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Eukaryotic Wobble Uridine Modifications Promote a Functionally Redundant Decoding System▽

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The translational decoding properties of tRNAs are modulated by naturally occurring modifications of their nucleosides. Uridines located at the wobble position (nucleoside 34 [U_{34}]) in eukaryotic cytoplasmic tRNAs often harbor a 5-methoxycarbonylmethyl (mcm^5) or a 5-carbamoylmethyl (ncm^5) side chain and sometimes an additional 2-thio (s^2) or 2′-*O*-methyl group. Although a variety of models explaining the role of these modifications have been put forth, their in vivo functions have not been defined. In this study, we utilized recently characterized modification-deficient *Saccharomyces cerevisiae* cells to test the wobble rules in vivo. We show that mcm^5 and ncm^5 side chains promote decoding of G-ending codons and that concurrent mcm^5 and s^2 groups improve reading of both A- and G-ending codons. Moreover, the observation that the mcm^5U_{34}- and some ncm^5U_{34}-containing tRNAs efficiently read G-ending codons challenges the notion that eukaryotes do not use U-G wobbling.

The universal genetic code consists of 64 triplets of which 61 represent different amino acids and 3 signal translation termination (37). The deciphering of the code led to the realization that it is degenerate; that is, most amino acids are represented by more than one codon. The presence of isoaccepting tRNAs, different tRNAs charged with the same amino acid, helped to explain the translation of the code. However, the number of tRNA species is always fewer than the 61 sense codons, suggesting that some tRNAs decode more than one triplet. This ability is due to the fact that the first base of the anticodon (position 34 of the tRNA, also called the wobble nucleoside) may pair with more than one base in the third position of the codon (11). At the time Crick presented his wobble hypothesis, it was not known that cytoplasmic tRNA almost never harbors an unmodified uridine (U) as a wobble nucleoside. Revised wobble hypotheses have since emerged to explain some unexpected results regarding the decoding properties of tRNAs with modified wobble uridines (1, 31, 47, 53, 54). These hypotheses are based mainly on translational experiments performed in vitro and by analyses of the modifications' influence on the structure of nucleosides, nucleotides, or anticodons.

The xm^5U type of modified wobble nucleosides, where x represents any of several different groups and m^5 stands for a methylene carbon directly bonded to the C-5 atom of the uracil moiety, can be found in organisms from all three domains of life (42, 47). These nucleosides can also harbor an additional 2-thio (xm^5s^2U) or a 2′-*O*-methyl group (xm^5Um). It is generally accepted that tRNA species containing xm^5U_{34}, $xm^5s^2U_{34}$, or xm^5Um_{34} residues do not read pyrimidine-ending codons (31, 53, 54). This is supported by the fact that these nucleosides are often found in tRNA species that decode in split codon boxes where the pyrimidine- and purine-ending codons are for different amino acids. The observation that a tRNA with an unmodified U_{34} can, under some circumstances, read codons ending with any nucleoside indicates that the xm^5U derivatives may have evolved to restrict wobbling (31, 53). However, more recent genetic and structural data have suggested that the inability to pair with the pyrimidine-ending codons may not, at least for $xm^5s^2U_{34}$-containing tRNAs, be a direct consequence of the modifications (20, 35). The models for how xm^5U derivatives affect pairing with purine-ending codons are more diverse, but the modifications are believed to allow either efficient interaction with both A and G or efficient pairing with A while simultaneously reducing pairing with G (29, 31, 35, 47, 52–54).

The lack of defined mutants in eukaryotes has led to a poor understanding of the role of the xm^5U derivatives found in this domain of life. However, in vitro translation systems have revealed that cytoplasmic tRNA species harboring xm^5U derivatives preferentially read codons ending with A (19, 33, 43, 50). These data, in combination with the fact that C_{34}-containing tRNAs are frequently found in eukaryotes, suggested that they may not use U-G wobbling (38, 47). This contrasts to xm^5U-derivative-containing tRNA species in organelles and prokaryotes, which often read codons ending with G. This apparent discrepancy has been proposed to be a consequence of different compositions of the xm^5 groups and/or differences in the ribosomes (47). In this study, we utilized recently characterized *Saccharomyces cerevisiae* mutants to analyze the in vivo decoding properties of tRNAs with or without the eukaryotic xm^5 side chains 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5). We also investigated the role of the s^2 group in tRNAs containing a wobble mcm^5s^2U residue. Our results show that many cytoplasmic tRNAs harboring an xm^5U derivative can read G-ending codons and that this ability is enhanced by the presence of the modifications.

MATERIALS AND METHODS

Yeast strains, media, and genetic procedures. The sources and genotypes of yeast strains used in this study are listed in Table 1. Yeast transformation (18),

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TABLE 1. Yeast strains used in this study

Yeast strain	Genotype	Source or reference
W303-1A	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*	16
W303-1B	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*	16
UMY2843	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 elp3*::*kanMX4*	32
UMY3297	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 trm9*::*kanMX4*	32
UMY3165	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tuc1*::*TRP1*	This study
UMY2893	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SUP4*	22
UMY2916	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SUP4 elp3*::*kanMX4*	22
UMY3104	*MAT***a**/*MAT*α *ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100*	This study
UMY2366	*MAT***a**/*MAT*α *ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 ade3*::*hisG/ade3*::*hisG can1-100/can1-100*	24
UMY3199	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tv*(*cac*)*d*::*TRP1*	This study
UMY3284	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tv*(*cac*)*h*::*TRP1*	This study
UMY3295	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tv*(*cac*)*d*::*TRP1 tv*(*cac*)*h*::*TRP1*	This study
UMY3296	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tv*(*cac*)*d*::*TRP1 tv*(*cac*)*h*::*TRP1*	This study
UMY3333	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tv*(*cac*)*d*::*TRP1 tv*(*cac*)*h*::*TRP1 elp3*::*kanMX4* p1725	This study
UMY2406	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 ade3*::*hisG can1-100 ts*(*cga*)*c*::*TRP1* p1280	This study
UMY3126	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ts*(*cga*)*c*::*TRP1* p1280	This study
UMY3127	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ts*(*cga*)*c*::*TRP1 elp3*::*kanMX4* p1280	This study
UMY3128	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tt*(*cgu*)*k*::*TRP1* p1244	This study
UMY3129	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tt*(*cgu*)*k*::*TRP1* p1244	This study
UMY3130	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tt*(*cgu*)*k*::*TRP1 elp3*::*kanMX4* p1244	This study
UMY3132	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tq*(*cug*)*m*::*TRP1* p1605	This study
UMY3133	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tq*(*cug*)*m*::*TRP1* p1605	This study
UMY3134	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tq*(*cug*)*m*::*TRP1 elp3*::*kanMX4* p1605	This study
UMY3345	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tq*(*cug*)*m*::*TRP1 trm9*::*kanMX4* p1605	This study
UMY3347	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tq*(*cug*)*m*::*TRP1 tuc1*::*TRP1* p1605	This study
UMY3322	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*i*::*TRP1*	This study
UMY3270	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*d*::*TRP1*	This study
UMY3329	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*i*::*TRP1 te*(*cuc*)*d*::*TRP1* p1723	This study
UMY3348	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*i*::*TRP1 te*(*cuc*)*d*::*TRP1* p1723	This study
UMY3350	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*i*::*TRP1 te*(*cuc*)*d*::*TRP1 elp3*::*kanMX4* p1723	This study
UMY3354	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*i*::*TRP1 te*(*cuc*)*d*::*TRP1 trm9*::*kanMX4* p1723	This study
UMY3352	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 te*(*cuc*)*i*::*TRP1 te*(*cuc*)*d*::*TRP1 tuc1*::*TRP1* p1723	This study
UMY3112	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 ade3*::*hisG can1-100 tr*(*ccu*)*j*::*TRP1*	This study
UMY3137	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tr*(*ccu*)*j*::*TRP1*	This study
UMY3136	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tr*(*ccu*)*j*::*TRP1 elp3*::*kanMX4* p1619	This study
UMY3358	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tr*(*ccu*)*j*::*TRP1 trm9*::*kanMX4*	This study
UMY3223	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tg*(*ccc*)*d*::*TRP1*	This study
UMY3226	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tg*(*ccc*)*o*::*URA3*	This study
UMY3303	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tg*(*ccc*)*d*::*TRP1 tg*(*ccc*)*o*::*URA3*	This study
UMY3304	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tg*(*ccc*)*d*::*TRP1 tg*(*ccc*)*o*::*URA3*	This study
UMY3320	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tg*(*ccc*)*d*::*TRP1 tg*(*ccc*)*o*::*URA3 elp3*::*kanMX4*	This study
UMY3360	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tg*(*ccc*)*d*::*TRP1 tg*(*ccc*)*o*::*URA3 trm9*::*kanMX4*	This study
UMY3195	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tp*(*agg*)*c*::*TRP1*	This study
UMY3293	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tp*(*agg*)*n*::*TRP1*	This study
UMY3342	*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tp*(*agg*)*c*::*TRP1 tp*(*agg*)*n*::*TRP1*	This study
UMY3343	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tp*(*agg*)*c*::*TRP1 tp*(*agg*)*n*::*TRP1*	This study
UMY3368	*MAT*α *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tp*(*agg*)*c*::*TRP1 tp*(*agg*)*n*::*TRP1 elp3*::*kanMX4*	This study

media, and genetic procedures have been described previously (7). The *tuc1*Δ strain (UMY3165) was constructed as previously described (3). tRNA genes were deleted by a PCR-mediated strategy (6), replacing the tRNA gene with a selectable marker. Disruptions of tRNA genes were confirmed by PCR. The *tV*(*CAC*)*H* and *tE*(*CUC*)*I* genes were deleted in strain W303-1A. These strains were backcrossed once to W303-1B, confirming the viability of the deletion strain. The *tS*(*CGA*)*C*, *tT*(*CGU*)*K*, *tQ*(*CUG*)*M*, *tV*(*CAC*)*D*, *tG*(*CCC*)*D*, *tG*(*CCC*)*O*, *tE*(*CUC*)*D*, *tR*(*CCU*)*J*, t*P*(*AGG*)*C*, and *tP*(*AGG*)*N* genes were independently deleted in either UMY2366 or UMY3104. UMY3104 is a diploid formed between W303-1A and W303-1B. The heterozygous strains were allowed to sporulate, and the viability of deletion strains was determined from tetrads. If a tRNA species was essential, a plasmid carrying the corresponding wild-type tRNA gene was introduced into the heterozygous strain before sporulation. The *ade3*::*hisG* allele in strains derived from UMY2366 was eliminated in subsequent crosses. For the tRNA species encoded by two genes, the double mutant was obtained in tetrads from a cross between strains with a deletion of the respective tRNA gene. The *elp3*Δ (UMY2843), *tuc1*Δ (UMY3165), and *trm9*Δ (UMY3297) mutants were crossed to the appropriate tRNA deletion strains and the double/triple mutants obtained from tetrads. In instances where the lack of modification influenced the viability of a strain lacking a tRNA, a plasmid carrying the corresponding wild-type tRNA gene was utilized to generate a rescued double/triple mutant. In standard *S. cerevisiae* laboratory strains, the number of genes encoding a tRNA species can vary (8). Southern blot analyses of the wild-type strains confirmed that the gene copy numbers of *tP*(*AGG*) and *tV*(*CAC*) are 2 and 2, respectively.

Plasmid constructions. DNA manipulations, plasmid preparations, and bacterial transformations were performed according to standard protocols. Genes were PCR amplified using *Pwo* DNA polymerase (Roche Applied Science). Plasmid pRS316-*ADE3-tS*(*CGA*)*C* (p1280) was constructed by cloning a BglII/SacI fragment from pRS316-*tS*(*CGA*)*C* (*sup61*$^+$) (23) into the BamHI/SacI sites of pDD44 (kindly provided by S. Åström). Plasmid p1244 is a derivative of a YCp50 library plasmid (41) that contains the *tT*(*CGU*)*K* gene. A SphI fragment

was removed from the original plasmid, leaving a fragment that contains the *tT*(*CGU*)*K* gene and approximately 800 and 1,200 bp of upstream and downstream sequence, respectively. The *tV*(*CAC*)*D*, *tQ*(*CUG*)*M*, *tE*(*CUC*)*D*, and *tR*(*CCU*)*J* genes were separately PCR amplified from W303-1B and cloned into pRS316 (44) utilizing restriction sites present within the oligonucleotides. This generated the plasmids pRS316-*tV*(*CAC*)*D* (p1725), pRS316-*tQ*(*CUG*)*M* (p1605), pRS316-*tE*(*CUC*)*D* (p1723), and pRS316-*tR*(*CCU*)*J* (p1619). The oligonucleotides used were the following: for *tV*(*CAC*)*D*, 5′-CTCTGGATCCACGT ACTTCCTGTAAGGAGC-3′ and 5′-CTCTCTCGAGGGAATGTACTTTCTG TAGGGC-3′; for *tQ*(*CUG*)*M*, 5′-GAACGGATCCTATCAGGCTCTCAGAA GGC-3′ and 5′-GAACCTCGAGAATATGACCAATCGGCGTGTG-3′; for *tE*(*CUC*)*D*, 5′-TTGAATTCTGGCCTGTATTTTCTATATTCC-3′ and 5′-TTC TCGAGCCCCCTAGAAGCATAGTTTTGT-3′; for *tR*(*CCU*)*J*, 5′-CATAGGA TCCTCGAGGAGAACTTCTAGTA-3′ and 5′-CCACGAATTCGGAGCAAT ATCGTACGCCAC-3′. The *tS*(*UGA*)*P* gene was PCR amplified from strain W303-1B, followed by addition of an A overhang by *Taq* DNA polymerase (Roche Applied Science). This fragment was cloned into the pGEM^{R}-T Easy Vector (Promega) generating p1456. The oligonucleotides used were 5′-TCTC GAATTCAAGTTCAGTATGCCATAGGTGC-3′ and 5′-CTCATCTAGACA GAATGAGCGGATGTTTCA-3′. The high-copy-number *LEU2* plasmid pRS425-*tS*(*UGA*)*P* (p1477) was constructed by cloning an ApaI/SpeI fragment from p1456 into the corresponding sites of pRS425 (10). Plasmid pRS425-*tT*(*UGU*)*G1* (p1779) was constructed by cloning an XhoI/SpeI fragment PCR amplified from W303-1B into the corresponding sites of pRS425. The oligonucleotides used were 5′-TTCTCGAGGCTAATTGGCGATCGTTTA-3′ and 5′-AAACTAGTGAAAGCAAATATCTGGCCTTC-3′. The pRS425-*tQ*(*UUG*) (pABY1499) and pRS425-*tE*(*UUC*) (pABY1479) plasmids have previously been described (32).

RNA analyses. Single tRNA species were prepared and analyzed as previously described (4, 22). Steady-state tRNA levels were determined as described previously by using total RNA isolated by the hot phenol method (2, 24). Aminoacylation levels were determined from exponentially growing cells at 30°C. Cells were harvested by pouring the cultures into tubes containing an equivalent volume of ice, followed by centrifugation. All subsequent steps were performed in the cold using prechilled reagents. Cells were washed with water and resuspended in 300 μl of buffer A (0.1 M NaAc [pH 4.5], 10 mM EDTA) followed by addition of ~0.3 g of glass beads and 300 μl of buffer A-equilibrated phenol-chloroform-isoamylalcohol (25:24:1) solution. The mixture was vortexed for two times for 1 min each time with 1 min on ice between steps. Following centrifugation, the RNA in the aqueous layer was precipitated by addition of 3 volumes of ethanol and incubation at −20°C. RNA pellets were dissolved in 10 mM NaAc (pH 4.5)–1 mM EDTA buffer. Polyacrylamide gel electrophoresis was performed essentially as described previously (48) by applying 10 μg of total RNA on an 8% polyacrylamide–8 M urea–0.1 M NaAc (pH 5.0) gel. After electroblotting to Zeta-Probe membranes (Bio-Rad), ^{32}P-labeled oligonucleotides complementary to the tRNA of interest were used as probes. Signals in Northern blotting experiments were detected and quantified by phosphorimaging using a Storm imaging system and ImageQuant software.

Nonsense suppression assay. The *LEU2* derivatives of pUKC815, pUKC817, pUKC818, and pUKC819 (45, 46) were separately transformed into wild-type (W303-1B), *SUP4* (UMY2893), and *SUP4 elp3*Δ (UMY2916) cells. Six independent clones from each transformation were grown in synthetic complete (SC)-Leu medium to an optical density at 600 nm (OD_{600}) of ~0.85. Cells were harvested, and the β-galactosidase activity determined in protein extracts as previously described (7).

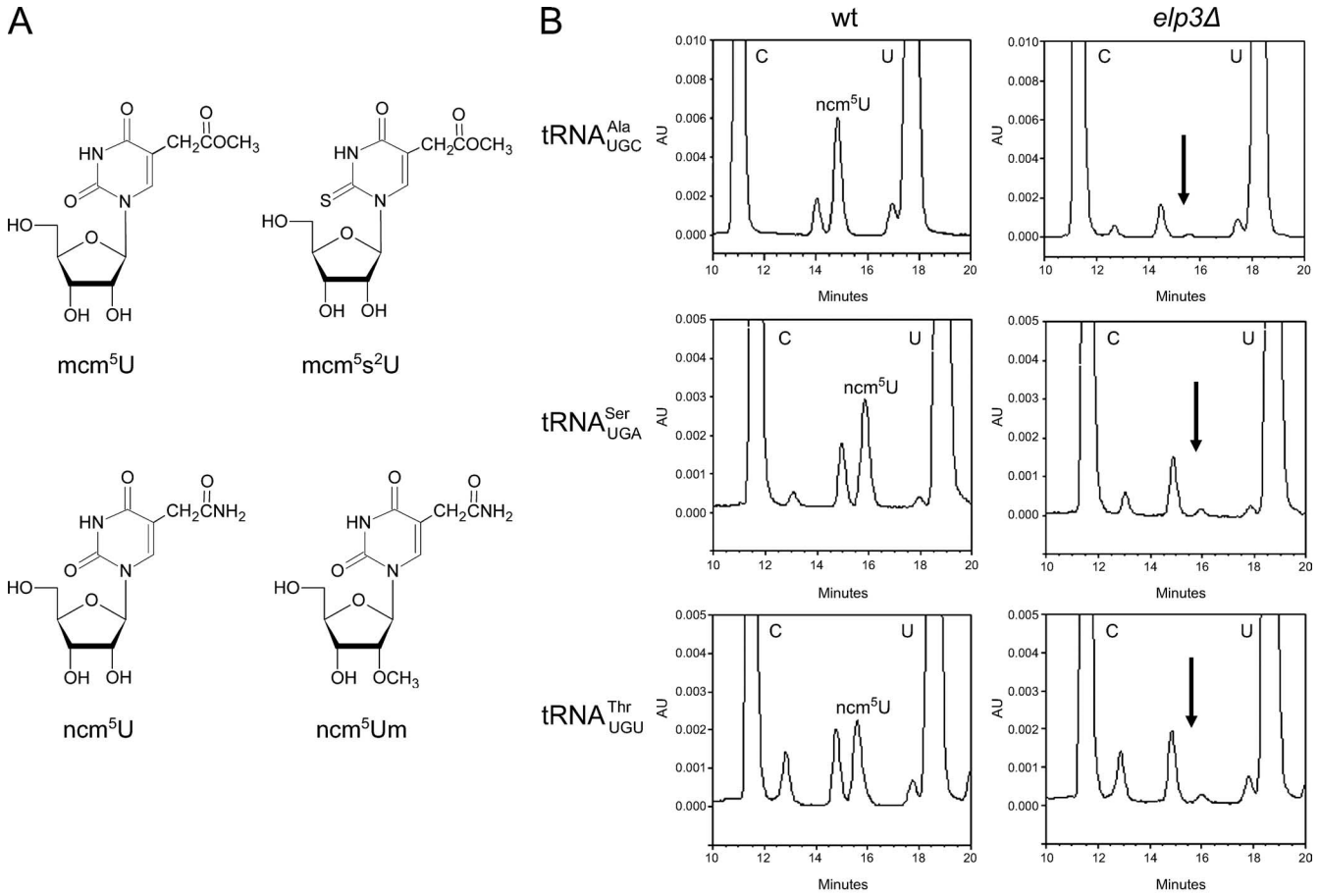
RESULTS

Lack of a wobble mcm^5, ncm^5, or s^2 group does not influence the steady-state or aminoacylation level of tRNA. In *S. cerevisiae*, approximately 274 nuclear-encoded tRNA genes code for the 42 different cytoplasmic tRNA species that are responsible for the decoding of the 61 sense codons (21, 39). The number of genes coding for individual tRNA species varies between 1 and 16, with a good correlation between gene copy number and intracellular tRNA levels (39). The identity of the nucleoside at position 34 is known for 10 of the 13 *S. cerevisiae* tRNA species that in the primary transcript contain a U at this position (25, 32). Of these, one contains an unmodified U ($tRNA^{Leu}_{UAG}$), and one contains a pseudouridine (Ψ; $tRNA^{Ile}_{ΨAΨ}$) residue. The remaining eight tRNA species contain ncm^5U, ncm^5Um, mcm^5U, or mcm^5s^2U (Fig. 1A). Since the nature of the wobble nucleoside in $tRNA^{Ala}_{UGC}$, $tRNA^{Ser}_{UGA}$, and $tRNA^{Thr}_{UGU}$ was not clear, we purified these tRNA species from a wild-type strain. The purified tRNAs were degraded to nucleosides, and their composition analyzed by high-performance liquid chromatography (HPLC). Based on retention time and UV absorption spectra, all three tRNA species were found to contain ncm^5U (Fig. 1B and data not shown). Thus, the eleven tRNA species that contain a wobble mcm^5 or ncm^5 side chain are as follows: $tRNA^{Arg}_{mcm^5UCU}$, $tRNA^{Gly}_{mcm^5UCC}$, $tRNA^{Lys}_{mcm^5s^2UUU}$, $tRNA^{Gln}_{mcm^5s^2UUG}$, $tRNA^{Glu}_{mcm^5s^2UUC}$, $tRNA^{Val}_{ncm^5UAC}$, $tRNA^{Ser}_{ncm^5UGA}$, $tRNA^{Pro}_{ncm^5UGG}$, $tRNA^{Thr}_{ncm^5UGU}$, $tRNA^{Ala}_{ncm^5UGC}$, and $tRNA^{Leu}_{ncm^5UmAA}$ (Fig. 1C).

We previously showed that the six subunits in the elongator complex (Elp1p to Elp6p) are all required for the formation of mcm^5 and ncm^5 side chains at the wobble position (22). In an *elp3* mutant, the formation of the side chain at position 5 of the wobble uridine is abolished in all 11 tRNA species that normally contain such groups (22, 32) (Fig. 1B). The *TUC1* and *TUC2* genes (previously known as *NCS6* and *NCS2*) were recently shown to be required for formation of the s^2 group in $mcm^5s^2U_{34}$-containing tRNA species (3, 14). The formation of s^2 and mcm^5 groups occurs independently of each other (3, 14, 22), which means that *elp3*Δ and *tuc1*Δ mutants can be used to dissect their individual functions. Although the wobble mcm^5/ncm^5 and s^2 groups are likely to influence the decoding properties of tRNA, it was conceivable that their absence could generate tRNAs that are destabilized and/or inefficiently aminoacylated (17, 25). To address this concern, we determined the steady-state and in vivo aminoacylation levels for each of the affected tRNA species in wild-type, *elp3*Δ, and *tuc1*Δ cells. The analyses revealed that the absence of an mcm^5, ncm^5, or s^2 group at the wobble position does not reduce abundance or aminoacylation of any of the hypomodified tRNA species (Fig. 2 and Tables 2 and 3). These data suggest that *elp3*Δ and *tuc1*Δ mutants can be utilized to study the decoding properties of tRNA species lacking a wobble mcm^5, ncm^5, or s^2 group.

A wobble mcm^5 side chain improves decoding of G-ending codons. Two *S. cerevisiae* tRNA species, $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ contain mcm^5U at the wobble position (Fig. 1C). The $tRNA^{Arg}_{mcm^5UCU}$ species decodes in the split codon box AGN, where the AGA and AGG codons are for arginine, whereas $tRNA^{Gly}_{mcm^5UCC}$ decodes in the glycine family codon box GGN. A C_{34}-containing tRNA species complementary to the G-ending codon is present in both the AGN and GGN box ($tRNA^{Arg}_{CCU}$ and $tRNA^{Gly}_{CCC}$) (Fig. 1C), indicating that $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ do not have to read these codons. Consistent with this suggestion, the $tRNA^{Arg}_{mcm^5UCU}$ species was able to read the AGA but not the AGG codon in an in vitro translation system (50).

We previously showed that lack of the mcm^5 group in the *SUP4*-encoded ochre suppressor tRNA, where the primary anticodon sequence is UUA, abolished suppression of the *ade2-1* (UAA) and *can1-100* (UAA) nonsense alleles in vivo (22). These results implied that the function of the wobble modification in $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ may be to improve reading of their respective A-ending codons. To test this hypothesis, we used a strategy that involved reducing the copy



C

codon	anticodon	amino acid	codon	anticodon	amino acid	codon	anticodon	amino acid	codon	anticodon	amino acid
UUU	-	Phe	UCU	IGA	Ser	UAU	-	Tyr	UGU	-	Cys
UUC	GmAA		UCC	-		UAC	GΨA		UGC	GCA	
UUA	**ncm^{5}UmAA**	Leu	UCA	**ncm^{5}UGA**		UAA	-	n.a.	UGA	-	n.a.
UUG	m^{5}CAA		UCG	CGA		UAG	-		UGG	CmCA	Trp
CUU	-	Leu	CCU	AGG	Pro	CAU	-	His	CGU	ICG	Arg
CUC	GAG		CCC	-		CAC	GUG		CGC	-	
CUA	UAG		CCA	**ncm^{5}UGG**		CAA	**mcm^{5}s^{2}UUG**	Gln	CGA	-	
CUG	-		CCG	-		CAG	CUG		CGG	CCG	
AUU	IAU	Ile	ACU	IGU	Thr	AAU	-	Asn	AGU	GCU	Ser
AUC	-		ACC	-		AAC	GUU		AGC		
AUA	ΨAΨ		ACA	**ncm^{5}UGU**		AAA	**mcm^{5}s^{2}UUU**	Lys	AGA	**mcm^{5}UCU**	Arg
AUG	CAU	Met	ACG	CGU		AAG	CUU		AGG	CCU	
GUU	IAC	Val	GCU	IGC	Ala	GAU	-	Asp	GGU	-	Gly
GUC	-		GCC	-		GAC	GUC		GGC	GCC	
GUA	**ncm^{5}UAC**		GCA	**ncm^{5}UGC**		GAA	**mcm^{5}s^{2}UUC**	Glu	GGA	**mcm^{5}UCC**	
GUG	CAC		GCG	-		GAG	CUC		GGG	CCC	

FIG. 1. Eleven *S. cerevisiae* tRNA species contain an xm^{5}U derivative at the wobble position. (A) Structures of mcm^{5}U, mcm^{5}s^{2}U, ncm^{5}U, and ncm^{5}Um. (B) The indicated tRNA species was isolated from either wild-type (UMY2893) or *elp3*Δ (UMY2916) cells, and their nucleoside compositions were analyzed by HPLC. The part of the chromatogram between 10 and 20 min is shown in each case. The arrow indicates expected retention time of ncm^{5}U (right panels). The small peak at this position represents an unrelated compound with an UV absorption spectrum different from that of ncm^{5}U. (C) The genetic code and distribution of cytoplasmic *S. cerevisiae* tRNAs. The anticodon sequences of the 42 different tRNA species (1 initiator and 41 elongator tRNAs) are indicated (21, 25, 32, 39). For anticodons with an uncharacterized RNA sequence, the primary sequence is shown. The initiator and elongator tRNA^{Met} species have identical anticodon sequences. The wobble rules suggest that an inosine (I_{34}) residue allows paring with U, C, and sometimes A. A tRNA with a G or its 2′-*O*-methyl derivative (Gm) at the wobble position should read U- and C-ending codons. Presence of a C_{34} residue or its 5-methyl (m^{5}C) or 2′-*O*-methyl (Cm) variant should only allow pairing with G. The pseudouridine (Ψ)-containing tRNA^{Ile} is presumably unable to pair with the methionine AUG codon. The anticodons containing an xm^{5}U derivative are shown in bold. In tRNA$^{Pro}_{A66}$, the A_{34} residue is most likely modified to I_{34} (see text). AU, absorbance units; wt, wild type.

number of the genes coding for tRNA$^{Gly}_{mcm^5UCC}$ and combined that with an *elp3*Δ allele. We deleted two of the three genes coding for tRNA$^{Gly}_{mcm^5UCC}$, which resulted in a strain with no apparent growth defect but with about a twofold decrease in the tRNA$^{Gly}_{mcm^5UCC}$ level (data not shown). Introduction of the *elp3*Δ allele into this strain did not generate a synergistic growth defect (data not shown), indicating that the wobble mcm^5 group does not affect the ability of a wild-type tRNA,

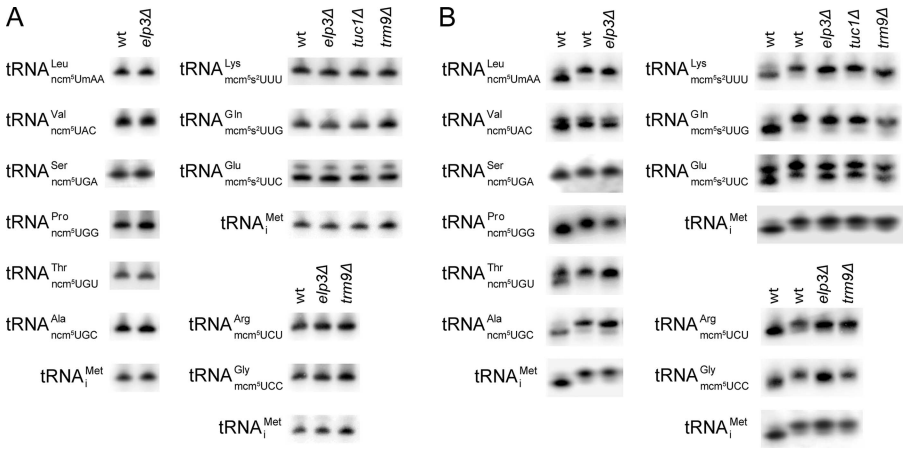


FIG. 2. Hypomodification induced by *elp3*Δ, *tuc1*Δ, or *trm9*Δ alleles does not reduce abundance or aminoacylation of any of the affected tRNA species. Northern blot analyses of total RNA isolated under either basic (A) or acidic (B) conditions from wild-type (W303-1B), *elp3*Δ (UMY2843), *tuc1*Δ (UMY3165), or *trm9*Δ (UMY3297) cells. After polyacrylamide gel electrophoresis and transfer under appropriate conditions, blots were probed for the U_{34}-containing tRNA of interest using ^{32}P-labeled oligonucleotides. To control for loading and indirect effects on aminoacylation, the blots were also probed for the C_{34}-containing initiator methionine tRNA ($tRNA_i^{Met}$). (A) Steady-state tRNA levels. (B) Aminoacylation levels. The first lane in each individual blot in panel B represents a deacylated wild-type RNA sample. The symbols for the U_{34}-containing tRNAs indicate the anticodon sequence of wild-type cells. The *elp3*Δ strain lacks the mcm^5 and ncm^5 side chains whereas the *tuc1*Δ strain lacks the s^2 groups. Cells deleted of *TRM9* lack the esterified methyl group of mcm^5 side chains. wt, wild type.

i.e., a nonsuppressor tRNA, to read the cognate A-ending codon.

The viability of a strain with a deletion of the single copy of $tRNA^{Arg}_{CCU}$ (27) [Fig. 3A, *tR*(*CCU*)*J*] or the two $tRNA^{Gly}_{CCC}$ genes [Fig. 3B, *tG*(*CCC*)*D* and *tG*(*CCC*)*O*] shows that $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ are capable of pairing with their corresponding G-ending codons. We will hereafter use the systematic tRNA gene names in figures since they indicate both the one-letter amino acid abbreviation and the primary sequence of the anticodon. To further assess the role of the mcm^5 group, we introduced an *elp3*Δ allele into a strain lacking either $tRNA^{Arg}_{CCU}$ or $tRNA^{Gly}_{CCC}$. Interestingly, synergistic growth defects were observed in both cases, suggesting that the presence of an mcm^5 side chain promotes reading of G-ending codons (Fig. 3A and B).

The observation that the wobble mcm^5 group in wild-type tRNAs seems to preferentially affect decoding of G-ending codons prompted us to reexamine the effect of the modification on the *SUP4*-encoded suppressor tRNA. This was accomplished by utilizing a plasmid-borne nonsense suppression assay, which is based on a low-copy-number plasmid carrying a *PGK1-LacZ* fusion (45, 46). The three nonsense suppression reporter constructs have one of the stop codons (UAA, UAG, or UGA) placed in frame at the junction of the *PGK1* and

TABLE 2. Steady-state levels of tRNAs with hypomodified wobble uridines

tRNA species[a]	Relative tRNA level[b]		
	*elp3*Δ	*tuc1*Δ	*trm9*Δ
$tRNA^{Leu}_{ncm^5UmAA}$	0.99 ± 0.13	ND	ND
$tRNA^{Val}_{ncm^5UAC}$	0.83 ± 0.04	ND	ND
$tRNA^{Ser}_{ncm^5UGA}$	0.97 ± 0.11	ND	ND
$tRNA^{Pro}_{ncm^5UGG}$	0.99 ± 0.20	ND	ND
$tRNA^{Thr}_{ncm^5UGU}$	0.85 ± 0.11	ND	ND
$tRNA^{Ala}_{ncm^5UGC}$	0.93 ± 0.12	ND	ND
$tRNA^{Arg}_{mcm^5UCU}$	0.95 ± 0.10	ND	0.79 ± 0.12
$tRNA^{Gly}_{mcm^5UCC}$	1.09 ± 0.07	ND	0.87 ± 0.08
$tRNA^{Gln}_{mcm^5s^2UUG}$	0.98 ± 0.09	1.04 ± 0.12	0.90 ± 0.13
$tRNA^{Lys}_{mcm^5s^2UUU}$	0.96 ± 0.27	1.02 ± 0.14	0.88 ± 0.27
$tRNA^{Glu}_{mcm^5s^2UUC}$	1.02 ± 0.13	1.06 ± 0.17	0.80 ± 0.15

[a] The tRNA symbol indicates the wild-type anticodon sequence.

[b] The signal of respective tRNA species was normalized to the $tRNA_i^{Met}$ signal and expressed relative to the corresponding value in the wild-type (wt) strain, which was set at 1. The values represent the average from the experiment shown in Fig. 2A and two additional independent experiments. The standard deviation is indicated. ND, not determined.

TABLE 3. In vivo aminoacylation levels of tRNAs with hypomodified wobble uridines

tRNA species[a]	In vivo aminoacylation level (%)[c]							
	Experiment 1				Experiment 2			
	wt	*elp3*Δ	*tuc1*Δ	*trm9*Δ	wt	*elp3*Δ	*tuc1*Δ	*trm9*Δ
$tRNA^{Leu}_{ncm^5UmAA}$	83	87	ND	ND	58	67	ND	ND
$tRNA^{Val}_{ncm^5UAC}$	88	92	ND	ND	86	86	ND	ND
$tRNA^{Ser}_{ncm^5UGA}$	61	61	ND	ND	82	76	ND	ND
$tRNA^{Pro}_{ncm^5UGG}$	69	76	ND	ND	46	52	ND	ND
$tRNA^{Thr}_{ncm^5UGU}$	80	82	ND	ND	67	68	ND	ND
$tRNA^{Ala}_{ncm^5UGC}$	80	79	ND	ND	53	62	ND	ND
$tRNA^{Arg}_{mcm^5UCU}$	67	68	ND	65	39	45	ND	50
$tRNA^{Gly}_{mcm^5UCC}$	84	87	ND	92	75	73	ND	80
$tRNA^{Gln}_{mcm^5s^2UUG}$	83	81	82	80	60	62	59	65
$tRNA^{Lys}_{mcm^5s^2UUU}$	79	81	86	84	61	56	63	61
$tRNA^{Glu}_{mcm^5s^2UUC}$[b]	85	83	85	83	94	92	91	94
$tRNA_i^{Met}$	72	71	71	68	58	59	56	54

[a] The tRNA symbol indicates the wild-type anticodon sequence.

[b] Two different aminoacylated species were observed using the $tRNA^{Glu}_{mcm^5s^2UUC}$ probe. The aminoacylation level for $tRNA^{Glu}_{mcm^5s^2UUC}$ was probably overestimated since the signal for the slower-migrating nonaminoacylated form is in close proximity to the signals for the two aminoacylated forms.

[c] wt, wild type; ND, not determined.

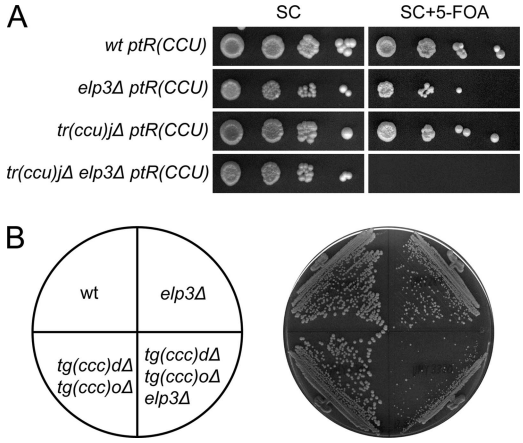


FIG. 3. The wobble mcm^5 side chain in $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ improves reading of G-ending codons. (A) Wild-type (W303-1B), *elp3*Δ (UMY2843), *tr*(*ccu*)*j*Δ (UMY3137), and *elp3*Δ *tr*(*ccu*)*j*Δ (UMY3136) cells carrying the low-copy-number *URA3* plasmid pRS316-*tR*(*CCU*)*J* were grown in liquid SC medium for 24 h. The cells were serially diluted, spotted onto SC plates and SC plates containing 5-fluoroorotic acid (5-FOA), and incubated for 3 days at 30°C. On 5-FOA-containing medium, only the cells that lost the *URA3* plasmid were able to grow (5). The *tR*(*CCU*)*J* gene codes for $tRNA^{Arg}_{CCU}$. (B) The wild-type (W303-1B), *elp3*Δ (UMY2843), *tg*(*ccc*)*d*Δ *tg*(*ccc*)*o*Δ (UMY3304), and *tg*(*ccc*)*d*Δ *tg*(*ccc*)*o*Δ *elp3*Δ (UMY3320) strains were streaked on a YEPD (yeast extract, peptone, dextrose) plate and incubated at 30°C for 2 days. The *tG*(*CCC*) genes code for $tRNA^{Gly}_{CCC}$. wt, wild type.

LacZ sequences. Thus, nonsense suppression can be quantified by determining the β-galactosidase activity in cells harboring a nonsense reporter construct and then comparing that to the activity in cells containing the control construct, which lacks a premature stop codon (45, 46). Analyses of wild-type, *SUP4*, and *SUP4 elp3*Δ strains transformed with the respective plasmid revealed that the presence of the *SUP4* allele caused read-through of the UAA and, to a lesser extent, the UAG codon (Table 4). As predicted from our previous results (22), lack of the mcm^5 group decreased suppression of the UAA codon. Interestingly, the influence of the mcm^5 group was even larger on the UAG codon (Table 4). These results validate our genetic approach and suggest that the function of an mcm^5U_{34} residue is not primarily to improve reading of the A-ending codons but to improve pairing with the codon ending with G.

Influence of mcm^5 and s^2 groups on the decoding properties of tRNA. The three mcm^5s^2U-containing tRNA species, $tRNA^{Gln}_{mcm^5s^2UUG}$, $tRNA^{Lys}_{mcm^5s^2UUU}$, and $tRNA^{Glu}_{mcm^5s^2UUC}$, decode in split codon boxes where the pyrimidine-ending codons are for another amino acid (Fig. 1C). In each of these codon boxes a C_{34}-containing tRNA species complementary to the G-ending codon is present ($tRNA^{Gln}_{CUG}$, $tRNA^{Lys}_{CUU}$, and $tRNA^{Glu}_{CUC}$), which is consistent with the suggestion that $mcm^5s^2U_{34}$-containing tRNA species preferentially decode triplets ending with A (33, 43, 54).

To examine the role of $mcm^5s^2U_{34}$ residues, strains with deletions of the single copy $tRNA^{Gln}_{CUG}$ [*tQ*(*CUG*)*M*] or the two $tRNA^{Glu}_{CUC}$ [*tE*(*CUC*)*I* and *tE*(*CUC*)*D*] genes were constructed. These strains were nonviable unless rescued by a wild-type copy of the respective tRNA gene on a plasmid (49) (Fig. 4A), suggesting that $tRNA^{Gln}_{mcm^5s^2UUG}$ and $tRNA^{Glu}_{mcm^5s^2UUC}$ are unable to read the codons ending with G. The evidence presented above that the mcm^5 group in $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ promoted reading of the AGG and GGG codons implied that the inability of mcm^5s^2U-containing tRNA species to read G-ending codons might be caused by a restricting effect of the s^2 group. However, introduction of a *tuc1*Δ allele, which abolishes formation of the s^2 but not the mcm^5 group (3), did not suppress the inviability of a strain lacking $tRNA^{Gln}_{CUG}$ or $tRNA^{Glu}_{CUC}$ (Fig. 4A). Moreover, introduction of an *elp3*Δ allele into a strain lacking either the $tRNA^{Gln}_{CUG}$ or the $tRNA^{Glu}_{CUC}$ species did not overcome the requirement for the rescuing plasmid (Fig. 4A). Thus, neither the mcm^5 nor the s^2 group in mcm^5s^2U-containing tRNA species is solely responsible for the inability to read G-ending codons.

We recently showed that the phenotypes of a strain lacking wobble mcm^5/ncm^5 and/or s^2 groups can be suppressed by increased dosage of the genes coding for the $tRNA^{Lys}$ and $tRNA^{Gln}$ species that normally contain mcm^5s^2U (3, 14). This prompted us to investigate whether the lethality of a strain lacking $tRNA^{Gln}_{CUG}$ or $tRNA^{Glu}_{CUC}$ could be suppressed by increased expression of the relevant U_{34}-containing tRNA species. Increased expression of the $tRNA^{Glu}$ species did not suppress the inviability of a strain lacking $tRNA^{Glu}_{CUC}$, regardless of whether the tRNA contained $mcm^5s^2U_{34}$, mcm^5U_{34}, or s^2U_{34} (Fig. 4B). However, overexpression of $tRNA^{Gln}_{mcm^5s^2UUG}$ suppressed the need for $tRNA^{Gln}_{CUG}$ in an otherwise wild-type background (Fig. 4B). This suppression was not observed in an *elp3*Δ or *tuc1*Δ background (Fig. 4B), suggesting that the mcm^5 and s^2 groups cooperatively improve pairing with G.

The wobble ncm^5 side chain in $tRNA^{Val}_{ncm^5UAC}$ is not required for efficient decoding of the A-ending codon. An ncm^5U_{34} residue is present in $tRNA^{Val}_{ncm^5UAC}$, $tRNA^{Ser}_{ncm^5UGA}$, $tRNA^{Pro}_{ncm^5UGG}$, $tRNA^{Thr}_{ncm^5UGU}$, and $tRNA^{Ala}_{ncm^5UGC}$, which all decode in family codon boxes (Fig. 1C). To assess the influence of a wobble ncm^5 group on the ability to read the A-ending codon, we reduced the copy number of the genes coding for $tRNA^{Val}_{ncm^5UAC}$ or $tRNA^{Ser}_{ncm^5UGA}$ and combined that with an *elp3*Δ allele. The strains with a deletion of one of the two genes coding for $tRNA^{Val}_{ncm^5UAC}$ or two of the three genes for $tRNA^{Ser}_{ncm^5UGA}$ showed no apparent growth defect although the steady-state level was reduced to about 50% ($tRNA^{Val}_{ncm^5UAC}$) or 30% ($tRNA^{Ser}_{ncm^5UGA}$) of that in wild type (data not shown). Introduction of the *elp3*Δ allele into any of these strains did not generate a synergistic growth defect (data not shown), indicating that an ncm^5U_{34}

TABLE 4. The wobble mcm^5 group in an ochre suppressor tRNA promotes reading of UAA and UAG stop codons

Termination codon	% Read-through[a]			Reduction (*n*-fold) in read-through (value for *SUP4* /value for *SUP4 elp3*)
	wt	*SUP4*	*SUP4 elp3*Δ	
UAA	0.10 ± 0.02	29.49 ± 9.03	11.31 ± 1.63	2.6
UAG	0.12 ± 0.03	1.39 ± 0.24	0.31 ± 0.04	4.5
UGA	0.10 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	1.0

[a] The β-galactosidase activity from cells harboring the indicated nonsense reporter construct was expressed as a percentage of the activity obtained in the corresponding cells carrying the control construct. The values represent the averages from six independent transformants. The standard deviation is indicated.

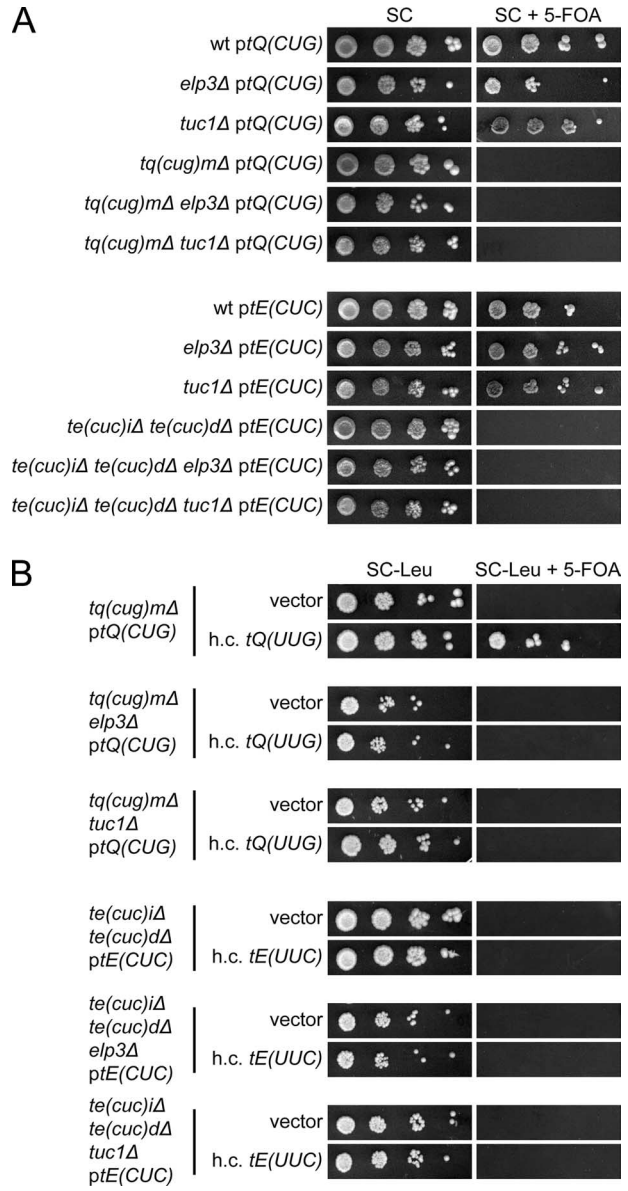


FIG. 4. Neither the mcm^5 nor the s^2 group restricts $tRNA^{Gln}_{mcm^5s^2UUG}$ and $tRNA^{Glu}_{mcm^5s^2UUC}$ to A-ending codons. (A) The appropriate strains (W303-1B, UMY2843, UMY3165, UMY3133, UMY3134, UMY3347, UMY3348, UMY3350, and UMY3352) carrying the indicated low-copy-number *URA3* plasmid were grown in liquid SC medium for 24 h. The cells were serially diluted, spotted onto SC plates and SC plates containing 5-fluoroorotic acid (5-FOA), and incubated at 30°C for 3 days. The *tQ*(*CUG*) and *tE*(*CUC*) genes code for $tRNA^{Gln}_{CUG}$ and $tRNA^{Glu}_{CUC}$, respectively. (B) The *tq*(*cug*)Δ, *te*(*cuc*)Δ, and their *elp3*Δ or *tuc1*Δ derivatives from panel A were transformed with an empty high-copy-number (h.c.) *LEU2* vector (10) or the same plasmid harboring a gene coding for the relevant U_{34}-containing tRNA [*tQ*(*UUG*) or *tE*(*UUC*)]. The transformants were grown in SC-Leu medium for 24 h, serially diluted, spotted onto SC-Leu plates and SC-Leu plates containing 5-FOA, and incubated at 30°C for 3 days. wt, wild type.

residue does not improve reading of A-ending codons. However, both the valine (GUN) and serine (UCN) family boxes harbor a tRNA species with an inosine (I) at the wobble position ($tRNA^{Val}_{IAC}$ and $tRNA^{Ser}_{IGA}$) (Fig. 1C). Since I_{34}-containing tRNAs may read not only codons ending with U and C but also those ending with A (11), it seemed possible that a synergistic growth defect could be masked by the ability of $tRNA^{Val}_{IAC}$ or $tRNA^{Ser}_{IGA}$ to read its respective A-ending codon (GUA and UCA). We cannot exclude this possibility for the UCA codon since a strain lacking the three genes coding for $tRNA^{Ser}_{ncm^5UGA}$ is viable (data not shown). In contrast, a strain with deletions of both $tRNA^{Val}_{ncm^5UAC}$ genes is inviable (data not shown), showing that $tRNA^{Val}_{IAC}$ cannot efficiently decode the GUA codon. This observation in combination with the above-mentioned lack of synergistic growth defect suggests that the ncm^5 side chain in $tRNA^{Val}_{ncm^5UAC}$ does not improve reading of the cognate A-ending codon.

A wobble ncm^5 side chain influences the ability to read G-ending codons. Two of the five family codon boxes where ncm$^5U_{34}$-containing tRNAs decode lack a tRNA with an anticodon complementary to the G-ending codon (proline CCN and alanine GCN boxes) (Fig. 1C), showing that an ncm^{5}U-containing tRNA is able to read such codons. However, a C_{34}-containing tRNA species with an anticodon complementary to the G-ending codon ($tRNA^{Ser}_{CGA}$, $tRNA^{Thr}_{CGU}$, and $tRNA^{Val}_{CAC}$) is present in the remaining three codon boxes (Fig. 1C). It was previously shown that the single-copy genes coding for $tRNA^{Ser}_{CGA}$ [*tS*(*CGA*)*C*] and $tRNA^{Thr}_{CGU}$ [*tT*(*CGU*)*K*] are essential for viability (9, 15). In contrast, a strain with deletions of the two genes coding for $tRNA^{Val}_{CAC}$ [*tV*(*CAC*)*D* and *tV*(*CAC*)*H*] is viable although with a growth defect (Fig. 5A). These data suggested that the ncm$^5U_{34}$-containing $tRNA^{Ser}_{ncm^5UGA}$ and $tRNA^{Thr}_{ncm^5UGU}$ do not efficiently read the UCG and ACG codons, whereas $tRNA^{Val}_{ncm^5UAC}$ can read the GUG codon.

To investigate the role of the ncm^5 group in $tRNA^{Ser}_{ncm^5UGA}$, $tRNA^{Thr}_{ncm^5UGU}$, and $tRNA^{Val}_{ncm^5UAC}$, we constructed strains lacking the C_{34}-containing $tRNA^{Ser}_{CGA}$, $tRNA^{Thr}_{CGU}$, or $tRNA^{Val}_{CAC}$ that harbored a wild-type copy of the respective C_{34}-containing tRNA gene on a plasmid. Introduction of the *elp3*Δ allele into a strain with a deletion of the $tRNA^{Val}_{CAC}$ genes generated a requirement for the plasmid (Fig. 5A), showing that the ncm^5 group in $tRNA^{Val}_{ncm^5UAC}$ is important for reading the GUG codons. Lack of $tRNA^{Ser}_{CGA}$ or $tRNA^{Thr}_{CGU}$ was lethal both in an *ELP3*$^+$ and an *elp3*Δ background (Fig. 5A), suggesting that neither the modified nor the unmodified $tRNA^{Ser}_{ncm^5UGA}$ and $tRNA^{Thr}_{ncm^5UGU}$ can efficiently read their respective G-ending codons. However, we previously showed that increased dosage of a gene coding for $tRNA^{Ser}_{ncm^5UGA}$ suppresses the lethality of a strain deficient for $tRNA^{Ser}_{CGA}$ (24) (Fig. 5B). Similarly, increased dosage of a $tRNA^{Thr}_{ncm^5UGU}$ gene suppressed the lethality of a strain lacking $tRNA^{Thr}_{CGU}$ (Fig. 5B). These dosage suppressions were not observed in an *elp3*Δ background (Fig. 5B), suggesting that a wobble ncm^5 group improves reading of G-ending codons also for these tRNAs. Based on these results, we conclude that the presence of the ncm^5 side chain improves reading of G-ending codons but that features other than the modification status of the wobble uridine determine the efficiency.

The wobble ncm^5 side chain in $tRNA^{Pro}_{ncm^5UGG}$ does not prevent reading of pyrimidine-ending codons. A tRNA with an unmodified U_{34} residue may, under some circumstances, pair with a codon ending with any nucleoside (31, 53). Interestingly, the only yeast tRNA species ($tRNA^{Leu}_{UAG}$) shown to have an unmodified wobble uridine (40) was able to read all four CUN

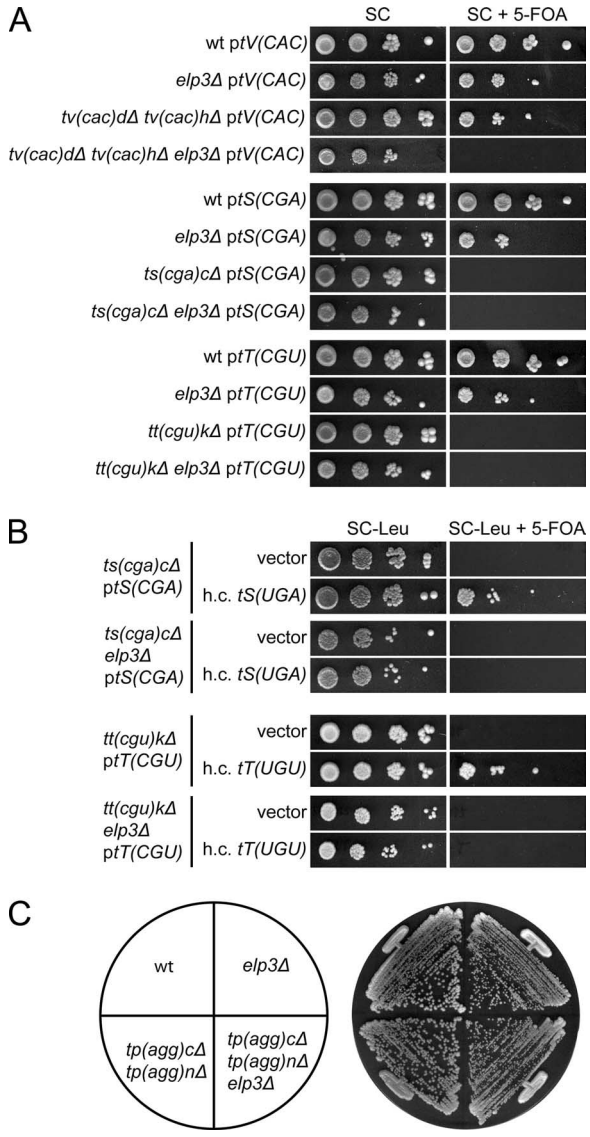


FIG. 5. A wobble ncm^5 side chain improves reading of codons ending with G but does not restrict the tRNA to purine-ending codons. (A) The appropriate strains (W303-1B, UMY2843, UMY3296, UMY3333, UMY3126, UMY3127, UMY3129, and UMY3130) carrying the indicated low-copy-number *URA3* plasmid were grown in liquid SC medium for 24 h. The cells were serially diluted, spotted onto SC plates and SC plates containing 5-fluoroorotic acid (5-FOA), and incubated at 30°C for 3 days. The *tV*(*CAC*), *tS*(*CGA*), and *tT*(*CGU*) genes code for $tRNA^{Val}_{CAC}$, $tRNA^{Ser}_{CGA}$, and $tRNA^{Thr}_{CGU}$, respectively. (B) The *ts*(*cga*)*c*Δ, *tt*(*cgu*)*k*Δ, and their *elp3*Δ derivatives from panel A were transformed with an empty high-copy-number (h.c.) *LEU2* vector (10) or the same plasmid harboring a gene coding for the relevant U_{34}-containing tRNA [*tS*(*UGA*) or *tT*(*UGU*)]. The transformants were grown in SC-Leu medium for 24 h, serially diluted, spotted onto SC-Leu plates and SC-Leu plates containing 5-FOA, and incubated at 30°C for 3 days. (C) The wild-type (W303-1B), *elp3*Δ (UMY2843), *tp*(*agg*)*c*Δ *tp*(*agg*)*n*Δ (UMY3343), and *tp*(*agg*)*c*Δ *tp*(*agg*)*n*Δ *elp3*Δ (UMY3368) strains were streaked on a YEPD (yeast extract, peptone, dextrose) plate and incubated at 30°C for 2 days. The *tP*(*AGG*) genes code for $tRNA^{Pro}_{AGG}$. wt, wild type.

codons in vitro (19, 51). Accordingly, a strain with a deletion of the gene encoding the other tRNA species in this box ($tRNA^{Leu}_{GAG}$) (Fig. 1C) is viable (data not shown). In an attempt to address whether this indiscriminate decoding is a unique feature of $tRNA^{Leu}_{UAG}$ or whether it would be a feature of other tRNAs harboring an unmodified U_{34}, we utilized the distribution of tRNA species in the proline family codon box (Fig. 1C). In this box, only two tRNA species are present, one contains ncm^{5}U ($tRNA^{Pro}_{ncm^5UGG}$), and the other has in the primary sequence an A at the wobble position. That an unmodified A_{34} is almost never found in tRNA and that I_{34} is present in the corresponding tRNA species in higher eukaryotes suggest that the A_{34} residue in the yeast species is also deaminated to I_{34} (28). Unexpectedly, a strain with deletions of the two $tRNA^{Pro}_{AGG}$ genes [*tP*(*AGG*)*C* and *tP*(*AGG*)*N*] was viable with no apparent growth defect (Fig. 5C), indicating that the ncm$^5U_{34}$-containing $tRNA^{Pro}_{ncm^5UGG}$ can read all four codons. Moreover, introduction of an *elp3*Δ allele did not generate a synergistic growth defect (Fig. 5C), suggesting that the ncm^5 group in $tRNA^{Pro}_{ncm^5UGG}$ has no influence on the ability to read the pyrimidine-ending codons.

The esterified methyl group of mcm^5 side chains has a modest effect on decoding. The biosynthesis of mcm^5 and ncm^5 side chains is likely to involve many steps and gene products (22). The last step in the formation of mcm^5 side chains is dependent on the Trm9 protein, which in vitro catalyzes formation of the methylester using *S*-adenosyl-methionine as the donor and tRNAs with a cm^5 group at U_{34} as substrates (26). Although, a *trm9*Δ strain was shown to lack methyl-esterified nucleosides, the identity of the hypomodified wobble nucleoside in the mutant was not clear (26). Analysis of the $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Glu}_{mcm^5s^2UUC}$ species isolated from a *trm9*Δ strain revealed that they did not contain the expected Trm9p substrates, cm^{5}U and cm^{5}s^{2}U, but that they contained ncm^{5}U and ncm^{5}s^{2}U (data not shown). It is not clear whether these nucleosides represent intermediates in the mcm^5 biosynthesis pathway or whether they reflect shunting of cm^5-modified nucleosides to the pathway responsible for ncm^5 formation. In analogy with the *elp3* mutant, no obvious reduction in abundance or aminoacylation level was observed in a *trm9*Δ strain (Fig. 2 and Tables 2 and 3).

A strain with a deletion of the *TRM9* gene does not show the same severe growth defect as *elp3*Δ or *tuc1*Δ mutants (Fig. 6 and data not shown). Furthermore, introduction of a *trm9*Δ allele does not prevent an ochre suppressor tRNA, which normally contains mcm^{5}U, from reading ochre stop codons (32). Consistent with this, we saw no or small synergistic effects when a *trm9*Δ allele was introduced into a strain containing only one $tRNA^{Gly}_{mcm^5UCC}$ gene or a strain deleted of the C_{34}-containing $tRNA^{Arg}_{CCU}$ or $tRNA^{Gly}_{CCC}$ gene (Fig. 6A and data not shown). Nevertheless, introduction of a *trm9*Δ allele into the *tuc1*Δ strain generated a small synergistic growth defect (data not shown), suggesting that the methyl ester is important for tRNA species that also contain an s^2 group at the wobble uridine. Although increased dosage of a $tRNA^{Gln}_{mcm^5s^2UUG}$ gene suppressed the need for the C_{34}-containing $tRNA^{Gln}_{CUG}$ in a *trm9*Δ background, the strain grew at a slower rate than the corresponding *TRM9*$^+$ strain, implying that the ncm^{5}s$^2U_{34}$-containing $tRNA^{Gln}$ is less capable of reading the CAG codon (Fig. 6B). We conclude that the lack of the esterified methyl group of mcm^5 side chains has a modest effect on the decoding properties of tRNA.

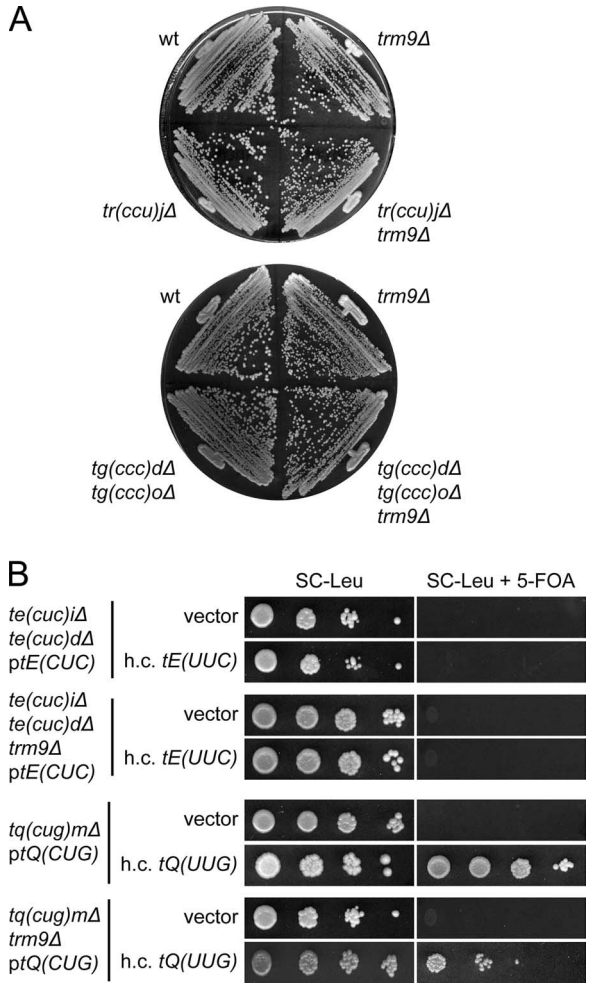


FIG. 6. Influence of the esterified methyl group of mcm^5 sidechains on the decoding properties of tRNA. (A) The indicated strains (W303-1B, UMY3297, UMY3137, UMY3358, UMY3304, and UMY3360) were streaked on a YEPD (yeast extract, peptone, dextrose) plate and incubated at 30°C for 2 days. (B) The indicated strains (UMY3348, UMY3354, UMY3134, or UMY3345) were transformed with an empty high-copy-number (h.c.) *LEU2* vector (10) or the same plasmid harboring a gene coding for the relevant U_{34}-containing tRNA [*tE*(*UUC*) or *tQ*(*UUG*)]. The transformants were grown in SC-Leu medium for 24 h, serially diluted, spotted onto SC-Leu plates and SC-Leu plates containing 5-fluoroorotic acid (5-FOA), and incubated at 30°C for 2 days. wt, wild type.

DISCUSSION

Analyses of the distribution of cytoplasmic tRNA species in eukaryotes have shown that they normally contain both U_{34}- and C_{34}-containing isoacceptors, suggesting that A- and G-ending codons are decoded by distinct tRNAs (13, 34, 38, 39). This observation suggested that eukaryotic organisms may not use U-G wobbling, since this would make the C_{34}-containing tRNA species functionally redundant (38). Further support for this view comes from the fact that the U_{34} residues are normally modified to an xm^5U derivative, which might restrict wobbling, and from the observation that tRNA species harboring such derivatives were unable to efficiently read G-ending codons in vitro (19, 33, 43, 50, 54). Here, we provide a comprehensive analysis of the decoding properties of tRNA species harboring an xm^5U derivative. In contrast to previous suggestions, we show that many eukaryotic U_{34}-containing tRNA species read G-ending codons (Fig. 7) and that the presence of an xm^5U derivative promotes this ability. These results also show that the ability of an xm^5U_{34}-containing tRNA to read the G-ending codon cannot be predicted from whether the codon box includes a C_{34}-containing tRNA.

An mcm^5U_{34} residue promotes decoding of G-ending codons. Based on an in vitro translation system, it was suggested that the yeast $tRNA^{Arg}_{mcm^5UCU}$ species is able to decode the AGA but not the AGG codon (50). These results are consistent with the notion that the mcm^5 group may prevent U-G wobbling. However, the observation that strains lacking the C_{34}-containing $tRNA^{Arg}_{CCU}$ or $tRNA^{Gly}_{CCC}$ species are viable (27) (Fig. 3A) shows that the mcm^5U_{34}-containing $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ are able to read the AGG and GGG codons in vivo. In fact, we found that the wobble mcm^5 group in $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Gly}_{mcm^5UCC}$ promotes reading of their respective G-ending codons (Fig. 3). A possible explanation for the contradictory results in vivo and in vitro is that the mcm^5 group at U_{34} may not significantly influence intrinsic differences in the efficiency by which the tRNA reads the A- or G-ending codon, i.e., it may only be possible to detect reading of the complementary codon in vitro. It cannot, however, be excluded that the inability of $tRNA^{Arg}_{mcm^5UCU}$ to read the AGG codon in vitro was caused by the fact that that *Escherichia coli* and not *S. cerevisiae* ribosomes were used in the translation system (50).

We did not detect a decrease in the ability of the hypomodified $tRNA^{Gly}_{mcm^5UCC}$ to read the GGA codon. This result contrasted our previous finding that the mcm^5 group in an ochre suppressor tRNA was required for suppression of the *ade2-1* and *can1-100* alleles (22). By utilizing a nonsense suppression reporter system, we confirmed that the modification in the suppressor tRNA improved reading of UAA codons (Table 4). However, the relative influence of the modification was even larger on the UAG codon, providing further support for the notion that an mcm^5U_{34} residue preferentially improves reading of the G-ending codon. It remains to be determined if the effect observed on the UAA codon is applicable to A-ending sense codons or if it is caused by the fact that the modification is present in an atypical context, i.e., in an altered anticodon.

An $mcm^5s^2U_{34}$ residue promotes decoding of A- and G-ending codons. The presence of an $mcm^5s^2U_{34}$ residue was originally proposed to allow the tRNA to efficiently read the cognate A-ending codon and simultaneously reduce the ability to pair with the G-ending codon (43, 54). However, more recent data have suggested that a mcm^5s^2U-containing tRNA may read both A- and G-ending codons (35). We have found here that neither $tRNA^{Gln}_{mcm^5s^2UUG}$ nor $tRNA^{Glu}_{mcm^5s^2UUC}$ can efficiently read its respective G-ending codon in vivo (Fig. 4A). However, $tRNA^{Gln}_{mcm^5s^2UUG}$ but not $tRNA^{Glu}_{mcm^5s^2UUC}$ can read the codon if its expression is increased (Fig. 4B). This ability of $tRNA^{Gln}_{mcm^5s^2UUG}$ required both the mcm^5 and s^2 groups, indicating that they cooperatively improve pairing with the CAG codon (Fig. 4B).

We previously showed that the growth defects of strains lacking mcm^5/ncm^5 and/or s^2 groups are suppressed by increased expression of $tRNA^{Gln}_{mcm^5s^2UUG}$ and $tRNA^{Lys}_{mcm^5s^2UUU}$ (3, 14).

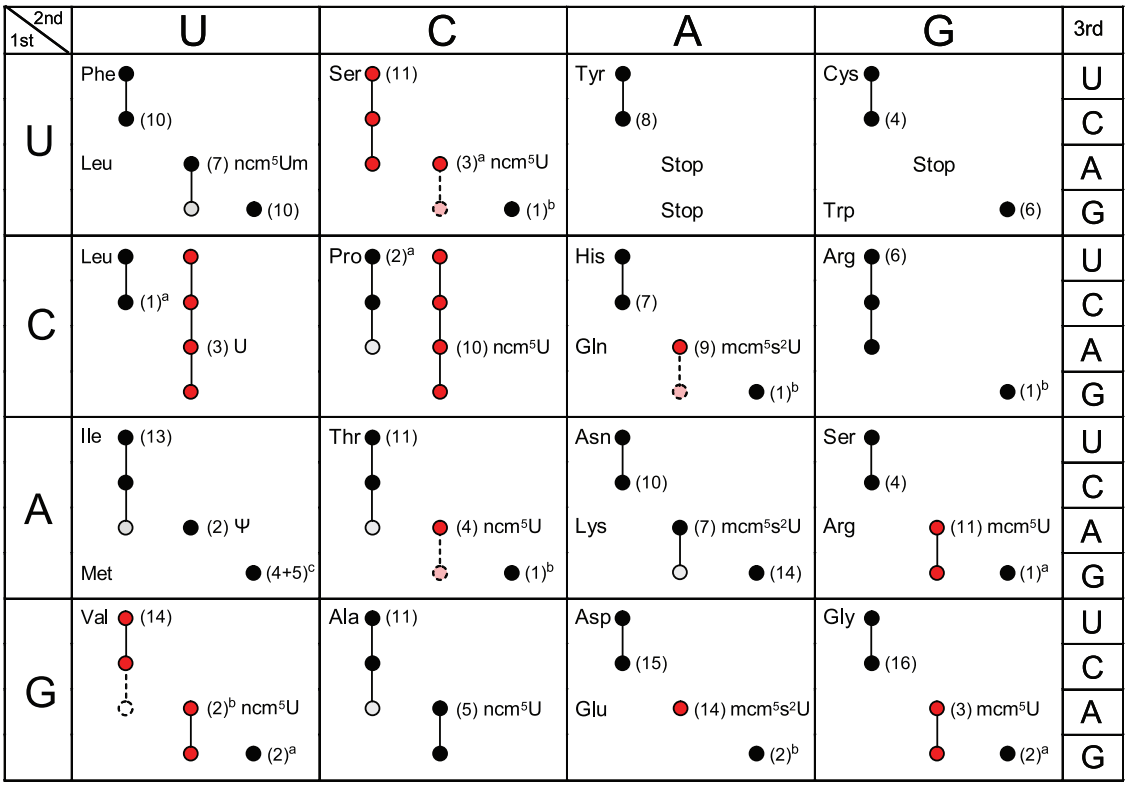


FIG. 7. The genetic code and decoding abilities of individual tRNA species. Codons read by a tRNA are indicated by circles and connecting lines. Red and pink circles represent tRNA species for which the decoding properties were investigated in this article. Pink circles connected with a dashed line indicate that the tRNA species reads the codon only when it is overexpressed. The empty dashed circle for $tRNA^{Val}_{IAC}$ is shown only to indicate that this inosine-containing tRNA species does not efficiently read the GUA codon. The nucleoside at the wobble position is given for the 13 wobble uridine-containing tRNA species. Black and gray circles represent decoding abilities predicted by the wobble hypothesis, the revised wobble rules, and the distribution of tRNA species. A gray circle indicates that the tRNA species is less likely to read the codon. The number of genes coding for a tRNA species is indicated next to the circle for the complementary codon. The following qualifications apply: a superscript a indicates that the gene(s) encoding the tRNA is nonessential; a superscript b indicates that the gene(s) encoding the tRNA is essential; where two values are given (superscript c) four genes code for $tRNA^{Met}_{i}$, and five code for $tRNA^{Met}_{m}$.

These data suggested that the lack of a wobble mcm^5 and/or s^2 group in $tRNA^{Gln}_{mcm^5s^2UUG}$ and $tRNA^{Lys}_{mcm^5s^2UUU}$ caused a reduced functionality and that this defect can be counteracted by increasing the tRNA levels. However, these studies did not determine if it was the A- or G-ending Gln and Lys codons that are poorly translated in the modification-deficient cells. The finding that the hypomodified $tRNA^{Gln}_{mcm^5s^2UUG}$ cannot read the CAG codon even when it is overexpressed indicates that the phenotypes are caused by poor translation of the A-ending codons (Fig. 4B). This also suggests that the relevant function of an $mcm^5s^2U_{34}$ residue is to improve reading of the codon ending with A. These results are consistent with a role for the modifications in promoting an appropriate anticodon conformation (12), which would improve pairing with both the A- and G-ending codon.

An ncm^5U_{34} residue promotes decoding of G-ending codons. An ncm^5U_{34} residue is found in tRNAs species decoding in codon boxes where all four codons specify the same amino acid (Fig. 7). Our results suggest that the function of the ncm^5 modification is to improve reading of G-ending codons and that any influence on reading of the A-ending codons is small (Fig. 5 and data not shown). However, the evidence that $tRNA^{Ser}_{ncm^5UGA}$ and $tRNA^{Thr}_{ncm^5UGU}$ are only able to read their respective G-ending codons when they are overexpressed suggests that features other than the modification status of the wobble uridine determine the decoding efficiency. Consistent with this hypothesis, the hypomodified forms of $tRNA^{Pro}_{ncm^5UGG}$ and $tRNA^{Ala}_{ncm^5UGC}$ must be able to read their respective G-ending codons efficiently since the major growth defect of an *elp3*Δ mutant is not caused by a reduced functionality of these tRNA species (14), even though there are no C_{34}-containing tRNA species for the proline CCG and alanine GCG codons (Fig. 7).

The observation that $tRNA^{Pro}_{ncm^5UGG}$ can read the four CCN codons in vivo (Fig. 5C) implied that the ncm^5U_{34} residue might extend the wobble capacity of the tRNA, analogous to the modified wobble nucleoside uridine-5-oxyacetic acid (cmo^5U) in bacteria. In fact, the *Salmonella enterica* $tRNA^{Pro}_{cmo^5UGG}$ species has been shown to read all four proline codons, and the modification promoted decoding of the CCU and CCC codons (36). However, the ability of yeast $tRNA^{Pro}_{ncm^5UGG}$ to read the CCU and CCC codons is independent of the ncm^5 group (Fig. 5C), implying that pairing with these codons involves a two-out-of-three interaction (30). The observation that the presence of a wobble ncm^5 group does not prevent pairing with pyrimidine-ending codons

provides a possible explanation to the wobble 2′-*O*-methyl group found in the only ncm^5-containing tRNA that decodes in a split codon box (Fig. 7). The $tRNA^{Leu}_{ncm^5UmAA}$ species was shown in an in vitro translation system to preferentially read the UUA codon, which is presumably due to the influence of the 2′-*O*-methyl group on the conformation of the wobble nucleoside and/or the anticodon (19, 31). Moreover, the observation that the tRNAs that normally harbor an mcm^5 group contain an ncm^5 group in a *trm9*Δ strain may provide a clue to the specific function of the esterified methyl group of mcm^5 side chains. In four of the five codon boxes where mcm^5-containing tRNA species decode, the pyrimidine-ending codons code for another amino acid (Fig. 7). It is therefore feasible that a mcm^5 group, in contrast to an ncm^5 group, would prevent misreading of codons ending with U or C and thereby improve the fidelity of translation. On the other hand, the minor growth defect of a *trm9*Δ mutant (Fig. 6 and data not shown) suggests that any influence on translational fidelity is relatively small.

Concluding remarks. The in vivo roles of modified wobble nucleosides are poorly understood. By utilizing genetic approaches, we have discovered several important features of eukaryotic xm^5U_{34} derivatives: (i) the presence of an mcm^5U_{34}, ncm^5U_{34}, or $mcm^5s^2U_{34}$ residue improves the ability of tRNA to read G-ending codons; (ii) an $mcm^5s^2U_{34}$ residue enhances the ability to decode the A-ending codon; (iii) an ncm^5U_{34} residue does not restrict tRNA to purine-ending codons; and (iv) the importance of a wobble modification depends on its context, which presumably includes structural attributes of the tRNA as well as properties of the codon-anticodon interaction. Although these general features are likely to be conserved in eukaryotes, the apparent requirement for the modifications in any given organism is likely to be influenced by variations in tRNA sequences, intracellular tRNA levels, and distribution of tRNA species.

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