A conserved modified wobble nucleoside (mcm^5s^2U) in lysyl-tRNA is required for viability in yeast

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ABSTRACT

Transfer RNAs specific for Gln, Lys, and Glu from all organisms (except *Mycoplasma*) and organelles have a 2-thiouridine derivative (xm^5s^2U) as wobble nucleoside. These tRNAs read the A- and G-ending codons in the split codon boxes His/Gln, Asn/Lys, and Asp/Glu. In eukaryotic cytoplasmic tRNAs the conserved constituent (xm^5-) in position 5 of uridine is 5-methoxycarbonylmethyl (mcm^5). A protein (Tuc1p) from yeast resembling the bacterial protein TtcA, which is required for the synthesis of 2-thiocytidine in position 32 of the tRNA, was shown instead to be required for the synthesis of 2-thiouridine in the wobble position (position 34). Apparently, an ancient member of the TtcA family has evolved to thiolate U34 in tRNAs of organisms from the domains Eukarya and Archaea. Deletion of the *TUC1* gene together with a deletion of the *ELP3* gene, which results in the lack of the mcm^5 side chain, removes all modifications from the wobble uridine derivatives of the cytoplasmic tRNAs specific for Gln, Lys, and Glu, and is lethal to the cell. Since excess of the unmodified form of these three tRNAs rescued the double mutant *elp3 tuc1*, the primary function of mcm^5s^2U34 seems to be to improve the efficiency to read the cognate codons rather than to prevent mis-sense errors. Surprisingly, overexpression of the mcm^5s^2U-lacking $tRNA^{Lys}$ alone was sufficient to restore viability of the double mutant.

Keywords: modification; mcm^5s^2U; tRNA; wobble nucleoside; translation

INTRODUCTION

Transfer RNA from all organisms contains modified nucleosides, which are derivatives of the four major nucleosides adenosine (A), guanosine (G), cytosine (C), and uridine (U). So far, about 100 different modified nucleosides in RNA have been characterized (http://medstat.med.utah.edu/RNAmods/). Two positions in tRNA—the wobble position (position 34) and the nucleoside next to and 3′ of the anticodon (position 37)—are frequently modified (Björk 1998). Modifications in these two positions appear to be required for proper decoding of the message. When tRNAs have uridine (U) in the wobble position, this U residue is almost universally modified; i.e., an unmodified U is normally not present in the wobble position. When tRNAs containing modified uridines in the wobble position read codons of the split codon boxes, which code for more than one amino acid, the wobble position modifications not only contain an addition to the 5-carbon of U, but also have other features by which they may be grouped into three general categories: (1) those that are thiolated at the 2-carbon of U; (2) those that contain a ribose methylation; and (3) those that have no additional modification other than on the 5-carbon (Fig. 1). All tRNAs that fall into the first of these groups, i.e., those that are thiolated, are specific for the split codon boxes that contain the codons for Gln, Lys, and Glu (Fig. 2). These tRNAs contain a uridine (U35) 3′ and adjacent to the wobble position, since they decode codons containing an A as second nucleoside of the codon (denoted AII); (NI, NII, and NIII denote the first, second, and third nucleoside of the codon; and N34, N35, and N36 denote the first, second, and third nucleoside of the anticodon; thus, N34 [wobble nucleoside] pairs with NIII, N35 with NII, and N36 with NI). Note that in all organisms (except *Mycoplasma*) and in all organelles studied to date there are no tRNAs containing U35 reading codons ending in A that are not thiolated at the wobble uridine. Apparently, tRNAs with U34–U35 in their anticodons make poor interaction with the A-rich codons, and therefore require a modification of the wobble nucleoside. If this interpretation is correct, we would predict that an organism having an unmodified U as

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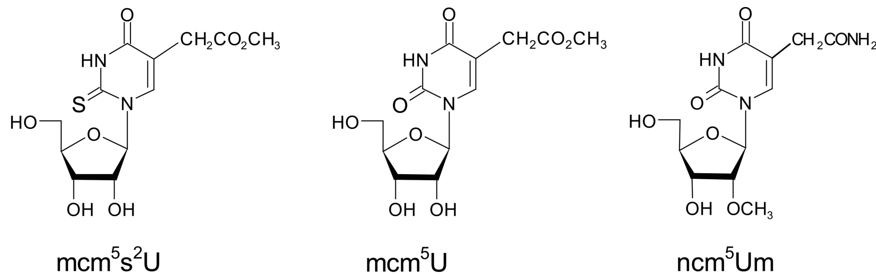

FIGURE 1. Modified wobble uridines present in yeast tRNA reading twofold degenerate codon boxes (Fig. 2). 5-methylcarboxymethyl-2-thiouridine (mcm^{5}s^{2}U), 5-methylcarboxy-methyl-uridine (mcm^{5}U), and 5-carbamoylmethyl-2′-*O*-methyluridine (ncm^{5}Um).

wobble nucleoside in these tRNAs would be detrimental to cell growth.

Transfer RNAs having a U34–U35 in their anticodons are specific for Gln, Lys, and Glu codons, and have a modification of the type xm^{5}s^{2}U (x = any substitution) in the wobble position. In bacteria, these tRNAs have 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U34) as the wobble nucleoside. The *mnmE* and *mnmA* genes are required for the synthesis of the side chain at position 5 and sulfur at position 2, respectively (Björk and Hagervall 2005). The mnm^{5}s^{2}U nucleoside is also present in tRNA from Archaea, but so far, mnm^{5}s^{2}U34 has not been identified as a wobble nucleoside in any purified tRNA species from organisms of this domain of life, although there is strong circumstantial evidence that this is the case (Gupta 1984; McCloskey et al. 2001). In eukaryotes, the corresponding tRNAs have 5-methoxycarbonylmethyl-2-thiourdine (mcm^{5}s^{2}U34) as wobble nucleoside. In yeast, the synthesis of the side chain at position 5 requires several proteins including Elp3p (Huang et al. 2005). In order to test the above-mentioned prediction that an unmodified U as wobble nucleoside is detrimental to yeast, it was necessary to first identify a gene whose product is required for the insertion of sulfur at position 2 of U in the wobble position of these tRNAs. Although yeast has a homolog (*MTU1*) to the bacterial *mnmA* gene, this gene is not involved in the thiolation of cytoplasmic tRNA, but only in mitochondrial tRNA (Umeda et al. 2005). The product of the bacterial gene *ttcA* is required in the synthesis of 2-thiocytidine (s^{2}C) at position 32 in a few tRNAs species (Jäger et al. 2004). We identified a gene (*TUC1*) in the yeast *Saccharomyces cerevisiae* that is similar to the bacterial *ttcA* gene, although no s^{2}C32 is present in yeast tRNA. We could show that rather than synthesizing s^{2}C32, the *TUC1* gene is required for the thiolation of U in the wobble position of cytoplasmic tRNAs specific for Gln, Lys, and Glu. A double mutant (*elp3::KanMX4 tuc1::TRP1*), which lacks both the s^2 and mcm^5 groups, cannot survive at a normal level of these tRNA species. However, an excess level of hypomodified tRNA$^{Lys}_{UUU}$ restored viability of the double mutant, although growth was very poor. Overexpression of hypomodified tRNA$^{Gln}_{UUG}$, but not of tRNA$^{Glu}_{UUC}$, together with overexpressed hypomodified tRNA$^{Lys}_{UUU}$ improved growth, but not to that of the wild-type strain. Our results suggest that the major role of the modification of wobble U is to enable tRNAs specific for Gln and Lys to read their cognate codons more efficiently rather than to increase their accuracy, since increased concentration of these tRNAs, still having an unmodified U in the wobble position, rescued growth of the double mutant (*elp3::KanMX4 tuc1::TRP1*).

Second position of codon

First position of codon (5′ end)	U	C	A	G	Third position of codon (3′ end)
U	G PHE; ncm^{5}Um LEU; C	I SER; U^b; C	G TYR STOP	G CYS STOP TRP; C	U C A G
C	G U LEU	I PRO; ncm^{5}U	G HIS; mcm^{5}s^{2}U^a GLN; C	I ARG; C	U C A G
A	I ILE; Ψ MET; C	I THR; U^b; C	G ASN; mcm^{5}s^{2}U LYS; C	G SER; mcm^{5}U ARG; C	U C A G
G	I VAL; ncm^{5}U; C	I ALA; U^b	G ASP; mcm^{5}s^{2}U GLU; C	G GLY; mcm^{5}U^a; C	U C A G

FIGURE 2. Modified nucleosides in the wobble position and the coding capacities of the corresponding tRNAs. Letters outside the box, to the *left*, at *top*, and to the *right* indicate the first, second, and third position of the codon, respectively. Circles connected by a line, or a single circle, represent one tRNA species. A filled circle indicates the capability of that tRNA to base pair with a particular codon, either by Watson–Crick or by wobble according to the current wobble rules (Yokoyama and Nishimura 1995). An open circle suggests a restricted base pairing. To the *right* of each tRNA, the modification at positions 34 (the wobble position) is shown. Data compiled from Johansson and Byström (2005). Thus, tRNA specific for Lys, having mcm^{5}s^{2}U as wobble nucleoside, pairs well with AAA (●) but less well with AAG (○). (*A*) Presence of mcm^{5}s^{2}U in tRNA^{Gln} and mcm^{5}U in tRNA^{Gly} as wobble nucleosides has been determined (Lu et al. 2005) (M.J.O. Johansson, pers. comm.) (*B*) tRNAs specific for Ser, Thr, and Ala have ncm^{5}U, which synthesis is dependent on *ELP3* (M.J.O. Johansson, pers. comm.).

RESULTS

The yeast protein Ygl211p, resembling the bacterial s^2C32-forming protein TtcA, is required for s^2U34 formation in cytoplasmic yeast tRNA

The bacterial TtcA protein, which is required for the synthesis of s^2C at position 32 of a subset of tRNAs in *Escherichia coli*, belongs to a protein family with members from all three phylogenetic domains (Jäger et al. 2004). These proteins can be divided into two phylogenetic groups, where bacterial TtcA homologs are in Group 1 and eukaryotic and archaeal homologs are in Group 2 (Fig. 3). Blast analysis with *E. coli* TtcA toward the *S. cerevisiae* genome revealed that the protein coded by the *YGL211* gene appears to be a homolog of the *E. coli* TtcA, suggesting that the yeast Ygl211p is involved in the synthesis of s^2C32 in yeast tRNA (Ygl211p has 23% identical and 46% conserved amino acids compared with *E. coli* TtcA). However, no biochemical evidence exists for the presence of s^2C in *S. cerevisiae*. If s^2C was in fact present in yeast tRNA at a level found in tRNA from *E. coli*, it should be readily identifiable, yet is never observed in bulk tRNA samples (data not shown). Thus, it is likely that the Ygl211p is not involved in the synthesis of s^2C32, but rather in some other sulfur transfer reaction.

The *MTU1* gene has been demonstrated to be required for the synthesis of the s^2 group of mnm^5s^2U34 in mitochondria (Umeda et al. 2005). Intriguingly, absence of Mtu1p does not affect the synthesis of mcm^5s^2U34 in cytoplasmic tRNA, despite the fact that this gene is extremely similar to MnmA from *E. coli*, which is required for the synthesis of s^2U derivatives in this organism (Umeda et al. 2005; data not shown). Because the s^2C32 and s^2U34 modifications both involve 2-carbon thiolations of nucleosides in the anticodon loop of tRNAs, as well as the remarkably similar primary sequences of TtcA and Ygl211p (Fig. 3), *YGL211* was considered as a candidate for the gene required for the synthesis of s^2U in yeast cytoplasmic tRNAs. We have renamed *YGL211* as *TUC1* (thiolation of uridine in cytoplasmic tRNA).

We analyzed both a strain from the yeast deletion collection (Research Genetics) and a deletion strain with a disruption covering the *TUC1* ORF that we constructed in the W303-1B strain background. The results from these two strains were indistinguishable. Growth of the *tuc1::TRP1* strain was discernibly different from that of the wild-type strain cultivated on YEPD agar plates at 30°C (Fig. 4). HPLC analysis of nucleosides obtained from bulk tRNA from wild-type and *tuc1::TRP1* strains revealed two specific changes in the base composition of the mutant tRNA when monitored at 254 nm. First, the presence of mcm^5s^2U is abolished (Table 1) with a concomitant increase in the levels of methylcarboxymethyluridine (mcm^5U). To improve the sensitivity of the analysis, $tRNA^{Glu}_{mcm5s2UUC}$ was purified from the *tuc1* mutant, but still no mcm^5s^2U was observed (data not shown). The only sulfur atoms previously known to exist in cytoplasmic yeast tRNA are in the context of mcm^5s^2U34 modifications present in $tRNA^{Gln}_{mcm5s2UUG}$, $tRNA^{Lys}_{mcm5s2UUU}$, and $tRNA^{Glu}_{mcm5s2UUC}$. In the absence of an s^2U sulfurtransferase, mcm^5U should accumulate on Gln, Lys, and Glu tRNAs, explaining the increased level of this modified nucleoside in the tRNA from the *tuc1::TRP1* strain (Table 1). This mcm^5U migrates close to and partially overlaps Um (Fig. 5). The disappearance of mcm^5s^2U34 in the *tuc1::TRP1* strain is more clearly visualized in the chromatogram by monitoring the absorbance at 314 nm (Table 1), as thiolated nucleosides generally absorb well at this wavelength, while nonthiolated nucleosides normally do not (Fig. 5). Additionally, at 314 nm it also becomes apparent that another compound with a shorter retention time than mcm^5s^2U is abolished in tRNA from the *tuc1::TRP1* strain (Fig. 5; Table 1). This compound is far less abundant than the standard modifications in yeast tRNA and its appearance is not observed at 254 nm as it is masked by more prominent compounds. It is clearly a thiolated derivative whose formation is *TUC1* dependent, and it has been tentatively identified as an intermediate (cm^5s^2U) in the synthesis of mcm^5s^2U (Fig. 5). *ELP3* is required for the formation of the modifications at the 5-carbon of U34 in tRNA (Huang et al. 2005). Accordingly, cm^5s^2U was also absent in the *elp3::KanMX4* mutant, further supporting that this compound is an intermediate in the synthesis of mcm^5s^2U (Table 1). Thus, this *TUC1*- and *ELP3*-dependent compound appears to be an intermediate in the biosynthesis of mcm^5s^2U34. As expected, we observed the appearance of s^2U in the *elp3::KanMX4* mutant, as has also been shown before (Huang et al. 2005). We conclude that the Tuc1p protein is required for the thiolation step in the synthesis of mcm^5s^2U34 in yeast tRNA, although it resembles the bacterial s^2C32-forming enzyme, TtcA.

The double mutant *elp3::KanMX4 tuc1::TRP1* is not viable, but is rescued by overexpression of only hypomodified $tRNA^{Lys}_{UUU}$

The three cytoplasmic tRNAs, which are affected by the loss of the *TUC1* gene, are each members of split codon boxes and they decode codons of the general type NAA and can wobble onto NAG codons (Fig. 2). Because numerous genes have been demonstrated to be required for formation of the mcm^5 group of mcm^5s^2U (Huang et al. 2005), it should be possible to effectively strip the uridines in the wobble position in $tRNA^{Gln}_{mcm5s2UUG}$, $tRNA^{Lys}_{mcm5s2UUU}$, and $tRNA^{Glu}_{mcm5s2UUC}$ of both the mcm^5 and the s^2 modification by combining a deletion of one of these genes (e.g., *ELP3*) with a *TUC1* disruption. However, since such a *elp3tuc1*

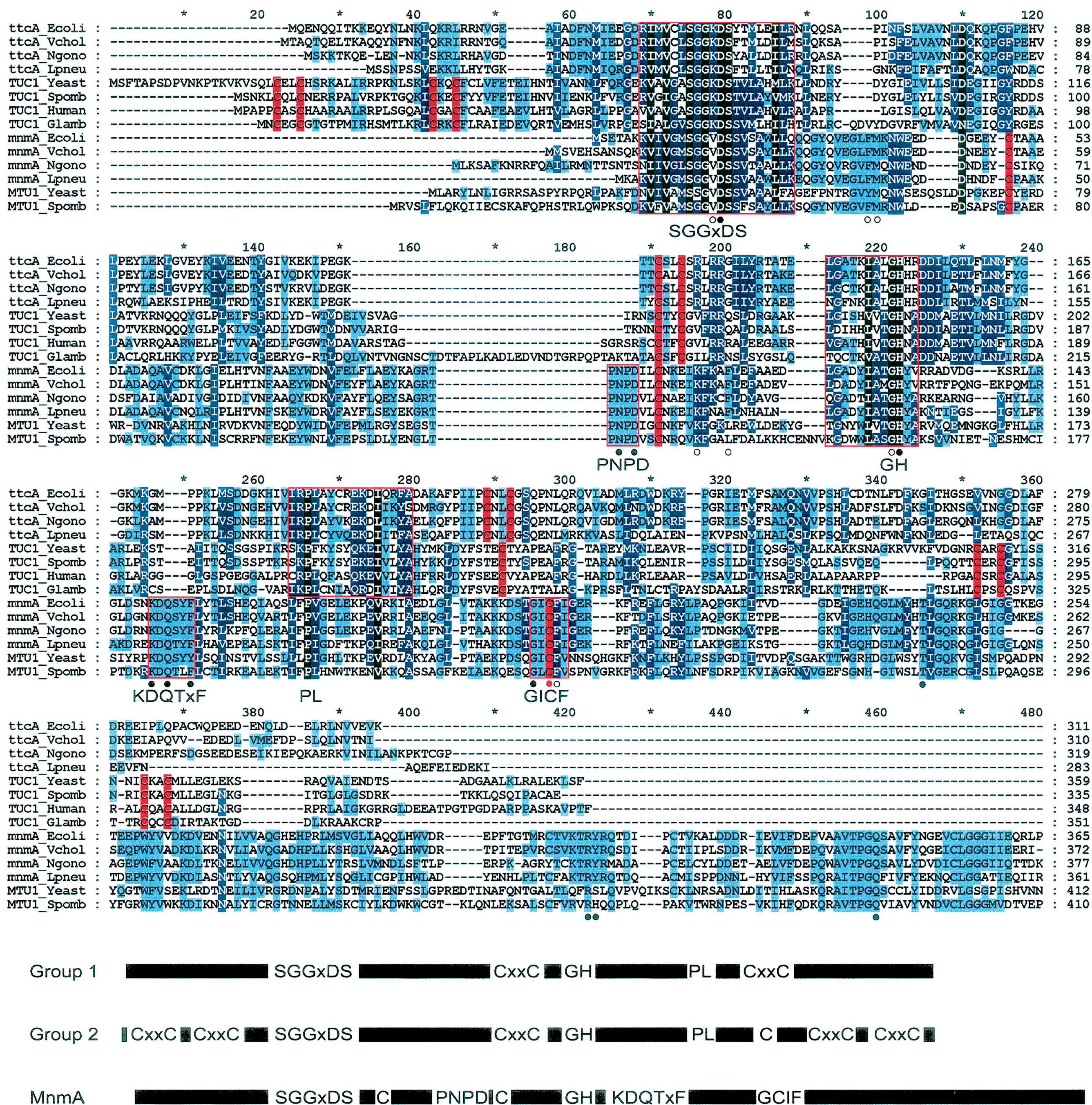

FIGURE 3. Sequence alignment of four TtcA and four Tuc1p homologs and six MnmA/Mtu1p proteins. Letters shaded black, dark-blue, and light-blue indicate that the indicated amino acid is found in this position in at least 100%, 55%, and 25% of the sequences in the alignment, respectively. Letters shaded red indicate conserved cysteine residues. Cys-X_1-X_2 -Cys motifs in the central domain of Groups 1 and 2 and in the flanking regions in Group 2 are, consequently, seen as two red stripes close to each other. Conserved regions thought to be important for binding and catalytic function are boxed in red. Amino acids identified by Numata et al. (2006) important for binding of the tRNA substrate (black dots) and for catalytic function (open circles), as well as the two sulphur coordinating cysteines (C102 and C199 in *E. coli* MnmA, red dots) are indicated. At *bottom* is shown a schematic comparison of Group 1 (TtcA) and Group 2 (Ygl211p, renamed to Tuc1p, see text) as suggested by Jäger et al. (2004). The central part of MnmA, which is required for the synthesis of s^2U34 in bacteria, is distinctly different from Groups 1 and 2 and is similar to the yeast protein Mtu1p, which is responsible for s^2U34 formation in mitochondria (Umeda et al. 2005). The central domains of both Group 1 and Group 2 consist of the PP-loop (SGGxDS) motif, a Cys-X_1-X_2-Cys motif, a GH motif, and a PL motif. In addition to the conserved central domain, Group 2 proteins have two Cys-X_1-X_2-Cys motifs on each side of the central domain. Note that MnmA has the PP-loop motif and GH motif, but otherwise is not similar to any of the TtcA family proteins, but instead has conserved regions absent in Groups 1/2 thiolases.

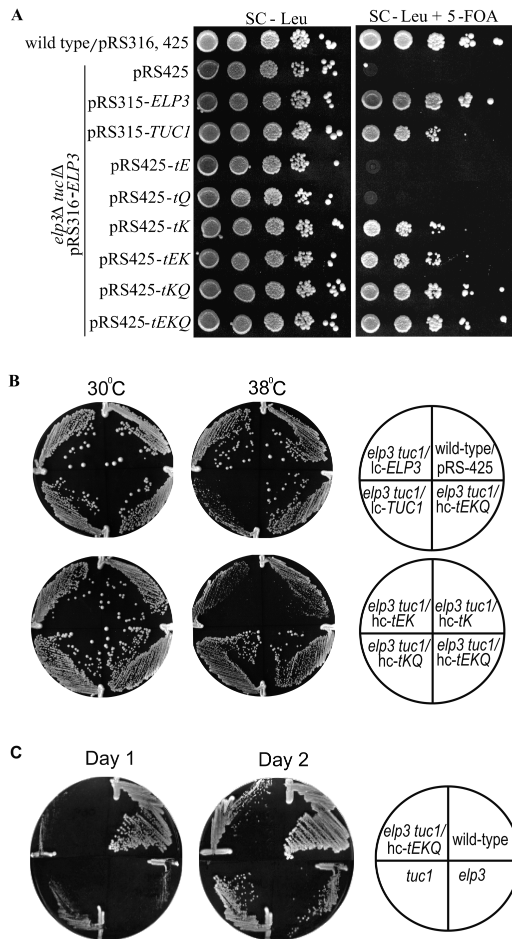

FIGURE 4. Increased expression of tRNAs normally having mcm^{5}s^{2}U suppresses lethality induced by lack of this modification of wobble uridines. (*A*) Strains containing the *elp3::KanMX4 tuc1::TRP1* mutations and carrying the *URA3* plasmid pRS316-*ELP3* and the *LEU2* plasmids pRS425, pRS315-*ELP3*, pRS-425-*tE(UUC)*, pRS425-*tQ(UUG)*, pRS425-*tK(UUU)*, pRS425-*tK(UUU)-tQ(UUG)*, pRS425-*tK(UUU)-tE(UUC)*, or pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)* (*tE*, *tK* and *tQ* indicate the genes for $\text{tRNA}^{\text{Glu}}_{\text{mcm5s2UUC}}$, $\text{tRNA}^{\text{Lys}}_{\text{mcm5s2UUU}}$, and $\text{tRNA}^{\text{Gln}}_{\text{mcm5s2UUG}}$, respectively), were grown in synthetic complete medium lacking leucine (SC-leu). Strains were diluted to 3×10^7 cells/mL, 10-fold serially diluted, and 5 μL was spotted onto either SC-leu plates or SC-leu plates containing 5-fluoroorotic acid (5-FOA). These plates were incubated at 30°C for 2 and 3 d, respectively. Cells containing a *URA3* plasmid are unable to grow on 5-FOA-containing medium, with the result that these strains only contain the indicated *LEU2* vectors when plated on 5-FOA plates (Boeke et al. 1984). (*B*) Growth of wild-type (W303-1B) and of the double mutant *elp3::KanMX4 tuc1::TRP1* containing plasmids harboring genes encoding various tRNAs on SC-plates lacking leucine. Cells were picked from SC-Leu+5-FOA plates (Fig. 4A) and restreaked on SC plates lacking Leu. Plates were incubated at 30°C or at 38°C for 2.5 d. Plasmid pRS315 and pRS425 are denoted lc (low copy) and hc (high copy), respectively. (*C*) Growth of wild-type (W303–1B), *tuc1::TRP1* (YMP006), *elp3::KanMX4* (UMY2843), or the double mutant *tuc1::TRP1*, *elp3::KanMX4*/ pRS425-*tEKQ* (UMY3400) on rich medium (YEPD). Plasmid pRS425-*tEKQ* is denoted hc-*tEKQ* and its presence results in overexpression of $\text{tRNA}^{\text{Glu}}_{\text{mcm5s2UUC}}$, $\text{tRNA}^{\text{Lys}}_{\text{mcm5s2UUU}}$, and $\text{tRNA}^{\text{Gln}}_{\text{mcm5s2UUG}}$. Growth at 30°C was scored after 1 or 2 d.

double mutant might not be viable, the double mutant was generated in a cross where it was rescued by a wild-type *ELP3* gene located on a *URA3* plasmid. This strain, which also is *leu2*, was transformed with a *LEU2* vector having *ELP3*, *TUC1*, or no chromosomal gene inserted (empty vector). To investigate whether or not these strains could survive without the *URA3* plasmid carrying the *ELP3* gene, they were grown on a medium containing 5-fluoro-orotic acid (5-FOA). These cells, being Ura$^+$, i.e., having the *URA3* plasmid, are unable to grow on a medium containing 5-FOA (Boeke et al. 1984). Growth of cells was obtained on the 5-FOA plates in the presence of the *LEU2* plasmids harboring the wild-type allele of *TUC1* or *ELP3*, but not together with the *LEU2* plasmid without the chromosomal gene insertion (empty vector) (Fig. 4). Thus, the double mutant is not viable. Recently, it has been shown that overexpression of $\text{tRNA}^{\text{Lys}}_{\text{mcm5s2UUU}}$ and $\text{tRNA}^{\text{Gln}}_{\text{mcm5s2UUG}}$ counteracted the various phenotypes induced by the *elp3::KanMX4* mutation (Esberg et al. 2006). This result prompted us to test whether a double mutant was viable in the presence of plasmid harboring the genes for the three tRNAs normally having mcm^{5}s^{2}U. Indeed, stable constructs were also obtained if tRNAs specific for Gln, Lys, and Glu were overexpressed when the *elp3::KanMX4* and *tuc1::TRP1* mutations were combined (Fig. 4). Presence of the plasmid harboring the genes for $\text{tRNA}^{\text{Gln}}_{\text{mcm5s2UUG}}$, $\text{tRNA}^{\text{Lys}}_{\text{mcm5s2UUU}}$, and $\text{tRNA}^{\text{Glu}}_{\text{mcm5s2UUC}}$ resulted in over-expression of these tRNAs (Fig. 6). However, the strain (UMY3400) overexpressing these tRNAs grew less well than the wild-type strain W303-1B (Fig. 4). Thus, even an excess of the hypomodified forms (see below) of the tRNA normally having mcm^{5}s^{2}U did not fully counteract the growth defect mediated by the lack of mcm^{5}s^{2}U.

To further evaluate whether or not overexpression of all three tRNAs normally having mcm^{5}s^{2}U as wobble nucle-oside was required for viability, plasmids with different combinations of genes encoding the three mcm^{5}s^{2}U-containing tRNAs were introduced into the double mutant containing the *URA3* plasmid harboring the *ELP3* gene (Fig. 4). When plasmids containing only one of the genes for these three tRNAs was present in conjunction with the complementing *URA3 ELP3* plasmid, only cells containing the plasmid harboring the gene for $\text{tRNA}^{\text{Lys}}_{\text{mcm5s2UUU}}$ resulted in colonies on the 5-FOA plates. Thus, such cells could grow in the absence of the complementing *ELP3* gene (Fig. 4). Overexpressed hypomodified $\text{tRNA}^{\text{Gln}}_{\text{UUG}}$, but not overexpressed hypomodified $\text{tRNA}^{\text{Glu}}_{\text{UUC}}$, together with overexpressed hypomodified $\text{tRNA}^{\text{Lys}}_{\text{UUU}}$ stimulated growth (Fig. 4). However, even excess of all three tRNAs did not still restore growth to that of the wild-type level on rich medium (Fig. 4). We conclude that viability of the double mutant requires excess of only hypomodified $\text{tRNA}^{\text{Lys}}_{\text{UUU}}$. Moreover, excess of hypomodified $\text{tRNA}^{\text{Gln}}_{\text{UUG}}$ together with excess of hypomodified $\text{tRNA}^{\text{Lys}}_{\text{UUU}}$ further

TABLE 1. HPLC analysis of modified nucleosides of tRNAs from wild-type and various mutants defective in the synthesis of mcm^5s^2U

Compounds	W303-1B	UMY2843	YMP006	UMY3400
ncm^5U[a]	0.029	<0.001[b]	0.030	<0.001[b]
mcm^5U[a]	0.022	N/D[c]	0.058	N/D[c]
mcm^5s^2U[a]	0.022	<0.001[b]	<0.001[b]	<0.001[b]
ncm^5UmA[a]	0.017	<0.001[b]	0.018	<0.001[b]
Ψ[a]	1.0	1.0	1.0	1.0
cm^5s^2U[d]	0.106	<0.01[b]	<0.01[b]	<0.01[b]
s^2U[d]	<0.01	0.043	<0.01[b]	<0.01[b]
mcm^5s^2U[d]	0.500	<0.01[b]	<0.01[b]	<0.01[b]
ac^4C[d]	1.0	1.0	1.0	1.0

Analyzed tRNAs were from strains: W303-1B (wt), UMY2843 (*elp3::KanMX4*), YMP006 (*tuc1::TRP1*), and UMY3400 (*elp3::KanMX4 tuc1::TRP1*/ pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*).
[a]Analysis of ncm^5U, mcm^5U, mcm^5s^2U, and ncm^5UmA at 254 nm using Ψ as internal standard (AU of Ψ: W303-1B = 1.65×10^6; UMY2843 = 2.61×10^6; YMP006 = 1.69×10^6; and YMU3400 = 1.75×10^6).
[b]In all cases, except for mcm^5U, a straight base line was observed where the indicated compounds should migrate (c.f. Fig. 5 in the case of strain UMY3400). At 254 and 314 nm, a level of 0.001 and 0.01, respectively, should have been easily detected.
[c]Figure 5 shows that mcm^5U is not well separated from Um. The values given for strains W303-1B and YMP006 are from a manual integration, but contributions from overlapping Um could not be completely avoided. (N/D) No peak was observed at the retention time of mcm^5U observed in tRNA from the wild type (see figure).
[d]Analysis of cm^5s^2U, s^2U, and mcm^5s^2U at 314 nm using ac^4C as an internal standard (AU of ac^4C: W303-1B = 41, 258; UMY2843 = 55, 836; YMP006 = 32, 506; UMY3400 = 36, 929).

stimulated growth of the double mutant *elp3::KanMX4 tuc1::TRP1*.

The double mutant *elp3::KanMX4 tuc1::TRP1* in the presence of excess hypomodified $tRNA^{Gln}_{UUG}$, $tRNA^{Lys}_{UUU}$, and $tRNA^{Glu}_{UUC}$ lacks mcm^5s^2U

As stated above, it was not possible to obtain the double mutant *elp3::KanMX4 tuc1::TRP1* except in the presence of excess hypomodified $tRNA^{Gln}_{UUG}$, $tRNA^{Lys}_{UUU}$, and $tRNA^{Glu}_{UUC}$ as in strain UMY3400. HPLC analysis of nucleosides obtained from bulk tRNA purified from this strain revealed five specific changes in nucleoside composition (Table 1). First, the disappearance of all mcm^5s^2U from tRNA, as expected; second, the absence of mcm^5U; third, the absence of ncm^5U; fourth, the disappearance of a compound with a retention time slightly longer than t^6A (Fig. 5). We suggest that this compound is the dinucleotide ncm^5UmpA originating from $tRNA^{Leu}$, which has the anticodon sequence $ncm^5UmpApA$. The 2′-*O*-methylation to the sugar of this nucleoside renders it less susceptible to endonucleolytic cleavage by nuclease P1 and a significant fraction of dinucleotides is retained using our digestion procedure. The complete digestion product, ncm^5Um, is masked by other more prominent peaks in the total tRNA digest. Fifth, the disappearance of the intermediate is tentatively identified as cm^5s^2U above (Table 1). These five nucleosides were all expected to disappear in the *elp3::KanMX4 tuc1::TRP1* double mutant, as each one is abolished in one or both of the single mutants *elp3::KanMX4* or *tuc1::TRP*. It should also be noted that the residual s^2U present in the *elp3::KanMX4* mutant is absent in the *elp3::KanMX4 tuc1::TRP1* double mutant (Table 1).

Aminoacylation and stability of $tRNA^{Glu}_{mcm5s2UUC}$, $tRNA^{Gln}_{mcm5s2UUG}$, and $tRNA^{Lys}_{mcm5s2UUU}$ are not affected by hypomodification

Modifications in the wobble position may in some cases improve the aminoacylation of tRNA (Giege et al. 1998). Therefore, the severe phenotype of the double mutant might be explained by low concentration of aminoacylated hypomodified tRNAs. However, this is not the case, since hypomodified $tRNA^{Gln}_{UUC}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Lys}_{UUU}$ had the same charging levels as the ones observed in the wild type (Table 2). The mutation in *elp3::KanMX4* abolishes the synthesis of all xm^5U derivatives (Fig. 2) (Huang et al. 2005), and such a modification deficiency does not influence the charging of these tRNAs (data not shown). Moreover, the wobble nucleosides in $tRNA^{Glu}_{mcm5s2UUC}$, $tRNA^{Gln}_{mcm5s2UUG}$, and $tRNA^{Lys}_{mcm5s2UUU}$ from yeast do not influence the aminoacylation in vitro, although the wobble nucleoside mnm^5s^2U34 in the corresponding tRNAs from *E. coli* is an identity element in aminoacylation (Giege et al. 1998).

Defects in tRNA may also influence its stability; and the severe growth phenotype induced by the hypomodification might be caused by extensive degradation of these tRNAs, resulting in low steady-state levels of hypomodified $tRNA^{Glu}_{UUC}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Lys}_{UUU}$. To test the stability of the hypomodified tRNAs in the double mutant, we compared the steady state levels of these tRNAs overproduced in the wild-type background (strain UMY3463) with the overproduced levels of the hypomodified tRNAs in the double mutant (strain UMY3400). The steady-state levels of the hypomodified tRNAs were not, in any case, lower than the corresponding fully modified tRNA, suggesting

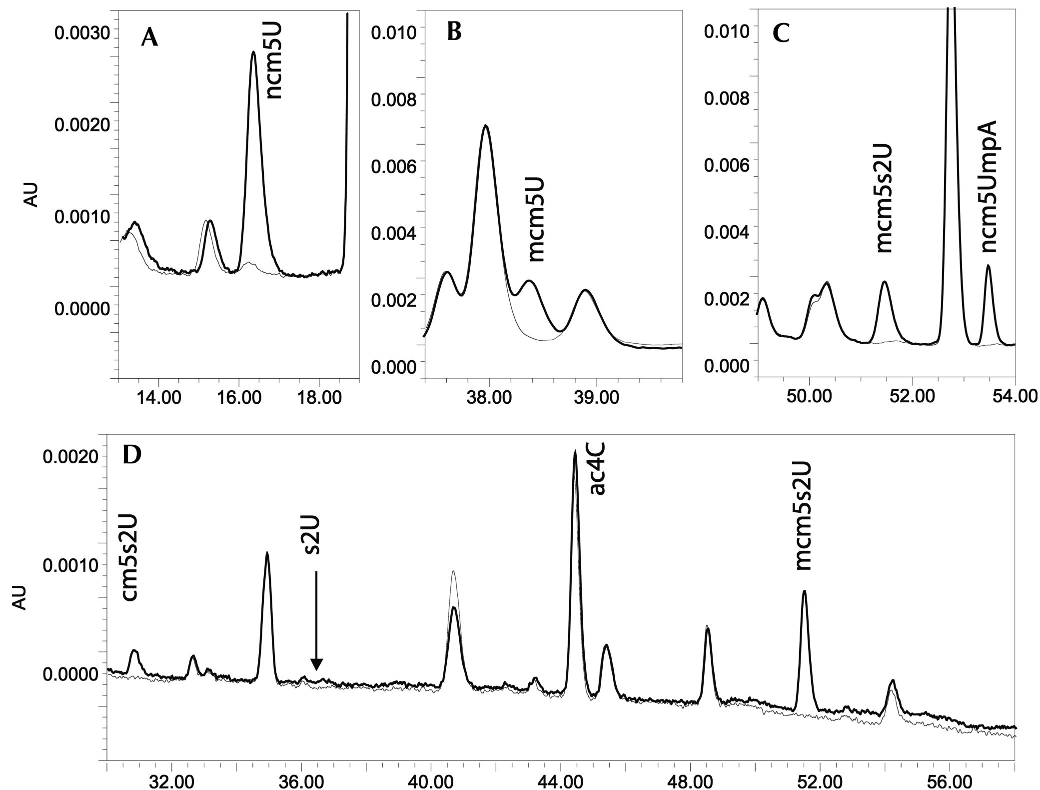

FIGURE 5. HPLC analysis of modified nucleosides of tRNA from W303-1B (wild-type; thick line) and from strain UMY3400 (*elp3::KanMX4 tuc1::TRP/pRS425-tE[UUC]-tK[UUU]-tQ[UUG]*) (*tE*, *tK* and *tQ* indicate the genes for $tRNA^{Glu}_{mcm5s2UUC}$, $tRNA^{Lys}_{mcm5s2UUU}$, and $tRNA^{Gln}_{mcm5s2UUG}$; thin line). Transfer RNA was degraded to nucleosides and the distribution of nucleosides was analyzed by HPLC. (*A–C*) The 13–19 min region, 37–41 min region, and 49.5–54 min region, respectively, of the HPLC chromatogram monitored at 254 nm. (*D*) The region 30–58 min was monitored at 314 nm. The expected position of s^2U is indicated at *bottom*, and this compound was observed only in tRNA from UMY2843 (*elp3::KanMX4*). The identities of cm^5s^2U and ncm^5s^2UmpA are tentative, since no markers for these compounds were available.

that the lack of this modification did not significantly reduce the stability of these tRNAs (Fig. 6).

We conclude that the induced severe growth phenotype due to lack of mcm^5s^2U is caused neither by poor aminoacylation nor by a reduced steady-state level of the hypomodified tRNAs. Therefore, the lethality caused by the two mutations should primarily be caused by a poor decoding of the message.

DISCUSSION

The identification of the *TUC1* gene as responsible for the formation of the sulfur group of the conserved wobble nucleoside mcm^5s^2U34 allowed us to construct a yeast strain devoid of both the mcm^5 and the s^2 modifications in $tRNA^{Gln}_{mcm5s2UUG}$, $tRNA^{Lys}_{mcm5s2UUU}$, and $tRNA^{Glu}_{mcm5s2UUC}$, which read codons in the His/Gln, Asn/Lys, and Asp/Glu split codon boxes, respectively (Figs. 2, 5; Table 1). This strain, which has an unmodified U as wobble nucleoside in these tRNAs, is not viable unless an excess of an unmodified form of one of these tRNAs, $tRNA^{Lys}_{mcm5s2UUU}$, is present (Fig. 4). The presence of excess hypomodified $tRNA^{Gln}_{UUG}$ together with hypomodified $tRNA^{Lys}_{UUU}$ stimulated growth further. However, an excess of the unmodified form of all three of these tRNAs did not restore the growth to that of the wild-type level, demonstrating that mcm^5s^2U deficiency exerts a severe growth defect even in the presence of excess hypomodified tRNA (Fig. 4). Importantly, neither the aminoacylation nor the stability of these tRNAs were affected by the hypomodification. Taken together, these results suggest that the primary effect of mcm^5s^2U34 is not to reduce mis-sense errors caused by reading the noncognate codons ending with U or C in the split codon boxes, but to improve the efficiency of reading the cognate codon ending with A (the G-ending codons are read also by an alternative tRNA) (Fig. 2).

A possible explanation for the nonviability of the double mutant *elp3::KanMX4 tuc1::TRP1* would be that Elp3p and Tuc1p have some uncharacterized function(s) beside being required for the synthesis of mcm^5s^2U. If so, deficiency in these unknown activities of these proteins would cause the nonviability of the double mutant and not the hypomodification of the tRNA. However, the fact that excess of the hypomodified form of the mcm^5s^2U-containing tRNAs counteracted the lethality induced by the mutations *elp3::KanMX4* and *tuc1::TRP1* argues against

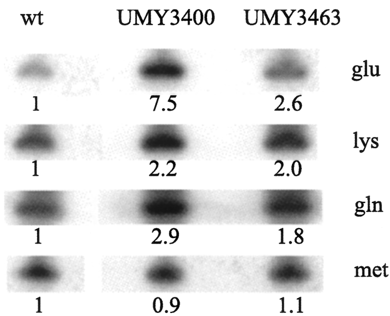

FIGURE 6. Levels of $tRNA^{Glu}_{mcm5s2UUC}$, $tRNA^{Gln}_{mcm5s2UUG}$, and $tRNA^{Lys}_{mcm5s2UUU}$ in strains W303-1B (wt), UMY3400 (*elp3::KanMX4 tuc1::TRP1*/pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*), and UMY3463 (*ELP3 TUC1*/pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*). The tRNA levels were monitored by Northern hybridization using radioactive oligonucleotides complementary to nucleotides 57–72 of $tRNA^{Gln}$, 57–73 of $tRNA^{Lys}$, 52–72 of $tRNA^{Glu}$, and 30–49 of $tRNA^{Met}$. Use of the last oligonucleotide, which monitors the levels of a tRNA not having mcm^5s^2U, served as controls for how much material was loaded on the gel. The numbers below each lane represent the levels relative to the level of the corresponding tRNAs in strain W303-1B (wild type).

such an explanation and rather suggests that it is a translational defect due to mcm^5s^2U deficiency that causes nonviability of the double mutant *elp3::KanMX4 tuc1::TRP1*.

Formation of s^2U34 in tRNA constitutes an interesting example of convergent evolution. This modification is present in hypermodified form in tRNAs specific for, $tRNA^{Gln}$, $tRNA^{Lys}$, and $tRNA^{Glu}$ from all organisms except *Mycoplasma* and in all organelles tested to date. With the discovery that *TUC1* is required for the synthesis of s^2U34, it becomes evident that it may serve the same role as MnmA in bacteria. The yeast Tuc1p, which thiolates U34, is more similar to the bacterial TtcA, which thiolates C32, than to MnmA (Fig. 3). Thus, it appears unlikely that Tuc1p has been derived from MnmA, even though MnmA is universally present in Bacteria and Eukarya; but rather, it has evolved from an earlier form of the nonessential bacterial protein TtcA, which makes the different, although related, tRNA modification s^2C32.

Whereas modification at position 5 of U is present in many tRNAs, the thiolation of U is only present in tRNAs reading the A- and G-ending codons in the His/Gln, Asn/Lys, and Asp/Glu split codon boxes, apparently making this modification of special importance to these tRNAs. Unlike tRNAs decoding in all other codon boxes, tRNAs decoding in the His/Gln, Asn/Lys, and Asp/Glu split codon boxes have U35. Since uracil is the base with the lowest stacking potential, the anticodon of the tRNAs reading the A- and G-ending codons in these split codon boxes are, when unmodified, inherently unstable, since the first two encoded nucleosides in the anticodon of these tRNAs are U34 and U35. Structural analyses using various model systems have revealed that the xm^5s^2 modification improves stacking and induces a proper conformation of the anticodon loop adapting it to efficient decoding (Yokoyama et al. 1979, 1985; Houssier et al. 1988; Kumar and Davis 1997; Grosjean et al. 1998; Ashraf et al. 1999; Sundaram et al. 2000; Durant et al. 2005; Agris et al. 2006). The lethality induced by lack of mcm^5s^2U, as observed, supports this suggestion and demonstrates the pivotal role that this conserved modification has on the viability of yeast. Interestingly, an excess of hypomodified $tRNA^{Lys}_{UUU}$ by itself restored viability to the *elp3::KanMX4 tuc1::TRP1* double mutant (Fig. 4), suggesting that the mcm^5s^2U modification is of special importance to this tRNA. The reason for the pivotal role of mcm^5s^2U in this tRNA is most likely because it is the only tRNA having the anticodon sequence U34–U35–U36, which, unmodified, has a poor stacking capacity and is highly flexible. In fact, the unmodified $tRNA^{Lys}_{UUU}$ does not form a canonical anticodon loop unless it contains mcm^5s^2U (Ashraf et al. 1999; Durant et al. 2005).

Although the mcm^5 group by itself has only limited impact on the remodeling of the anticodon, and thereby its stability, compared with the s^2 group (Durant et al. 2005), lack of the mcm^5 modification in the *elp3::KanMX4* mutant reduced growth more than that caused by the s^2 deficiency in the *tuc1::TRP1* mutant (Fig. 4). An explanation of this apparent inconsistency may be that the various xm^5 groups are present in many more tRNAs than the s^2 group is (Fig. 1), and the final phenotypic outcome may be an additive effect by the xm^5 deficiency of all of the xm^5-containing tRNAs. This also explains why overexpression of unmodified $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Glu}_{UUC}$ did not fully suppress the growth phenotype to that exhibited by the wild type.

TABLE 2. Relative levels of aminoacylated tRNA

Strain	$tRNA^{Glu}_{mcm5s2UUC}$	$tRNA^{Gln}_{mcm5s2UUG}$	$tRNA^{Lys}_{mcm5s2UUU}$
W303-1B (wild type)	**1.0**	**1.0**	**1.0**
UMY2843 (*elp3::KanMX4*)	1.0	1.0	0.9
YMP006 (*tuc1::TRP1*)	0.9	0.9	0.9
UMY3400 (*elp3::KanMX4 tuc1::TRP1*/pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*)	1.0	1.0	1.0

Average of two close independent experiments. The charging levels of $tRNA^{Glu}_{mcm5s2UUC}$, $tRNA^{Gln}_{mcm5s2UUG}$, and $tRNA^{Lys}_{mcm5s2UUU}$ in the wild type were 87%, 87%, and 84%, respectively.

A unmodified U34 may base pair with UIII and CIII under certain conditions (Lim 1994; Yokoyama and Nishimura 1995), and structural data of the decoding center on the 30S ribosomal subunit show that there are no constraints precluding a U34–UIII or U34–CIII pairing (Murphy et al. 2004). Such decoding can be allowed in the family codon boxes because it would not induce any mis-sense errors. Similar decoding by U34 is not allowed in the split codon family boxes His/Gln, Asn/Lys, and Asp/Glu, since such base pairing would result in mis-sense errors (see Fig. 2). It is, therefore, reasonable to suppose that the ubiquitous presence of the xm^5s^2U modification in tRNA reading codons in these split codon boxes are there to prevent mis-sense errors. It follows that the major cause of the nonviability of the *elp3::KanMX4 tuc1::TRP1* double mutant could be the misreading of the U- and C-ending codons in the split codon boxes His/Gln, Asn/Lys, and Asp/Glu by U34 (Fig. 2) (as discussed above). Alternatively, the mcm^5 and s^2 modifications of the wobble uridine in these tRNAs may be present primarily to improve the decoding efficiency of the cognate codons. It is possible that a combination of both of these alternatives is in operation. One way to increase the reading efficiency of the cognate codons would be to increase the concentration of the corresponding hypomodified tRNAs, provided that such an increase of the aminoacylated tRNA would also result in a corresponding increase of the ternary complex. Indeed, excess of unmodified variants of the $tRNA^{Lys}_{mcm5s2UUU}$ encoded from a plasmid restored viability of the *elp3::KanMX4 tuc1::TRP1* double mutant (Fig. 4), and the presence of hypomodified $tRNA^{Gln}_{mcm5s2UUG}$ partially suppressed the poor growth obtained by the presence of $tRNA^{Lys}_{mcm5s2UUU}$ (Figs. 4). As stated above, complete suppression by over-expression of $tRNA^{Lys}_{UUU}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Glu}_{UUC}$ cannot be expected, since the *elp3* mutation also removes the xm^5 modification of eight other tRNA species (Fig. 1). We therefore suggest that the primary function of mcm^5s^2U34 is to improve the reading of the cognate codons rather than to prevent mis-sense errors, since an excess of unmodified tRNA specific for Gln, Lys, and Glu should increase rather than decrease misreading. A direct test for the hypothesis that these modifications should improve reading fidelity did not support such a role of the modifications (Hagervall et al. 1998). Moreover, deficiency of the wobble nucleoside 5-taurinomethyl-2-thiouridine (τm^5s^2U34) of mitochondrial $tRNA^{Lys}$, which induces the mitochondrial disease myoclonus epilepsy associated with ragged-red fibers (MERRF), reduces the efficiency of reading the cognate codons AAA/G (Yasukawa et al. 2001). These and other experiments (for review, see Björk and Hagervall 2005) support our suggestion that the primary function of mcm^5s^2U34 is to improve the reading of the cognate codons. This is also consistent with the postulated function of the xm^5s^2U modifications as deduced from structural data (see above) (Murphy et al. 2004; Durant et al. 2005). However, in certain organisms, some of these split codon boxes are read only with an xm^5s^2U34-containing tRNA (e.g., in the Asn/Lys and Asp/Glu split codon boxes in *E. coli* and *Salmonella enterica* Serovar Typhimurium). Thus, in these cases the efficiency of this modified nucleoside to wobble toward GIII must be sufficient, and no other tRNA is required. Yeast (Fig. 2), as well as many other organisms, has other tRNAs with C34 as wobble nucleoside, which efficiently read the G-ending codons in the His/Gln, Asn/Lys, and Asp/Glu split codon boxes, suggesting that in these organisms the ability of the xm^5s^2U34 to base pair with G is limited.

MATERIALS AND METHODS

Media and yeast strains

Media and genetic procedures used have been described (Burke et al. 2000). Yeast strains are listed in Table 3. A one-step gene replacement was performed to disrupt the *TUC1* gene (Brachmann et al. 1998). Oligonucleotides5′-TTTGGCGATGAGACGATATGG TAAGAGTAAAGCAAAGGA ACCGTCAGATTGTACTGAGAGTG CAC and 5′-ATTATGTTACGCTGCATTCTT CTACTGCGAGCTA TATATATGTCACTGTGCGGTATTTCACACCG were used to PCR amplify a DNA fragment containing the *TRP1* marker and 45 nt of *TUC1* flanking sequences on both sides. Strain UMY3373, in which both the *TUC1* and *ELP3* genes are disrupted and where the viability of the strain is dependent on an *ELP3* gene located on the *URA3*-based plasmid pRS316, was constructed in a cross between strains YMP006 and UMY2843. The diploid was transformed with plasmid pRS316-*ELP3* and in the concomitant tetrad analysis, offspring were identified that were $G418^R$, Trp^+, and Ura^+, which is indicative that the spore had the *elp3::KanMX4 tuc1::TRP1* alleles and harbored the pRS316-*ELP3* plasmid. The construction of the double mutant *elp3::KanMX4 tuc1::TRP1* harboring plasmids encoding various tRNA genes is described below.

Plasmid shuffling assay

In strain UMY3373, the endogenous *TUC1* and *ELP3* genes are disrupted and the viability of the strain is dependent on an *URA3*-based plasmid carrying the *ELP3* gene (pRS316-*ELP3*). These strains were transformed with an *LEU2* plasmid carrying no insert (pRS425), *ELP3* (pRS315-*ELP3*), *TUC1* (pRS315-*TUC1*), *tE(UUC)* (pRS425-*tE*), *tQ(UUG)* (pRS425-*tQ*), *tK(UUU)* (pRS425-*tK*), *tE(UUC) tK(UUU)* (pRS425-*tEK*), *tK(UUU) tQ(UUG)* (pRS425-*tKQ*), or *tE(UUC) tK(UUU) tQ(UUG)* genes (pRS425-*tEKQ*) (Lu et al. 2005). To investigate whether or not the strains were able to grow in the absence of the *URA3-ELP3* plasmid, strains were first grown in synthetic complete medium lacking leucine (SC-Leu), and thereafter serially diluted on SC-Leu plates and SC-Leu plates containing 5-fluoroorotic acid (5-FOA). Plates were incubated at 30°C. Cells maintaining the *URA3* plasmid are unable to grow on plates containing 5-FOA (Boeke et al. 1984). Strains capable of growing in the absence of the *URA3-ELP3*-rescuing plasmid were analyzed for growth at 30°C and 38°C.

Analysis of modified nucleosides in tRNA

Cells were grown in YEPD medium at 25°C to about OD_{600} of 1.0. In all cases, the cultures used for tRNA preparations were

TABLE 3. *Saccharomyces cerevisiae* strains

Strain	Genotype	Reference
W303-1B	MATα *trp1-1 ura3-1 leu2-3,112 ade2-1 his3–11,15 can1-100*	(Fiorentini et al. 1997)
UMY2843	MATα *elp3::KanMX4 trp1-1 ura3-1 leu2-3,112 ade2-1 his3-11,15 can1-100*	(Lu et al. 2005)
YMP006	(UMY3164) MATa *tuc1::TRP1 trp1-1 ura3-1 leu2-3,112 ade2-1 his3-11,15 can1-100*	This work
UMY3373	MATα *trp1-1 ura3-1 leu2-3,112 ade-–1 his3-11,15 can1-100 elp3::KanMX4 tuc1::TRP1*/pRS316-*ELP3*	This work
UMY3399	MATα *trp1-1 ura3-1 leu2-3,112 ade2-1 his3-11,15 can1-100 elp3::KanMX4 tuc1::TRP1*/pRS315-*ELP3*	This work
UMY3400	MATα *trp1-1 ura3–1 leu2-3,112 ade2-1 his3-11,15 can1-100 elp3::KanMX4 tuc1::TRP1/*pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*	This work
UMY3463	MATα *trp1-1 ura3-1 leu2-3,112 ade2-1 his3-11,15 can1-100*/pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*	This work

homogenous and no revertants or contaminants were observed, as judged from single-cell streak on agar plates at the time of harvest. Cells were washed and resuspended in 0.9% NaCl and 2 vol of phenol were added. The mixture was shaken for 30 min at room temperature and then 0.1 vol of chloroform was added and the mixture was shaken for an additional 15 min. The water and phenol phases were separated by centrifugation for 20 min at 10–15.000 rpm in a Sorvall GS3 rotor. Adding 2.5 vol of ethanol and 0.1 vol of 20% potassium acetate precipitated the tRNA in the water phase. Transfer RNA was extracted by 2 M of LiCl as described earlier (Avital and Elson 1969) and precipitated with ethanol. To remove excess salt, another ethanol precipitation was performed, and the resulting tRNA pellet was washed with 70% ethanol. Such tRNA preparation was dissolved in R200 buffer (10 mM Tris-H_3PO_4 at pH 6.3; 15% ethanol, 200 mM KCl) and applied to a Nucleobond column (AX500) equilibrated with the same buffer. The column was washed with 6 mL of R200 and 2 mL of R600 buffer (similar to R200 buffer, but containing 600 mM KCl) and this combined eluate was discarded. Transfer tRNA was then eluted with 7 mL of buffer R600 and precipitated with 0.7 vol of cold isopropanol. The precipitate was dissolved in water and precipitated with ethanol, the precipitate was washed twice with 80% ethanol, and dried. The dried tRNA was dissolved in water, heated to 95°C for 2.5 min, and quickly put on ice. A portion of it was degraded to nucleosides by nuclease P1, followed by treatment with bacterial alkaline phosphatase (Gehrke et al. 1982). The hydrolysate was analyzed by HPLC (Gehrke and Kuo 1990) using a Develosil C30 reverse-phase column (250 × 4.6 mm; Phenomex Ltd.).

Analysis of tRNA levels

Strains (W303-1B, wild type; UMY3400 (*elp3::KanMX4 tuc1::TRP1*/pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*), and UMY3463 (*ELP3 TUC1*/pRS425-*tE(UUC)-tK(UUU)-tQ(UUG)*) were grown in 10 mL of YEPD medium at 25°C to about OD_{600} of 1.0. Following centrifugation the pellet was suspended in 1 mL of ice-cold water. Cells were collected by centrifugation and resuspended in 400 μL of 10 mM Tris-EDTA, (pH 7.5). The same amount of acid phenol was added and the mixture was vigorously shaken for 10 sec and incubated for 45 min at 65°C with occasional shaking before the phases were separated by centrifugation. To the water phase, 400 μL of chloroform was added and the mixture was shaken, the water phase was transferred to a clean test tube, and 40 μL of 3 M sodium acetate (pH 5.3) and 1 mL of 100% ethanol was added. The precipitated RNA was washed once with 70% ethanol, centrifuged, and dissolved in 50 μL of water. About 5 μg of RNA was applied to an 8% polyacrylamide gel containing 8 M Urea in 89 mM Tris-borate buffer (pH 8.2) containing 2 mM EDTA. Following electrophoresis, the gel was transferred to a Zeta probe membrane and the RNA were UV cross-linked to the membrane. The tRNAs was detected by Northern hybridization using radioactive oligonucleotides complementary to nucleotides 57–72 of $tRNA^{Gln}_{mcm5s2UUG}$, 57–73 of $tRNA^{Lys}_{mcm5s2UUU}$, 52–72 of $tRNA^{Glu}_{mcm5s2UUC}$, and 30–49 of $tRNA^{Met}_{CAU}$. The last oligonucleotide, which monitors the levels of two tRNAs not having mcm^5s^2U, served as controls for how much materials was loaded on the gel.

Determination of aminoacylation of $tRNA^{Gln}_{mcm5s2UUG}$, $tRNA^{Lys}_{mcm5s2UUU}$, and $tRNA^{Glu}_{mcm5s2UUC}$ in vivo

Cells were grown in 30 mL of YEPD medium at 25°C (except for strain UMY2843, which was grown at 30°C) to about OD_{600} of 1.0, and cells were collected by centrifugation. Cells were resuspended in 1 mL of water, washed once with 1 mL of water, and finally resuspended in 500 μL of cold 0.1 M NaAc (pH 4–5) containing 10 mM EDTA. To the suspension of cells, 200 uL of glass beads and 500 μL of 25:24:1 phenol-chloroform-isoamyl-alcohol mixture were added and the mixture was vortexed four times for 1 min with 1 min on ice between the shaking. Following centrifugation, the supernatant was transferred to a new tube and RNA was precipitated by adding 3 vol of ethanol. The RNA was dissolved in 50 μL of 10 mM NaAc (pH 4.5) containing 1 mM EDTA. Half of the sample was diluted with an equal volume of 0.5 M Tris HCl (pH 9.0) and incubated for 20 min at 37°C. The deacylated and nontreated samples were run on an acid gel containing 8% polyacrylamide, 8 M urea, and 0.1 M NaAc (pH 5.0). RNA was transferred to Zeta probe membrane and the tRNA was detected as above.

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