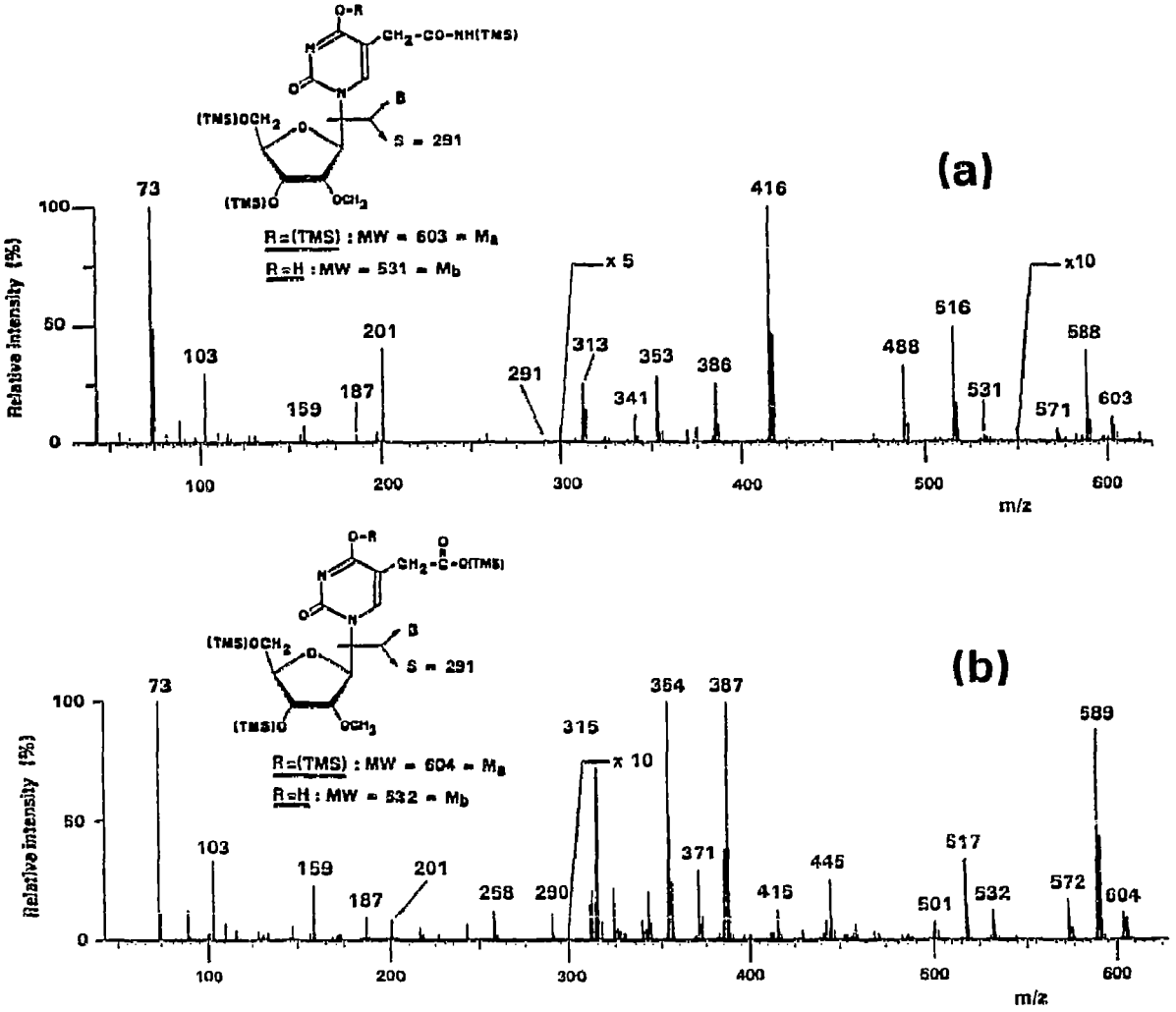

Fig. 3. Electron impact (EI) mass spectrum of the trimethylsilyl (TMS) derivative of U* nucleoside isolated from brewer's yeast $tRNA^{Leu}$ (U*AA) (a, upper panel), as compared to that of the (TMS) derivative of the compound resulting from acidic treatment of U* (b, lower panel). The structure for this new modified uridine U* as 2′-*O*-methyl-5-carbamoylmethyluridine (ncm^5Um) 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^5Um) 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^5Um) structure: (i) molecular ion M_a^+ 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 ($M_a - CH_3$), 572 ($M_a - CH_3OH$), 517 ($M_b - CH_3$); (iii) base ion peaks containing the intact base part B_a of the $(TMS)_4$ derivative plus portions of the sugar moiety, e.g. fragment ions at *m/z* 501 (B_a +188), 445 (B_a +132), 415 (B_a +102), 387 (B_a +74), 371 (B_a + 58), 354 (B_a +41), 315 (B_a +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^5Um into CH_2-COOH acid group carried by the resulting cm^5Um.

Table I

Fragment-ion series from electron impact mass spectroscopy of U* nucleoside (i.e. ncm^5Um) 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_a	Molecular ion $(TMS)_4$	–	–
588	$M_a - 15$	$M_a - CH_3$	–	–
571	$M_a - 32$	$M_a - CH_3OH$	–	–
488	$M_a - 115$	M_a −CON(TMS)	–	–
531	–	–	M_b	Molecular ion $(TMS)_3$
416	$M_a - 187$	$M_a - CH_3OH - C_3H_3CONH(TMS)$	$M_b - 115$	M_b −CON(TMS)
516	$B_a + 204$	$B_a + C_2H_2O_2(TMS)_2$	$M_b - 15$	$M_b - CH_3$
386	$B_a + 74$	$B_a + H + (TMS)$	–	–
353	$B_a + 41$	$B_a + C_2HO$	–	–
341	$B_a + 29$	$B_a + CHO$	–	–
313	$B_a + 1$	$B_a + H$	–	–
270	–	–	$B_b + 30$	$B_b + CH_2O$
291	S	Sugar moiety	S	Sugar moiety
259	S −32	S $-CH_3OH$	S −32	S $-CH_3OH$
201	S −90	S −(TMS)OH	S −90	S −(TMS)OH
187	S −104	S −H $-CH_2O(TMS)$	S −104	S−H $-CH_2O(TMS)$
159	S −132	$C_3H_3O_2(TMS)_2$	S −132	$C_3H_3O_2(TMS)_2$
103	–	$CH_2O(TMS)$	–	$CH_2O(TMS)$

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

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

Recently, the authentic ncm^5Um nucleoside was chemically synthesized, and its structure was characterized and rigorously established by protonated nuclear magnetic resonance (^1H 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^5Um 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^5Um exhibited identical chromatographic and UV-spectrometric properties.

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

3.4. *ncm^5Um 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^5UmAA) was used. But with [^{14}C]leucyl $tRNA^{Leu}$ (UAG), we obtained all three radioactive proteins, and with [^{14}C]leucyl $tRNA^{Leu}$ (m^5CAA), 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^5Um, or G, when it is m^5C.

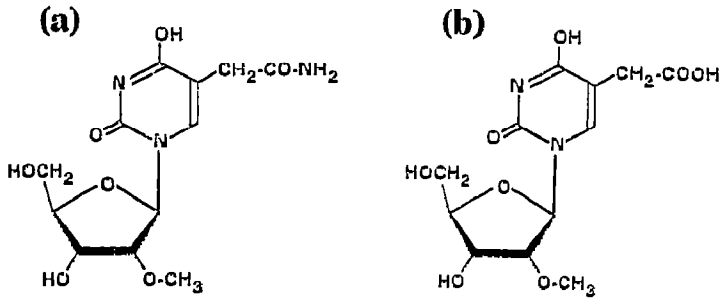

Fig. 4. Structures of (a) 2′-*O*-methyl-5-carbamoylmethyluridine (ncm^5Um), located at the first position of the anticodon in the third cytoplasmic $tRNA^{Leu}$ (ncm^5UmAA) isolated from brewer's yeast, and (b) 2′-*O*-methyl-5-carboxymethyluridine (cm^5Um), obtained by acidic treatment of ncm^5Um.

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^5Um). 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^5Um). At this time, the author predicted that the postulated ncm^5Um 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^5Um, 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^5Um, 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^5UmAA) decodes the codon UUA (where ncm^5Um base pairs with A), the $tRNA^{Leu}$ (m^5CAA) decodes the codon UUG (where m^5C 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^5Um 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.

Acknowledgements: The expert technical assistance of C. Fix is warmly acknowledged. We are also grateful to J.-L. Guyot who conducted the MS studies in the Centre Européen de Spectrométrie de Masse (Spiral Co., Couternon, France) managed by Dr. M. Prost. C.E.A. acknowledges FEBS for a Summer Fellowship, and EMBO for a short-term fellowship to visit the Biological Laboratory at Canterbury (UK) where the in vitro protein synthesis experiments were performed. This research had financial support from Association pour la Recherche sur le Cancer, Ministère de la Recherche et de l'Espace, Ligue Bourguignonne contre le Cancer, and Centre National de la Recherche Scientifique.

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