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Lack of Pseudouridine 38/39 in the Anticodon Arm of Yeast Cytoplasmic tRNA Decreases *in Vivo* Recoding Efficiency*

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Many different modified nucleotides are found in naturally occurring tRNA, especially in the anticodon region. Their importance for the efficiency of the translational process begins to be well documented. Here we have analyzed the *in vivo* effect of deleting genes coding for yeast tRNA-modifying enzymes, namely Pus1p, Pus3p, Pus4p, or Trm4p, on termination readthrough and +1 frameshift events. To this end, we have transformed each of the yeast deletion strains with a *lacZ-luc* dual-reporter vector harboring selected programmed recoding sites. We have found that only deletion of the *PUS3* gene, encoding the enzyme that introduces pseudouridines at position 38 or 39 in tRNA, has an effect on the efficiency of the translation process. In this mutant, we have observed a reduced readthrough efficiency of each stop codon by natural nonsense suppressor tRNAs. This effect is solely due to the absence of pseudouridine 38 or 39 in tRNA because the inactive mutant protein Pus3[D151A]p did not restore the level of natural readthrough. Our results also show that absence of pseudouridine 39 in the slippery $tRNA^{Leu}_{UAG}$ reduces +1 frameshift efficiency. Therefore, the presence of pseudouridine 38 or 39 in the tRNA anticodon arm enhances misreading of certain codons by natural nonsense tRNAs as well as promotes frameshifting on slippery sequences in yeast.

Most classes of cellular RNA (tRNA, mRNA, rRNA, and small nuclear RNA) from all organisms contain post-transcriptionally modified nucleotides. Among these molecules, tRNAs are generally the most modified and contain the largest number of different modified nucleotides (81 different structures reported to date, see Ref. 1). Although the function of most of these modified nucleotides remains unclear, the role of modified nucleotides in the anticodon loop of tRNA, especially at positions 34 and 37, begins to be well documented (reviewed in Ref. 2 and 3). These modifications usually improve the fidelity and efficiency of tRNA in decoding the genetic message in the correct frame on the ribosome.

Pseudouridine (Ψ),[1] an isomer of uridine, is by far the most frequently encountered modified nucleotide in tRNA. Indeed, Ψ is found almost universally at position 55 in the so-called TΨ loop and very often at position 13 in the D-arm and at positions 38 and 39 of the anticodon stem and loop of a large number of tRNA species from all organisms examined so far (Ref. 4 and also see www.uni-bayreuth.de/departments/biochemie/trna/). Depending on the species and the origin of the tRNA molecule, Ψ has been also found at several other positions (for review see Ref. 5). Pseudouridines in tRNA are formed post-transcriptionally by a family of enzymes called tRNA:Ψ-synthases (6, 7). In *Escherichia coli*, four tRNA:Ψ-synthases have been characterized so far: TruA, TruB, and TruC modify uridines at position 38–40 (8), 55 (9), and 65 (10), respectively. RluA catalyzes the formation of Ψ32 in tRNA and Ψ746 in 23 S rRNA (11). Disruption of *truB* or *rluA* has no discernible effect on exponential growth rate (as for the *truC* mutant) but confers a selective disadvantage in competition with wild-type cells. However, this phenotype for *truB* mutant is due to the absence of the protein TruB and not to the absence of Ψ55 *per se* (12). In *truA* mutant cells, which are unable to modify the uridines at positions 38, 39, and 40, a decrease in the rate of the aminoacyl-tRNA selection step during translation depending on the identity of the tRNA has been demonstrated (13). This observation could explain the pleiotropic phenotype of *truA* mutant cells, like derepression of *his*, *leu*, and *ilv* operons, reduction of growth, and polypeptide chain elongation rates (reviewed in Ref. 3). It has not been established whether these phenotypes are linked to the lack of Ψ in anticodon arm of the tRNA or to the absence of the TruA protein *per se*.

In *Saccharomyces cerevisiae*, four tRNA:Ψ-synthases have been also characterized. Pus1p catalyzes the formation of Ψ at positions 26, 27, 28, 34, 35, 36, 65, and 67 in cytoplasmic tRNA (14) as well as position 44 in U2 small nuclear RNA (15). Pus3p, Pus4p, and Pus6p act at positions 38, 39 (16), 55 (17), and 31 (18), respectively, in both cytoplasmic and mitochondrial tRNAs (Fig. 1). None of these identified tRNA:Ψ-synthases in yeast is essential. However, disruption of *PUS3* gene leads to a slower growth rate phenotype, especially at suboptimal tem-

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[1] The abbreviations used are: Ψ, pseudouridine; m^5C, 5-methylcytosine; eRF, eukaryotic release factor; TMV, tobacco mosaic virus.

peratures (16, 19). Moreover, combined disruptions of the *PUS1* and *LOS1* genes or the *PUS1* and *PUS4* genes cause lethality at increased temperatures (20, 21). Los1p has been characterized as the tRNA exportin in yeast and is required for the efficient nuclear export of spliced tRNAs (22, 23). Accordingly, nuclear export of the minor $tRNA^{Ile}_{UAU}$ and of a mutant $tRNA^{Gln}_{CUG}$, both substrates of Pus1p, is impaired in a disrupted *pus1* strain. Thus Pus1p is directly implicated in the nuclear export of at least some tRNA species (21).

One role of Ψ is to stabilize the conformation of RNA by enhancing local base stacking (Ref. 24 and reviewed in Ref. 25) and by coordinating a structured water molecule between its N1-H and the phosphate backbone (Ref. 26 and reviewed in Ref. 27). Changes in tRNA structure, such as those induced by defective modification, may affect the decoding properties of tRNA. For example, lack of Ψ35 in yeast and plant $tRNA^{Tyr}_{G\Psi A}$ (28, 29) and Ψ38/39/40 in several *E. coli* tRNAs (Ref. 30 and reviewed in Ref. 31) affect the misreading, readthrough, and/or +1 frameshift event(s).

Less is known concerning the enzymatic formation of 5-methylcytosine (m^5C) in tRNA. In *S. cerevisiae*, one single gene product (Trm4p) catalyzes the formation of m^5C at positions 34, 40, 48, and 49 in various cytoplasmic tRNAs (32) (Fig. 1). In *E. coli*, none of the tRNAs sequenced so far harbors this modified nucleotide (4). The role of m^5C in tRNA is largely ignored, except for the unique m^5C40 in yeast $tRNA^{Phe}_{GmAA}$, which plays an important role in the correct spatial organization of the anticodon arm and the formation of a Mg^{2+}-binding pocket (Ref. 33 and reviewed in Ref. 34). Likewise, the unique m^5C34 at the wobble position of the anticodon loop in yeast SUP53 $tRNA^{Leu}_{m5CUA}$ affects the efficiency of amber UAG codon suppression (35).

We have previously described a dual gene reporter that allows the analysis of the effect of *cis*- and *trans*-acting factors on translational recoding events, like readthrough and frameshifting (36). This *in vivo* system has now allowed us to test the role of specific modified nucleotides in tRNA, formed by Pus1p, Pus3p, Pus4p, and Trm4p, on the efficiency of stop codons readthrough and +1 frameshifting in yeast. We have found that the absence of pseudouridine at positions 38 or 39 causes a detectable defect on these recoding events.

MATERIALS AND METHODS

Yeast Strains and Plasmids—The *S. cerevisiae* strains used in this study are W303 (*MAT**a**, ade2, his3-11, 15, leu2-3, trp1-1, ura3-1*), FY73 (*Mat**a**, his-3Δ200, ura3-52*), BY4742 (*Mat**a**, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*), 74-D694 [psi−] (*Mat**a**, ade 1-14, trp1-289, his3Δ200, leu2-3, 112, ura3-52,* [*psi*−]), 74-D694 [PSI+] (*Mat**a**, ade 1-14, trp1-289, his3Δ200, leu2-3, 112, ura3-52,* [PSI+]) and their respective derivative deleted mutants as indicated in Fig. 2 and Table II. Deleted strains for *TRM4* (32) and *PUS4* (17) were described previously. ResGen (Invitrogen, The Netherlands) provided the BY4742 *pus1*Δ strain. All mutant *pus3*Δ strains were from this study and are described below. These strains were grown in minimal media supplemented with the appropriate amino acids to allow maintenance of the different plasmids. Reporter plasmids were constructed by cloning a double-stranded oligonucleotide containing the recoding sequence in the unique *Msc*I site present between *lacZ* and *luc* genes of pAC99 (37). The list of the oligos is shown in Table I. All constructs were verified by sequencing the region of interest with an ABI310 automatic sequencer. These plasmids were transformed into the different yeast strains described above (refer to figure and table legends) by the lithium acetate method (38).

Quantification of Recoding Efficiency—Luciferase and β-galactosidase activities were assayed in the same crude extract as described previously (36). The assays were carried out using at least three independent transformants that were grown in the same conditions. Luciferase/β-galactosidase ratio obtained with test construct is normalized to the ratio obtained with the in-frame control (pAC-TQ for readthrough and pAC-TTy for frameshifting) and expresses readthrough or frameshift efficiency.

*Preparation of Disrupted pus3*Δ *Yeast Strains*—Yeast mutant strains bearing a deletion of the *PUS3* open reading frame were prepared by the one-step gene replacement approach (39). The kan^r gene was amplified from plasmid pF6A-kanMX2 by PCR using two oligonucleotides (CTCGAGGTGCCCACATGCAATCTTTACTGCCCTACTATAACCTC-CCTTGACAGCTGAAGCTTCGTACGC and GAAAAGAAATATAGTCT-TCAAGGTTATATTATACAGGTTTATATATTATTGCATAGGCCACT-AGTGGATCTG) complementary to the kanMX2 cassette and to 50 nucleotides 5′ upstream and 3′ downstream to the *PUS3* gene, respectively. The haploid yeast strains BY4742, 74-D694 [psi−] and 74-D694 [PSI+] were made competent by lithium acetate/PEG-4000 treatment and transformed by the purified PCR product. G418-resistant transformants were analyzed for correct integration by PCR amplification with specific oligonucleotides.

Cloning of the Wild-type PUS3 Gene and Site-directed Mutagenesis—The *PUS3* gene and its 5′- and 3′-UTR was amplified by PCR from total yeast genomic DNA using primers CCCGAGATTATCCCATTCCAAT-GAC and ATGAAAAGAAATATAGTCTTCAAGG. The purified PCR product was cloned at the unique *Sma*I site of the pRS315 vector. In order to inactivate Pus3p, a mutation was introduced using an Ex-Site™ PCR-based kit (Stratagene), to change an aspartate residue into an alanine (position 151 of the wild-type protein) with oligonucleotides gcagatgtggcagaacag**c**caagggagttagcgcc and ggcgctaactcccttg**g**ctgttctgc-cacatctgc, where c and g in small letters and bold indicate the point mutation. The sequence of these two *PUS3* genes was verified. The resulting plasmids were then transformed into the BY4742 *pus3*Δ strain.

^{32}P Labeling of $tRNA^{Gln}_{CUG}$, in Vitro Assay, and Analysis of Modified Nucleotides—The gene of the yeast $tRNA^{Gln}_{CUG}$ was amplified from plasmid pUN100-tQ using two complementary oligonucleotides, one bearing a T7 promoter sequence and the other the *Mva*I restriction site as described previously (21). *In vitro* transcription of the substrate $tRNA^{Gln}_{CUG}$ using [α-^{32}P]CTP and the *in vitro* enzymatic assay for testing formation of modified nucleotides in tRNA transcript were described previously (40). The yeast S10 extracts were used at 0.2 mg/ml final concentration. The modified synthetic [α-^{32}P]CTP-$tRNA^{Gln}_{CUG}$ transcript was phenol-extracted, precipitated, and redissolved in 50 mM NH_4 acetate, pH 4.6, for further hydrolysis with 0.1 units of RNase T2 (Sigma). Each hydrolysate was chromatographed in two-dimensions on TLC plates (Schleicher & Schuell; 10 × 10 cm) using the chromatographic solvent system N/N as described previously (40). Radioactive spots were revealed and quantified after exposure of the plates to a PhosphorImager screen (Amersham Biosciences).

Immunochemical Assay—Recombinant Pus3p was expressed in the *E. coli* BL21 (DE3) strain and purified from the inclusion bodies by extraction from a denaturing polyacrylamide gel.[2] Antibodies against Pus3p were prepared in rabbits by a standard immunization procedure after three injections of 0.25 mg of recombinant Pus3p per rabbit at 20-day intervals. S10 extracts from different strains (refer to legend of Fig. 3) were applied onto a 8% acrylamide gel and electrophoretically separated under denaturing conditions as described by Laemmli (69). Proteins were transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences). Blotting was performed in 25 mM Tris, 192 mM glycine buffer for 1 h at 80 V. For immunochemical detection the membrane was saturated by blocking agent (low fat milk) and incubated with the polyclonal antibodies against Pus3p. The membrane was then incubated with the anti-rabbit IgG-alkaline phosphatase conjugate (Sigma). Alkaline phosphatase activity was revealed using substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma).

RESULTS

The Test Systems—Mature transfer tRNA contains a large variety of modified nucleotides. To test individually their importance for the accurate translation of the mRNA in *S. cerevisiae*, we checked two different processes as follows: codon-anticodon recognition by stop codon readthrough efficiency and processivity of the decoding process by the occurrence of frameshift events. Two model systems were used (Table I). The first one is based on the UAG readthrough of the TMV replicase cistron and allowed us to measure readthrough efficiency. This sequence contains a UAG termination codon embedded in a very peculiar nucleotide context and promotes a high level of spontaneous termination readthrough in yeast (36, 41). When

[2] F. Lecointe, unpublished results.

TABLE I
Schematic view of the reporter and plasmids used in this study

plasmids	sequences[a]
Termination readthrough	
pAC-TMV (UAG)	GGA ACA CAA **TAG** CAA TTA CAG G T Q **Stop** Q L Q
pAC-TGA	GGA ACA CAA **TGA** CAA TTA CAG G T Q **Stop** Q L Q
pAC-TAA	GGA ACA CAA **TAA** CAA TTA CAG G T Q **Stop** Q L Q
+1 frameshifting	
pAC-Ty1	GCT AGC ACA ***CTT AGG C***CG ATC A A S T L G R S
pAC-FST3	GCT AGC ACA ***CTT TGA C***AG ATC A A S T L D R S
pAC-FST4	GCT AGC ACA ***CCG TGA C***AG ATC A A S T P D R S
pAC-FST5	GCT AGC ACA ***GCG TGA C***AG ATC A A S T A D R S
Controls	
pAC-TQ for termination readthrough	*T->C* GGA ACA CAA *C*AG CAA TTA CAG G T Q Q Q L Q
pAC-TTy for +1 frameshifting	*ΔC* GCT AGC ACA TTA GGC CGA TCA A S T L G R S

[a] Sequences were cloned between *lacZ* and *luc* genes in plasmid pAC99. The sequence of the primary translation product (one-letter code system for amino acid) from each construct is indicated below the nucleotide sequence. For termination readthrough, stop codons are in bold. For +1 frameshifting, nucleotide sequences of the frameshift site are in bold and italic. The first expected codon in +1 frame is underlined, and the asterisk above a nucleotide corresponds to the nucleotide expected to be overlapped by the translation machinery. Change of T to C (T → C in italic) made in pAC-TMV and deletion of the first C (ΔC in italic) of the frameshift site in pAC-Tyl are indicated above the sequence, and plasmids obtained (pAC-TQ and pAC-TTy respectively), have been used as control plasmids. These plasmids have been all described (36, 42), except pAC-FST4 and pAC-FST5 that are from this study.

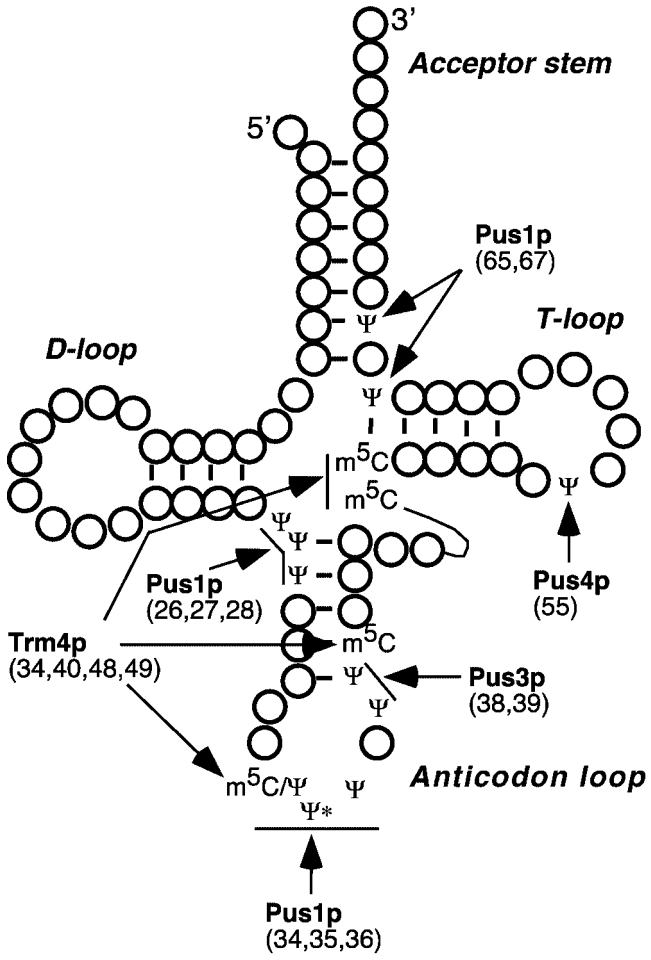

FIG. 1. **Type and location of modified nucleotides catalyzed by Pus1p, Pus3p, Pus4p, and Trm4p in cytoplasmic tRNAs.** Positions of modified nucleotides in tRNA are indicated in *parentheses* accordingly to the standard numbering convention (4). *Asterisk* (Ψ*) corresponds to the pseudouridine that is catalyzed by Pus1p and another yet unidentified pseudouridine synthase (14).

the UAG stop codon is replaced by UAA or UGA, high readthrough efficiencies were also obtained (42). The second system is based on the slippery sequence of the Ty1 retrotransposon, consisting of the heptanucleotide CUU AGG C, where AGG is a poorly recognized arginine codon. Slow decoding of this codon provides a translational pause that allows slippage of the $tRNA^{Leu}_{UAG}$ from the leucine codon CUU to the leucine codon UUA. This results in incorporation of the major $tRNA^{Gly}_{GCC}$ in the A+1-site of the ribosome (43).

The sequences described above were inserted between the *lacZ-luc* dual reporter of pAC99 (37), where *lacZ* is used as an internal reference for translation efficiency. With constructs pAC-TMV (UAG), pAC-TAA, and pAC-TGA (see Table I), ribosomes that terminate at the stop codon express only β-galactosidase, whereas those that read through the termination site also express luciferase. In pAC-Ty1, the *luc* gene is fused in the +1 frame downstream of the frameshift site making luciferase activity a measure of the efficiency of +1 frameshifting.

Only Deletion of the PUS3 Gene Affects Readthrough and +1 Frameshift Efficiencies—These constructs were used to transform various yeast strains that were defective for the activity of a given tRNA modification enzyme, namely the *pus1*Δ, *pus3*Δ, *pus4*Δ, and *trm4*Δ strains. The enzymes Pus1p, Pus3p, Pus4p, and Trm4p catalyze the formation of different modified nucleotides in yeast tRNA (see Fig. 1). Each transformed yeast strain was grown in minimal medium supplemented with the appropriate amino acids at 28 °C, and the ratios of luciferase/β-galactosidase activities were then determined. These results were normalized as described in Fig. 2 and compared with the ratios obtained with the corresponding wild-type strains. For each wild-type strain (indicated in *black* in Fig. 2), readthrough efficiencies of the three stop codons ranged between 13 and 25% for UAG, 8 and 11% for UGA and 5.5 and 7% for UAA, although the frameshift efficiencies ranged between 22 and 44%. These values are in good agreement with those obtained with other yeast strains (42) as well as with another system depending on a *lacZ* reporter for Ty1 frameshifting (43). The recorded efficiencies were not significantly different when wild-type strains are compared with their corresponding tRNA modification defective mutants (indicated in *white* in Fig. 2), except for the *pus3*Δ strain. Compared with the wild-type strain, the readthrough efficiency of the *pus3*Δ strain was reduced by a factor of 1.9, 2.2, or 1.4 on the stop codons UAG, UGA, and UAA, respectively. Moreover, the *pus3*Δ strain was the only mutant strain tested in this study that reduced the +1 frameshift efficiency on the Ty1 slippery site by a factor 1.8.

*The Reduction of Readthrough Efficiency in the pus3*Δ *Strain Is Solely Due to the Lack of Ψ38/39 in tRNA*—Because readthrough efficiency was directly influenced by the competition between eRF1 and the suppressor tRNA in the A-site of the ribosome (44) as well as by the presence of modified nucleotides in the anticodon arm (reviewed in Ref. 3), the effect observed

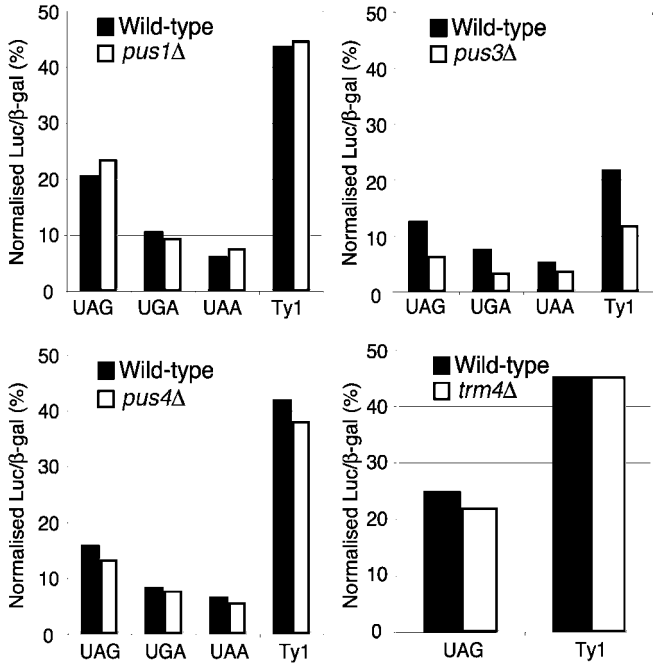

FIG. 2. **Effect of gene deletions *pus1*Δ, *pus3*Δ, *pus4*Δ, and *trm4*Δ on termination readthrough and Ty1 frameshifting.** Wild-type yeast strain BY4742, 74-D694[psi−], FY73, and W303 and their deleted derivative strain (*pus1*Δ, *pus3*Δ, *pus4*Δ, and *trm4*Δ, respectively, as indicated) were transformed with either pAC-TMV, pAC-TGA, pAC-TAA, pAC-TQ, pAC-Ty1, or pAC-TTy (see Table I) and grown at 28 °C to reach A_{600} = 1.5. Luciferase/β-galactosidase ratio obtained with pAC-TMV, pAC-TGA, pAC-TAA, or pAC-Ty1 was normalized to the control ratio (100%) obtained with the in-frame control (pAC-TQ for readthrough and pAC-TTy for frameshifting). The normalized luciferase/β-galactosidase ratio (in %) expresses readthrough efficiency on UAG, UGA, or UAA stop codon and frameshifting on Ty1-programmed frameshift site. The assays were carried out using at least three independent transformants. The S.E. of all data presented in this work is <10%.

with the *pus3*Δ strain suggested that the absence of pseudouridine in position 38 or 39 in yeast natural suppressor tRNA (no tRNA in yeast harbors Ψ at both position 38 and 39, see Ref. 4) caused a reduction in their ability to decode a stop codon. To verify that this effect was actually due to the absence of Ψ38 or Ψ39 and not to the absence of the Pus3p protein *per se*, we introduced a point mutation that changed the aspartate residue in position 151 into alanine (*pus3*[D151A]). This aspartate residue is part of a characteristic motif among various RNA-pseudouridine synthases (6) and in particular in *E. coli* TruA (aspartate residue 60) which catalyzes the formation of Ψ38/39/40 in bacterial tRNA. Its mutation into alanine has been shown to abolish the activity of the TruA enzyme (45).

We first verified that Pus3[D151A]p, when expressed in the *pus3*Δ strain, was unable to catalyze the formation of Ψ38 or Ψ39 in tRNA. S10 extracts prepared from the wild-type and *pus3*Δ strains as well as from the *pus3*Δ strain expressing the mutant Pus3[D151A]p protein were used to test pseudouridine formation in an *in vitro* transcribed and [α-^{32}P]CTP-labeled yeast $tRNA^{Gln}_{CUG}$. This tRNA normally bears several modified nucleotides, among them are pseudouridines at positions 28, 38, and 55. These pseudouridines can be all formed *in vitro* and identified as a single radiolabeled spot on thin layer cellulose plates after two-dimensional chromatography of RNase T2 hydrolysates (21). As shown in Fig. 3, *panel A*, the S10 extracts from the wild-type strain modified this tRNA giving rise to 2.1 mol of pseudouridine per mol of tRNA after 1 h of incubation. In contrast, the extract from the *pus3*Δ strain, which is unable to modify the uridines at positions 38 or 39 (16), produced only 1.3 mol of pseudouridine per mol of tRNA. Exactly the same result was obtained with the extract derived from the *pus3*Δ cells carrying the *pus3*[D151A] gene, confirming that the Pus3[D151A]p protein is catalytically inactive. This was not due to the instability of the mutant Pus3[D151A]p protein because it was expressed normally, as verified by Western blot analysis (Fig. 3, *panel B*).

The *pus3*Δ strain was transformed with a pRS315 centromeric plasmid harboring either the mutant *pus3*[D151A] or the wild-type *PUS3* gene under its natural promoter, and the growth rates of the resulting strains were compared. By taking into account that the slow growth phenotype of the *pus3*Δ strain was particularly strong at suboptimal growth temperature (16), the growth rate was measured at 39 °C. From the results shown in Fig. 3 (*panel C*), generation times of 730 and 190 min can be estimated for the *pus3*Δ and wild-type strains, respectively. When the wild-type *PUS3* gene was expressed in the *pus3*Δ strain, the wild-type growth rate was restored (*black squares* in Fig. 3, *panel C*). In contrast, the mutant *pus3*[D151A] gene expressed from the same vector (*white squares*) was unable to complement the growth defect of the *pus3*Δ strain. This demonstrates that the slow growth phenotype of *pus3*Δ strain is due to the absence of Ψ38 or Ψ39 in the anticodon arm of tRNA.

Next, we tested the readthrough efficiency in the wild-type BY4742 strain, the corresponding *pus3*Δ strain, and *pus3*Δ expressing wild-type Pus3p or Pus3[D151A]p. These four strains were cotransformed with the pAC-TMV vector (UAG stop codon, see Table I). The results are shown in Fig. 4. Clearly, the readthrough efficiency of the wild-type strain (21.3%) was restored upon transformation of the *pus3*Δ strain with wild-type *PUS3* gene (20.3%), whereas the percentage of readthrough remained the same when *pus3*Δ was transformed with either empty pRS315 or the same plasmid harboring the *pus3*[D151A] gene (10.5 and 10.2%, respectively). The difference in the readthrough efficiencies on the UAG stop codon between wild-type [*psi*−] BY4742 (21.3%) and 74-D694[*psi*−] (12.7%, Fig. 2) probably reflects the difference of the genetic background between these two strains. However, the reduction in readthrough efficiency on the UAG stop codon was similar in the corresponding *pus3*Δ strains (by a factor 1.9 in the mutant 74-D694 and 1.8 in the mutant BY4742), indicating that the effect of the absence of pseudouridine 38 or 39 on readthrough is independent of a particular genetic background.

In addition, as for the growth rate, the ratio of readthrough efficiency in the wild-type strain over the readthrough efficiency in the *pus3*Δ strain was higher at 36 than 28 °C for each of the three stop codons (compare values in boldface italic in parentheses in Table II). However, the absolute values of readthrough efficiencies in each case were lower at 36 than at 28 °C. The effect of temperature on readthrough levels has been observed previously (37) with similar readthrough-promoting sequences.

Taken together, these results show that the reduction of readthrough efficiency in the *pus3*Δ strain was due solely to the absence of the catalytic activity of Pus3p, *i.e.* the lack of pseudouridine 38 or 39 in the anticodon arm of yeast natural suppressor tRNAs.

Lack of Ψ in $tRNA^{Leu}_{UAG}$ but Not in $tRNA^{Pro}_{U*GG}$ and $tRNA^{Ala}_{IGC}$ Affects +1 Frameshifting—The results obtained above (Fig. 2) show that among the four deletions tested (*pus1*Δ, *pus3*Δ, *pus4*Δ, and *trm4*Δ), only *pus3*Δ has an inhibitory effect on the programmed +1 frameshifting, using the pAC-Ty1 test system. This particular recoding system was demonstrated to depend on the interplay between three different tRNAs (Fig. 5, *panel A*) as follows: $tRNA^{Leu}_{UAG}$ bound at the decoding P-site of the

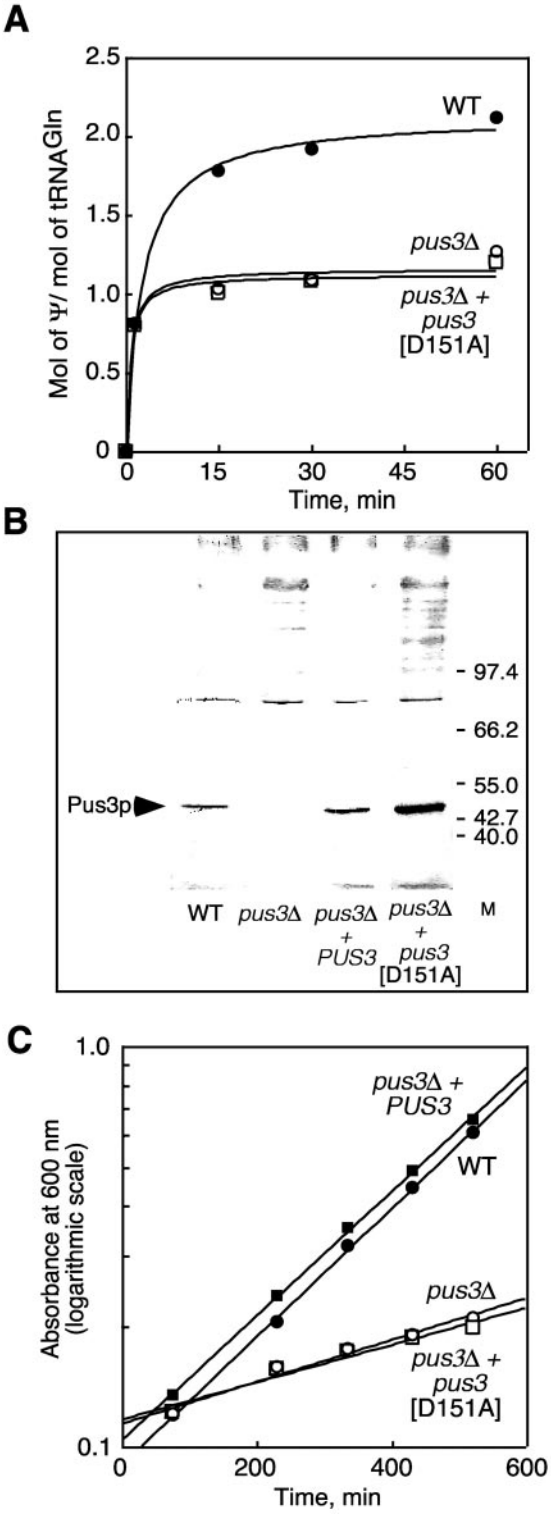

FIG. 3. **Thermosensitivity of the *pus3*Δ strain is due to the absence of Ψ38/Ψ39 in tRNA.** *Panel A*, *in vitro* time course formation of pseudouridine 28, 38, and 55 on T7-transcript of synthetic $tDNA^{Gln}_{CUG}$ gene (radiolabeled with [α-^{32}P]CTP) at 30 °C. Incubation was performed with S10 extracts prepared from a wild-type (*WT*, *filled circles*) and from a *pus3*Δ strain (*open circles*) both transformed with pRS315, and from a *pus3*Δ strain transformed with pRS315 containing the mutant *pus3*[D151A] (*open squares*). *Panel B*, Western blot analysis, using a Pus3p rabbit polyclonal antibody, of S10 extracts of wild-type (*WT*) and *pus3*Δ strains, both transformed with a pRS315 vector, and *pus3*Δ strain transformed with the same plasmid containing the *PUS3* or the mutant *pus3*[D151A] gene as indicated. *Arrow* indicates the Pus3p protein; *M* indicates molecular weight markers in thousands. *Panel C*, kinetics of growth at 39 °C in minimal media of the same strains as indicated in *panel B*.

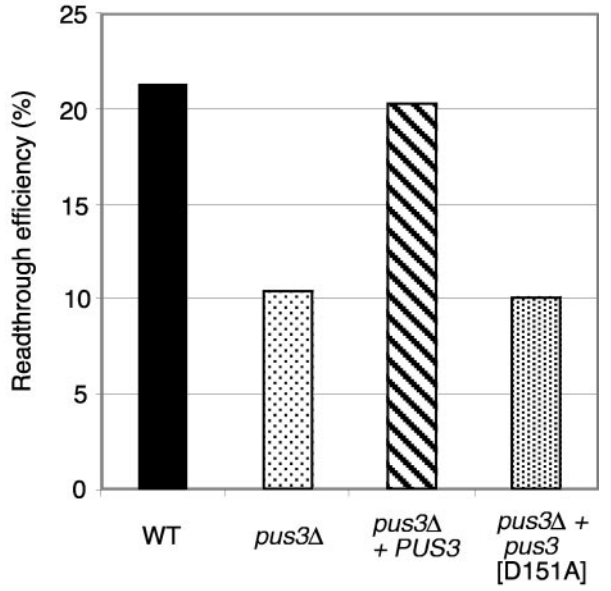

FIG. 4. **Effect of the absence of Ψ38/Ψ39 in tRNA on UAG termination readthrough.** Readthrough efficiency was measured at 28 °C using the pAC-TMV system. Data (expressed in %, see legend Fig. 2) correspond to those obtained with wild-type BY4742 (WT) strain transformed with a pRS315 vector and the BY4742 *pus3*Δ strain transformed with empty pRS315 or the same plasmid containing the *PUS3* gene or the mutant *pus3*[D151A] gene, as indicated.

ribosome, an incoming very minor $tRNA^{Arg}_{CCU}$ (46) that normally binds in the A-site (in-frame), and a major $tRNA^{Gly}_{GCC}$ (46) that can potentially bind to the +1 out-of-frame codon (43). It is important to note that the yeast $tRNA^{Leu}_{UAG}$, which harbors an unmodified uridine residue at the wobble position of its anticodon, can translate all six leucine codons *in vitro*, thus both CUU and UUA (47). Therefore, it has been proposed that at the frameshift site (Leu Arg/Gly)/(CUU-AGG-C), the near-cognate peptidyl-$tRNA^{Leu}_{UAG}$ forms 2 bp with leucine codon CUU and, subsequently, has a certain probability to slip +1 onto the other leucine codon UUA, probably during transient occupation of the A-site by the major glycyl-$tRNA^{Gly}_{GCC}$. The probability of such a frameshift event was shown to depend on the delay (pause) during translation due to the limiting amount of the incoming arginyl-$tRNA^{Arg}_{CCU}$ (Ref. 43 and reviewed in Ref. 48). Moreover, it has been shown recently (13, 30) that in a bacterial system the presence or absence of certain modified nucleotides within the anticodon loop of tRNA also affect the probability of a frameshift event, possibly by affecting the rate at which the incoming tRNA in the A-site will be recruited by the mRNA-peptidyl-tRNA ribosome complex or by affecting the probability of peptidyl-tRNA slippage in the P-site.

Inspection of the modified nucleotides content in yeast tRNA reveals that, among other modified nucleotides, Ψ55 (catalyzed by Pus4p) is present in all mature cytoplasmic tRNAs; Ψ27 (catalyzed by Pus1p), Ψ39 (catalyzed by Pus3p), and m^5C48 (catalyzed by Trm4p) are present in $tRNA^{Leu}_{UAG}$, whereas Ψ38 and m^5C49 (catalyzed by Pus3p and Trm4p, respectively) are present in $tRNA^{Gly}_{GCC}$ (4). $tRNA^{Arg}_{CCU}$ has not been sequenced so far; however, according to its gene sequence it contains U27, A38, G39, U48, and G49 (4). Therefore, one can expect the presence of Ψ27 in the corresponding mature tRNA (14) but not of Ψ38/39 or m^5C48/49. As shown in Fig. 2 and Table III (2nd line, 3rd column), the absence of pseudouridine 38 and/or 39 in the *pus3*Δ strain reduced +1 frameshift efficiency by a factor 1.8 (indicated in boldface italic in Table III) as compared with the wild-type strain, whereas the absence of the modifications catalyzed by Pus1p or Pus4p or Trm4p, from the three involved

TABLE II

Effect of the PUS3 gene deletion on termination readthrough at 28 or 36 °C in yeast strain BY4742

Wild-type (WT) BY4742 yeast strain and its *pus3*Δ derivative strain were transformed with one of the three plasmids harboring the test sequence as indicated. Stop codons are indicated in parentheses. Cells were grown at either 28 or 36 °C as indicated. The frequencies of readthrough are expressed in % (see legend Fig. 2). Numbers in bold and italic in parentheses indicate ratios of readthrough efficiency in the wild-type strain over the readthrough efficiency in the *pus3*Δ derivative strain.

Vector name (stop codon)	Readthrough efficiency in %			
	28 °C		36 °C	
	WT	*pus3*Δ	WT	*pus3*Δ
pAC-TMV (UAG)	20.0	10.9 (***1.8***)	10.6	4.0 (***2.7***)
pAC-TGA (UGA)	7.0	3.2 (***2.2***)	6.2	2.4 (***2.6***)
pAC-TAA (UAA)	5.7	2.9 (***2.0***)	3.1	1.4 (***2.2***)

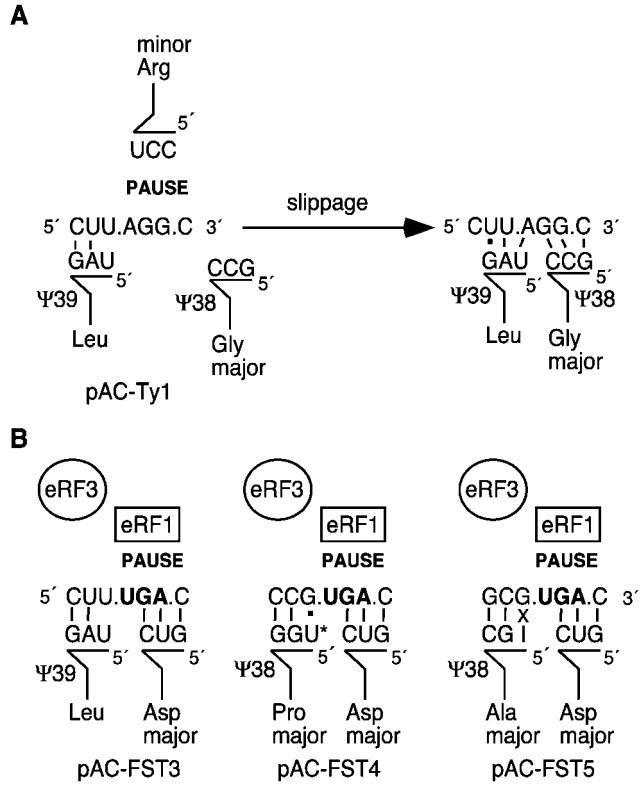

FIG. 5. **A model for the mechanism of programmed +1 frameshifting in *S. cerevisiae*.** *Panel A*, model of +1 frameshifting in recoding site from the yeast retrotransposon Ty1 (adapted from Ref. 51). The frameshift site is a heptamer CUU.AGG.C in which CUU is the slippery codon in the P-site decoded by $tRNA^{Leu}_{UAG}$, AGG is a poorly recognized codon by the minor $tRNA^{Arg}_{CCU}$ in the A-site and GG.C is a codon decoded by the major $tRNA^{Gly}_{GCC}$ in the A+1-site. Watson/Crick pairing is indicated by a *line* and a G.U type of wobble pairing by a *dot*. The presence of Ψ at position 38 or 39 is indicated next to the tRNA. *Panel B*, shifty stop constructs used in this study. *Symbols* are the same as above. UGA stop codon is indicated in *bold*. Purine-purine clash is indicated by a *X*, and *U** indicates an unknown modified uridine. *eRF1* and *eRF3* are the eukaryotic release factors.

tRNAs ($tRNA^{Leu}_{UAG}$, $tRNA^{Arg}_{CCU}$, and $tRNA^{Gly}_{GCC}$) had no effect on the efficiency of Ty1 + 1 frameshift process. However, because Ψ39 or Ψ38 was present in both $tRNA^{Leu}_{UAG}$ and $tRNA^{Gly}_{GCC}$ (see Fig. 5, *panel A*), the role of these pseudouridines in the Ty1 + 1 frameshift event may be obscured by a possible compensatory effect.

To clarify this problem, we used three new programmed +1 frameshift systems, pAC-FST3, pAC-FST4 ,and pAC-FST5 (Table I and Fig. 5, *panel B*). These constructs carry +1 "shifty stops," *i.e.* they consist of a frameshift-inducing codon followed by an in-frame termination opal codon UGA. The frameshift-inducing codons used in these constructs (CUU for leucine, CCG for proline, and GCG for alanine) were chosen because they promote the highest level of +1 frameshifting compared with other codons in yeast (49). According to previous work, the frameshift-inducing codons CUU, CCG, and GCG will be read by $tRNA^{Leu}_{UAG}$, $tRNA^{Pro}_{U*GG}$, and $tRNA^{Ala}_{IGC}$, respectively (43, 50). At least two of these three tRNAs are abundant (major) tRNAs in yeast ($tRNA^{Pro}_{U*GG}$ and $tRNA^{Ala}_{IGC}$, see Ref. 46). All three tRNAs are substrates for yeast Pus3p (16). Moreover, the tRNA that has to decode the +1 A-site of the ribosome is a major $tRNA^{Asp}_{GUC}$ lacking Ψ38 or Ψ39 (Fig. 5, *panel B*). This allowed us to determine the effect of the absence of pseudouridine only on the tRNA decoding the frameshift-inducing codons. The data obtained for the frameshift event expressed in percent for the wild-type 74-D694 [*psi*−] and its corresponding *pus3*Δ mutant are reported in Table III (2nd and 3rd columns). The results indicate that in the wild-type [*psi*−] strain, the level of frameshifting obtained with the pAC-FST constructs is very low compared with that observed with the pAC-Ty1 vector. This result confirms our earlier work with pAC-FST3 (42). However, using the pAC-FST5 (sequence GCG.UGA.C instead of CUU.UGA.C) in another yeast strain (387–1D), more than 30% frameshift efficiency has been reported (51). These differences are probably due in part to the different genetic backgrounds of the yeast strains used in the two studies. When the same experiments were performed in 74-D694[*psi*−] cells lacking Pus3p, a slight reduction of the frameshift efficiency was observed upon transformation with pAC-FST3, whereas no detectable effect was measured with either pAC-FST4 or pAC-FST5 (see numbers in boldface italic in the 3rd column of Table III).

We have demonstrated previously that restricting the availability of release factors in *S. cerevisiae* stimulates +1 frameshifting on the slippery site CUU.UGA.C (pAC-FST3, see Ref. 42), most probably by increasing the translational pause in the A-site. In order to increase +1 frameshift efficiency, we used the yeast strain 74-D694[PSI+], in which the eRF3 termination factor was partially depleted by aggregation. Readthrough efficiency on the UGA stop codon in the [PSI+] wild-type and *pus3*Δ strains (see pAC-TGA in Table III) was higher than that observed in the [*psi*−] strain (see also Fig. 2). However, the effect of the *PUS3* gene deletion was approximately the same because the readthrough efficiency was again reduced by a factor of 1.8. If the absence of Ψ39 in $tRNA^{Leu}_{UAG}$ had no effect on the frameshift mechanism in pAC-FST3 (CUU.UGA.C), an increase of the +1 frameshift efficiency with the system (Leu(*ψ*) RF/Asp)/(CUU-UGA-C) in a *pus3*Δ strain could be expected, possibly due to an increase of the pause in the A-site of the ribosome containing the opal UGA stop codon. In contrast, by using the 74-D694[PSI+] *pus3*Δ strain, we observed a clear "inhibitory" effect of the *PUS3* gene deletion on the +1 frameshift mechanism with pAC-FST3 but not with the two other systems tested ((Pro(*ψ*) RF/Asp)/(CCG-UGA-C), pAC-FST4; (Ala(*ψ*) RF/Asp)/(GCG-UGA-C), pAC-FST5, see numbers in boldface italic in the 5th column of Table III). Noteworthy, the [PSI+] trait does not modify the +1 frameshift efficiency on the Ty1 slippery site in the wild-type and *pus3*Δ strains (see PAC-Ty1 in Table III), indicating that aggregation of eRF3 does not

TABLE III
Effect of the PUS3 gene deletion on the +1 frameshift efficiency

Wild-type (WT) and mutant *pus3*Δ of 74-D694 [*psi*−] and 74-D694 [PSI+] strains were transformed with one of the five plasmids harboring the test sequence as indicated. +1 frameshifting and readthrough efficiencies were measured at 28 °C, and the data were expressed in % (see legend Fig. 2). Numbers in bold and italic in parentheses correspond to ratios of recoding efficiency in the wild-type strain over the recoding efficiency in the *pus3*Δ derivative strain. NA, not applicable.

Plasmids	+1 frameshifting in [*psi*−] strain, %		+1 frameshifting in [PSI+] strain, %		Readthrough in [PSI+] strain, %	
	WT	*pus3*Δ	WT	*pus3*Δ	WT	*pus3*Δ
pAC-Tyl	21.7	12.0 (***1.8***)	21.2	11.9 (***1.8***)		
pAC-FST3	1.2	0.9 (***1.3***)	9.3	3.6 (***2.6***)	NA	NA
pAC-FST4	0.6	0.6 (***1.0***)	4.1	4.5 (***0.9***)		
pAC-FST5	0.5	0.4 (***1.2***)	4.6	4.4 (***1.0***)		
pAC-TGA	NA		NA		49.1	27.0 (***1.8***)

interfere with the mechanism of Ty1 frameshifting that occurs at a run of sense codons.

These results show that an unmodified U39 in the slippery $tRNA^{Leu}_{UAG}$ reduces +1 frameshift efficiency, whereas an unmodified uridine residue at position 38 in the non-slippery $tRNA^{Pro}_{U*GG}$ or $tRNA^{Ala}_{IGC}$ has no effect on +1 frameshifting.

DISCUSSION

Naturally occurring tRNAs contain a variety of modified nucleotides. The role of these modified nucleotides is not always fully understood. However, some modified nucleotides in the anticodon arm of tRNA appear to improve the efficiency of the translational process (reviewed in Refs. 2 and 3). One way to study *in vivo* the role of modified nucleotides is the use of nonsense suppression assays in mutant strains lacking the gene coding for a given tRNA modification enzyme (see for example Refs. 52 and 53). In these assays, the efficiency of suppression of a stop codon is an indication of the translational activity of a suppressor tRNA.

Another way is the use of programmed frameshift assays, which allow the determination of the contribution of a modified nucleotide on reading frame maintenance (13, 30). These systems have been mainly used in prokaryotes, and only limited data are available for eukaryotic systems.

In this work, we used the dual gene reporter approach (36) to analyze the effect of *trans*-acting factors on translational recoding events in yeast. In particular, we studied the effect of the absence of a given tRNA modification enzyme on both programmed translational readthrough and +1 frameshift events, using yeast strains devoid of Pus1p, Pus3p, Pus4p, or Trm4p activity (see Fig. 1). In this *in vivo* system, only the tRNAs that are naturally present in yeast will lead to stop codon readthrough or +1 frameshifting. Identification of the so-called natural suppressors harboring a near-cognate anticodon is not easy, and in fact several concurrent near-cognate tRNAs can usually bind to a given stop codon, yet with different efficiencies (for review, see Ref. 54). In yeast, it has been clearly shown that three naturally occurring tRNAs, specific for tyrosine, tryptophan, and lysine, can suppress the amber UAG stop codon within a readthrough site closely related to that of TMV replicase, $tRNA^{Tyr}_{G\Psi A}$ being the most efficient, followed by $tRNA^{Lys}_{CUU}$, and then $tRNA^{Trp}_{CmCA}$ (55).

Inhibition of Pseudouridinylation at Position 38 or 39 Leads to Decreased Readthrough Efficiencies—Among other modified nucleotides (see Ref. 4), yeast $tRNA^{Tyr}_{G\Psi A}$ contains Ψ at positions 35, 39, 55, and 5-methylcytosine (m^5C) at position 48, $tRNA^{Trp}_{CmCA}$ contains Ψ at positions 26, 27, 28, 39, and 55, and $tRNA^{Lys}_{CUU}$ contains Ψ at positions 27, 39, and 55. In our experiments, the deletion of the *TRM4* gene does not affect readthrough efficiency on the UAG stop codon in the TMV context. Therefore, the absence of m^5C48 in the m^5C-containing $tRNA^{Tyr}_{G\Psi A}$ does not detectably affect its suppressor efficiency. However, the lack of an effect in *trm4*Δ cells may also result from stronger competition by the two other potential natural suppressors $tRNA^{Lys}_{CUU}$ and/or $tRNA^{Trp}_{CmCA}$. In yeast, all tRNA species contain Ψ at position 55 (except initiator tRNA), which is catalyzed by Pus4p. The absence of an effect on the efficiency of UAG, UGA, and UAA stop codon readthrough in the *pus4*Δ strain indicates that Ψ55 has no influence on the incorporation of these termination suppressors to the A-site of the ribosome. Alternatively, a possible effect may be compensated by the fact that the tRNAs present in the A- and the P-site both contain or lack Ψ55. Noteworthy, disruption of *PUS4* (as of *TRM4*) has no detectable effect on yeast cell growth (17, 32). Lack of TruB in *E. coli*, the homologue of the yeast Pus4p, does not affect the growth rate but confers a selective disadvantage to the mutant when it is competing against the wild-type strain. However, this effect was shown to be due to the absence of the protein itself rather than to the inhibition of Ψ55 synthesis in tRNA (12). We did not obtain an effect on the efficiency of stop codon readthrough with the yeast *pus1*Δ strain, despite the fact that the three natural yeast suppressors of UAG in the TMV context are all substrates of Pus1p. The presence of Ψ35 in $tRNA^{Tyr}_{G\Psi A}$ was shown to be necessary for efficient suppression *in vitro* of the UAG of TMV RNA (29), probably because it stabilizes the codon-anticodon interaction (56). However, formation of Ψ35 in $tRNA^{Tyr}_{G\Psi A}$ remains unaffected in a *pus1*Δ strain, although recombinant Pus1p can catalyze *in vitro* the formation of Ψ35 in a transcript of intron-containing $tRNA^{Tyr}_{G\Psi A}$ (14). This is because *S. cerevisiae* contains a yet unidentified tRNA: pseudouridine-35 synthase, which has overlapping specificity with Pus1p (14). The absence of a detectable effect on UAG stop codon readthrough efficiency within the TMV context after deletion of *PUS1* indicates that Ψ at positions 26–28 in $tRNA^{Trp}_{CmCA}$ and Ψ at position 27 in $tRNA^{Lys}_{CUU}$ does not influence their competition with $tRNA^{Tyr}_{G\Psi A}$ for reading UAG in the A-site of the ribosome. This conclusion does not contradict earlier work concerning the importance of the type of base pair at positions 27–43 of the anticodon helix on suppression readthrough of UAG stop codon by *E. coli* su7 G36 suppressor $tRNA^{Trp}_{CUG}$ (57). In this latter case, the *in vivo* suppression test involved suppressor $tRNA^{Trp}$, which contains the anticodon CUG. As a result, no competition should take place with other potential natural suppressor tRNA harboring a near-cognate anticodon. It is also possible that in yeast, the presence of Ψ instead of U at positions 26, 27, and/or 28, although probably stabilizing the anticodon arm (56), has no important influence on the accuracy of codon reading on eukaryotic ribosomes. Therefore, the only known function of the Pus1p-catalyzed modifications remains their involvement in the transport of tRNA from the nucleus to the cytoplasm (21).

Of the four mutant yeast strains we examined (*pus1*Δ, *pus3*Δ, *pus4*Δ, and *trm4*Δ), only deletion of the *PUS3* gene coding for the tRNA:pseudouridine-38/39 synthase affects cell growth (16). Here we have shown that the decreased growth rate of the *pus3*Δ strain is due to the absence of Ψ at position 38 or 39 in the anticodon arm of tRNA and not to the absence of

the Pus3p protein itself. Indeed, a Pus3[D151A]p, which is catalytically inactive, is unable to restore a wild-type growth rate at 39 °C. This control experiment was essential because the slow growth phenotype of various strains lacking a modification enzyme is not always correlated to the absence of the corresponding tRNA modifications, because the enzyme may be involved in additional functions besides modifying tRNA. For example, in *E. coli* the presence of the enzymes TruB or RluD, two pseudouridine synthases catalyzing Ψ formation at position 55 in tRNA (9) or Ψ at positions 1911, 1915, and 1917 in 23 S RNA (58), respectively, is required for efficient cell growth. Deletion of the genes coding for these enzymes led to growth defects. However, these phenotypes could be complemented by the corresponding mutated genes, which encoded inactive enzymes (12, 59). Likewise, for the dimethylase Dim1p, which catalyzes the formation of the conserved $m^2{}_6A$ at positions 1779 and 1780 on yeast 18 S rRNA (60), it was demonstrated that the lethality resulting from deletion of the *DIM1* gene was essentially due to a defect in pre-rRNA processing and not to the lack of $m^2{}_6A$ at positions 1779 and 1780 on the mature 18 S rRNA (61).

In yeast, our results clearly indicate that deletion of the *PUS3* gene reduces the readthrough efficiency on all of the three stop codons UAG, UAA, and UGA within a programmed readthrough TMV context. This reduction of readthrough efficiency is due to the absence of Ψ at position 38 or 39 in tRNA, and the effect becomes even more drastic after increasing the temperature of cell growth to 36 °C. Because all three yeast natural suppressors of the UAG stop codon harbor a pseudouridine at position 39, it can be reasonably concluded that this modification improves the efficiency of tRNA on decoding the UAG stop codon. The same conclusion was reached for Ψ at positions 38–40 catalyzed by TruA (also called HisT) for the suppression of the amber stop codon in bacteria. Indeed, the activity of *supE* amber suppressor, a derivative of $tRNA^{Gln}_{CUG}$ in *E. coli*, was shown to be almost abolished when Ψ at both positions 38 and 39 were lacking (53). Similarly, the absence of Ψ at position 39 in *supF*, a mutated suppressor $tRNA^{Tyr}_{UAG}$ in *Salmonella typhimurium*, reduced readthrough efficiency on UAG by about 40% (52). This effect seems to apply also on the misreading process. Indeed, during histidine starvation of *E. coli* truA mutants, a reduction of mistranslation efficiency of histidine codons has been observed. This effect is probably due to the absence of pseudouridine in the anticodon arm of tRNA (62). Noteworthy, the aminoacylation capability of $tRNA^{His}$ was not affected in a *truA* mutant (63), and pseudouridines at positions 38, 39, or 40 are not major identity elements for the aminoacylation systems analyzed so far (reviewed in Ref. 64). Altogether, these results demonstrate that the presence of pseudouridine(s) at positions 38, 39, and/or 40 of the anticodon arm in tRNA facilitates codon reading on the ribosome, probably by stabilizing the codon-anticodon interaction and that this property is important in both eukaryotic and prokaryotic cells.

Lack of Ψ38 or Ψ39 in tRNA Influences Ribosome Frameshifting—We have used the programmed Ty1 frameshift assay (Figs. 2 and 5) (36) to test the effect of selected tRNA modifications on maintenance of correct reading frame during the translation process in yeast. Again, our results indicate that, among the four gene deletions we tested, *pus1*Δ, *pus4*Δ, and *trm4*Δ do not affect +1 frameshift efficiency in the Ty1 retrotransposon context. From these results we conclude that absence of the universally conserved Ψ at position 55 in the three tRNAs involved (*i.e.* $tRNA^{Leu}_{UAG}$, $tRNA^{Arg}_{CCU}$, and $tRNA^{Gly}_{GCC}$, see Fig. 5) has no detectable effect on the efficiency of +1 frameshifting. The same conclusion can be reached for the lack of m^5C at position 48 in the variable loop of $tRNA^{Leu}_{UAG}$, m^5C at position 49 at the T-stem of $tRNA^{Gly}_{GCC}$, or the absence of Ψ27 in $tRNA^{Leu}_{UAG}$ and probably also in $tRNA^{Arg}_{CCU}$ (this mature tRNA has not been sequenced so far). Notably, a +1 frameshift suppressor $tRNA^{Ser}_{UGA}$ has been identified in yeast mitochondria (65). This mutant bears a C to U base change at position 42, thus leading to a G°U wobble at base pair 28–42 of the anticodon helix. As a consequence of this point mutation, U27 (5′-adjacent to G28) is no longer modified into Ψ27. It was concluded that the +1 frameshift suppressor phenotype, observed in mitochondrial ribosomes, might result from the absence of Ψ27 or from the base change at position 42, or both. However, what is true for the mitochondrial, prokaryote-like, translation machinery may not necessarily apply to the cytoplasmic translation process.

In contrast, deletion of *PUS3* caused a substantial reduction of +1 frameshift efficiency in the context of both pAC-Ty1 and pAC-FST3 (see Fig. 5 and Table III). According to previous results (43), the frameshift event observed with the programmed Ty1 sequence depends on the ability of peptidyl-$tRNA^{Leu}_{UAG}$ (a Ψ39-containing tRNA) to slip from the leucine codon CUU in the normal frame of the P-site to the leucine codon UUA in the P + 1-site. The probability of this event would depend on the translational pause due to the limiting amount of the incoming minor arginyl-$tRNA^{Arg}_{CCU}$. In the case of pAC-FST3, the shift from CUU to the phenylalanine codon UUU in the +1 frame has never been proved. By taking into account that the absence of Ψ39 in the slippery $tRNA^{Leu}_{UAG}$ evidently weakens the codon-anticodon interaction, as discussed above for the termination suppression phenomena, we now propose two non-exclusive alternative explanations for the effect observed in the *pus3*Δ strain. In the first case, the frequency of slipping of the U39-containing peptidyl-$tRNA^{Leu}_{UAG}$ is simply reduced, because the interaction of the UAG anticodon with the +1 near-cognate codon UUA (or UUU) in the P + 1-site of the ribosome is further weakened compared with the in-frame near-cognate codon CUU. In the second case, the reduced binding affinity of undermodified leucyl-$tRNA^{Leu}_{UAG}$ for codon CUU in the incoming A-site of the ribosome allows the minor $tRNA^{Leu}_{GAG}$ to be incorporated in place of $tRNA^{Leu}_{UAG}$. Despite the fact that this minor $tRNA^{Leu}_{GAG}$ harbors an unusual C33 instead of U33, it is able to compete with $tRNA^{Leu}_{UAG}$ for reading the leucine codons CUU and CUC (50). Once in the P-site, this non-slippery $tRNA^{Leu}_{GAG}$ will not allow the ribosome to shift from the normal to the +1-frame of the mRNA (50).

The two other frameshift systems we studied, pAC-FST4 and pAC-FST5, depend on a different mechanism. As pointed out before for the Ty3 frameshift system, peptidyl-$tRNA^{Ala}_{IGC}$, decoding the alanine GCG codon in the P-site stimulates an out-of-frame binding of a major tRNA in the A+1-site, without itself slipping on the mRNA (Ref. 50 and reviewed in Ref. 48). The same explanation prevails for peptidyl-$tRNA^{Pro}_{U*GG}$, decoding the proline CCG codon in the P-site of the ribosome (50). Indeed, these two tRNAs cannot slip in the +1 frame, because of a G*X*G clash in the middle of the codon-anticodon interaction. In this frameshift mechanism, the probability of frameshifting may not depend on the strength of the codon-anticodon interaction, as in the slippery Ty1 mechanism, but rather to other types of interactions. These may be between the two tRNAs, one in the P-site and the other in the A+1-site, and/or between tRNA and rRNA. Whatever the mechanism, the absence of an effect with pAC-FST4 and pAC-FST5 in the *pus3*Δ strain favors the idea that Ψ38 has no influence on this type of RNA-RNA interactions.

Unlike our observations with the yeast *pus3*Δ strain, mutation of the *truA* gene, the homologue of *PUS3*, in *S. typhi-*

murium has been shown to increase +1 frameshift efficiency of a shifty stop programmed recoding system. This effect was observed for a tRNA decoding the leucine codon CUA in the P-site of the ribosome (30). It is important to note that a different set and relative abundance of $tRNA^{Leu}$, harboring distinct patterns of modified nucleotides (including Ψ in the anticodon arm), exists in *E. coli* (and possibly in *S. typhimurium*) and in *S. cerevisiae* (66, 67). Therefore, it can be suggested that in *S. typhimurium* the lack of Ψ in the CUA cognate $tRNA^{Leu}$ allows another more frameshift-inducing competitor tRNA, possibly one of the other near-cognate $tRNA^{Leu}$, to be incorporated in the A-site of the ribosome. The competition between these two tRNAs in *S. typhimurium* would be analogous to the one we discussed above between $tRNA^{Leu}_{UAG}$ and $tRNA^{Leu}_{GAG}$ on the programmed Ty1 frameshift event in yeast, except that in our case the absence of Ψ39 in $tRNA^{Leu}_{UAG}$ might favor the incorporation of the non-frameshift inducing competitor $tRNA^{Leu}_{GAG}$ in the A-site of the eukaryotic ribosome.

Altogether, our results demonstrate that the presence of pseudouridine at position 38 or 39 in tRNA enhances termination readthrough and +1 frameshifting in yeast. It is also noticeable that the growth of *pus3*Δ mutant cells is impaired. These observations strongly support the idea that translational accuracy is optimal rather than maximal, pointing to a role of recoding events in the normal yeast cell physiology. Two chromosomal genes of *S. cerevisiae* are already known to be controlled by a recoding mechanism. The *EST3* gene, encoding a telomerase subunit, needs a +1 frameshift to be expressed (68), and readthrough of a UAG stop codon modulates the expression of the phosphodiesterase encoded by the *PDE2* gene (37). Furthermore, other genes controlled by such mechanisms are still possibly to be discovered.

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Lack of Pseudouridine 38/39 in the Anticodon Arm of Yeast Cytoplasmic tRNA Decreases *in Vivo* Recoding Efficiency

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