A reduced level of charged tRNA$^{Arg}_{mnm^5UCU}$ triggers the wild-type peptidyl-tRNA to frameshift

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ABSTRACT

Frameshift mutations can be suppressed by a variety of differently acting external suppressors. The +1 frameshift mutation *hisC3072*, which has an extra G in a run of Gs, is corrected by the external suppressor mutation *sufF44*. We have shown that *sufF44* and five additional allelic suppressor mutations are located in the gene *argU* coding for the minor tRNA$^{Arg}_{mnm^5UCU}$ and alter the secondary and/or tertiary structure of this tRNA. The C61U, G53A, and C32U mutations influence the stability, whereas the C56U, C61U, G53A, and G39A mutations decrease the arginylation of tRNA$^{Arg}_{mnm^5UCU}$. The T-10C mutant has a base substitution in the −10 consensus sequence of the *argU* promoter that reduces threefold the synthesis of tRNA$^{Arg}_{mnm^5UCU}$. The lower amount of tRNA$^{Arg}_{mnm^5UCU}$ or impaired arginylation, either independently or in conjunction, results in inefficient reading of the cognate AGA codon that, in turn, induces frameshifts. According to the sequence of the peptide produced from the suppressed -GGG-GAA-AGA- frameshift site, the frameshifting tRNA in the *argU* mutants is tRNA$^{Glu}_{mnm^5s^2UUC}$, which decodes the GAA codon located upstream of the AGA arginine codon, and not the mutated tRNA$^{Arg}_{mnm^5UCU}$. We propose that an inefficient decoding of the AGA codon by a defective tRNA$^{Arg}_{mnm^5UCU}$ stalls the ribosome at the A-site codon allowing the wild-type form of peptidyl- tRNA$^{Glu}_{mnm^5s^2UUC}$ to slip forward 1 nucleotide and thereby re-establish the ribosome in the 0-frame. Similar frameshifting events could be the main cause of various phenotypes associated with environmental or genetically induced changes in the levels of aminoacylated tRNA.

Keywords: frameshifting; arginine tRNA; suppression

INTRODUCTION

The genetic code contains 61 sense codons for only 20 amino acids. Therefore, one amino acid can be coded by several synonymous codons. However, codon usage in genes is not random (Nakamura et al. 2000) and correlates with the relative quantities of individual transfer RNAs. In genes encoding abundant proteins, codons corresponding to the major tRNAs are used (Ikemura 1981b). This arrangement of biased codon usage was suggested to maximize the speed and fidelity of translation. The presence of rare codons throughout the messenger RNA lowers its translation rate to some extent, but does not influence the expression level of the protein (Kurland 1991). Instead, protein synthesis is determined by the rate of translation initiation (Kurland 1991). In cases in which rare codons are close to the translation initiation site, they modulate protein

synthesis by stalling the ribosome and preventing entry of incoming ribosomes (Chen and Inouye 1990; Rosenberg et al. 1993). This mechanism has been suggested to regulate the expression of a group of essential genes for various cellular functions (Chen and Inouye 1994). Alternatively, if the level of a minor aminoacyl-tRNA is limiting, the cognate rare codon could lower the synthesis of the mature protein by triggering frameshift errors. Indeed, occurrence of two rare codons in tandem induces ribosomes to shift frequently (Spanjaard and van Duin 1988). This paper addresses how the changed concentration of a minor tRNA may induce frameshifts even at a single rare codon.

The genetic analysis of ribosomal reading frame maintenance is founded on the analysis of suppression of frameshift mutations. Frameshift mutations are created by either addition or deletion of nucleotides in the gene sequence, resulting in an out-of-frame translation of the mRNA and production of an aberrant protein. Correction of the phenotype is possible if the ribosome shifts the reading frame back to the original, as shown in Figure 1. Slow entry of a cognate tRNA to the A-site may induce a ribosomal pause, allowing the peptidyl-tRNA in the P-site to shift 1 nt either to the left (−1 frameshifting) or to the right (+1 frameshift-
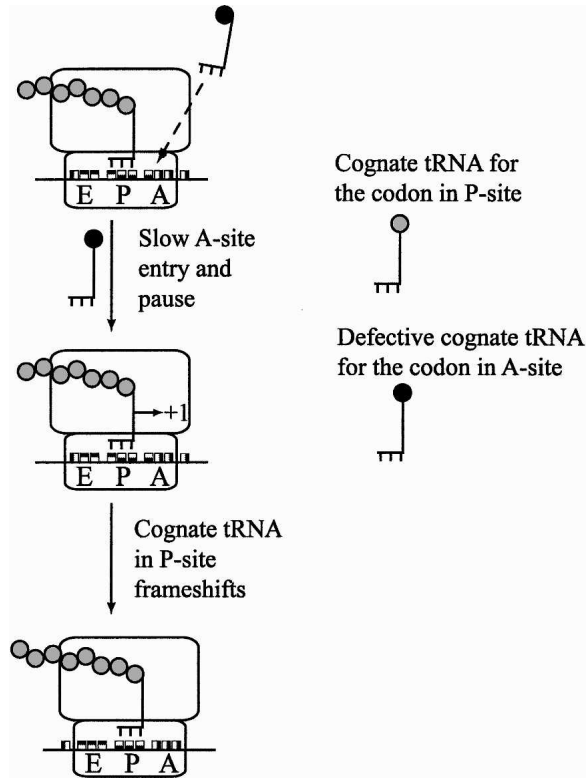

FIGURE 1. Frameshifting model. A defective cognate tRNA enters the A-site slowly, thereby inducing a stalled ribosome that allows the wild-type cognate peptidyl-tRNA to slip into the +1 frame. The pattern of the nucleotides in the E-, P-, and A-sites corresponds to the triplets found in the wild-type allele of the frameshift mutant.

ing) (Farabaugh 1996; Atkins et al. 2000; Gallant et al. 2000). The direction is determined by the ability to form at least two Watson-Crick base pairs with the codon in the new frame (Farabaugh and Björk 1999), although landing sites with less or no base-pairing can also be used (Herr et al. 2004). Peptidyl-tRNA can also change the reading frame because of the slow action of a release factor at a stop codon in the A-site (Weiss et al. 1990). Spontaneous frameshifting events are very infrequent, probably $<10^{-5}$ per codon (Kurland et al. 1996). However, the frequency can be affected by certain sequence contexts and/or by certain physiological conditions (Fu and Parker 1994; Barak et al. 1996; Schwartz and Curran 1997; Stahl et al. 2004); for example, upon entrance into stationary phase, frameshifting increases threefold to sixfold in *Escherichia coli* (Wenthzel et al. 1998). It has also been observed that both the deprivation of a particular aa-tRNA species and accumulation of abnormally high concentrations of other tRNA species increases frameshifting levels (Farabaugh 1996; Kurland et al. 1996; Atkins et al. 2000; Gallant et al. 2000).

Alteration in either tRNA structure or extent of modification can also cause translating ribosomes to frameshift (Björk et al. 1989; Farabaugh and Björk 1999; Urbonavicius et al. 2001) and thereby suppress frameshift mutations. In

1970 Riddle and Roth presented a collection of *Salmonella enterica* Serovar Typhimurium frameshift suppressors (Riddle and Roth 1970). The suppressors were classified by the frameshift mutations they suppressed, by their location on the chromosome, and by their dominance or recessiveness to the wild-type alleles (Riddle and Roth 1972a). Suppressor mutations in the *sufA* and *sufB* genes were dominant and affected the structure of two proline tRNAs later identified as tRNA$^{Pro}_{CGG}$ and tRNA$^{Pro}_{GGG}$, respectively (Riddle and Roth 1972b; Sroga et al. 1992). Mutations in *sufD* were also dominant and affected tRNA$^{Gly}_{CCC}$ (Riddle and Roth 1972b; Riddle and Carbon 1973). Since the recessive suppressor *sufF* corrected some of the same frameshift mutations as the dominant suppressor *sufD*, *sufF* was also inferred to elicit a change in a glycine tRNA. Consistent with this suggestion was the fact that glycine tRNA from the *sufF44* mutant had altered chromatographic properties (Riddle and Roth 1972b). Together with the recessiveness of *sufF44* to the wild-type allele, this evidence suggested that *sufF* might encode a tRNA-modifying enzyme acting on a glycine tRNA. It is now known that tRNA$^{Gly}_{mnm^5UCC}$ in *E. coli* has the modified uridine 5-methylaminomethyluridine (mnm^{5}U) as the wobble nucleoside (T. Suzuki, pers. comm.) and is similar to the homologous glycine tRNA in *Bacillus subtilis* (Sprinzl et al. 1998). Since deficiency in the related nucleoside 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) induces frameshifts (Urbonavicius et al. 2001), we hypothesized that *sufF* may encode an enzyme involved in the synthesis of mnm^{5}U. However, in contrast to our presumptions, we present evidence that the product of *sufF* is not a protein but a minor arginine tRNA. A mechanism by which this mutated tRNA suppresses a frameshift mutation located upstream of the codon AGA is proposed.

RESULTS

The frameshift mutation *hisC3072* is a G insertion in a run of Gs

All the frameshift suppressors described earlier (Riddle and Roth 1970) were grouped according to their suppressing specificity of different frameshift mutations. The *sufF* class only suppressed mutations *hisC3072* and *hisC3736*. The *hisC* gene from the *hisC3072* and *hisC3736* mutants was sequenced (see Table 1 for strains used), and it was found that they both had a G insertion (underlined) in a run of Gs: GGG GAA AGA (N)$_{87}$ UGA causing a shift in the reading frame (presented is the reading frame formed following the insertion). Thus, as anticipated, the mutation created a sequence consistent with a possible slip forward by a defective tRNA^{Gly}. In the mutants, the first 102 amino acids of the HisC protein were coded correctly (the full protein is 359 amino acids long), but due to the frameshift mutation, translation proceeded in the wrong frame until a stop

TABLE 1. Strains

Strain	Genotype	Source
LT2	Wild type	Riddle and Roth 1970
GT885	hisO1242 hisC3072	Riddle and Roth 1970
GT889	hisO1242 hisC3736	Riddle and Roth 1970
GT400	hisO1242 hisC3072 sufF44 (C61U) trpA8	Riddle and Roth 1970
GT5148	hisO1242 hisC3072 argU2343 (G53A)	This work
GT5130	hisO1242 hisC3072 purE::Tn10dTc	This work
GT5164	hisO1242 hisC3072 argU2343 fimC2529::Tn10dTc	This work
GT5162	hisO1242 hisC3072 purE8 argU2343 fimC2529::Tn10dTc	This work
GT5144	hisO1242 hisC3072 argU2341 (C32U)	This work
GT5150	hisO1242 hisC3072 argU2344 (C61U)	This work
GT5136	hisO1242 hisC3072 argU2342 (T-10C)	This work
GT5140	hisO1242 hisC3072 argU2346 (C56U)	This work
GT5141	hisO1242 hisC3072 argU2345 (G39A)	This work
GT6863	hisO1242 hisC3072 ttcA5<>kan	This work

codon was encountered. Cells carrying such a truncated HisC had a His⁻ phenotype.

The *sufF44* mutation is in the *argU* gene

The *sufF44* mutation is 27% linked to mutation in the *purE* gene that induces purine auxotrophy (Riddle and Roth 1972a). A transposon Tn10dTc insertion was isolated (strain GT5164) and was shown to be linked to both the *purE* and *sufF44* genes (data not shown). Direct sequencing

of chromosomal DNA isolated from GT5164 using Tn10 specific primers revealed that the Tn10dTc element was inserted into the gene *fimC*, encoding a periplasmic chaperone involved in the formation of type 1 fimbriae.

Since the *sufF44* mutation was recessive to the wild-type allele, it seemed possible to isolate a plasmid harboring the *sufF⁺* allele. Such a plasmid should change the phenotype of the *sufF44* mutant from His⁺ to His⁻. Thus, the *S. enterica* gene bank was introduced into His⁺ strain GT5164 (*hisO1242 hisC3072 argU2343 fimC2529*::Tn10dTc), and His⁻ transformants were screened. Plasmid DNA was purified from several His⁻ transformants, and the chromosomal inserts harbored by the plasmids were partially sequenced. The sequence obtained from clone GT5164/pTH67 showed that the 16.7-kb-long insert contained DNA from a region close to the *fimC* gene and contained a gene coding for tRNA$_{mnm^5UCU}^{Arg}$. To further investigate the role of tRNA$_{mnm^5UCU}^{Arg}$, plasmid pMW210 containing the tRNA$_{mnm^5UCU}^{Arg}$ gene from phage T4 cloned into the vector pACYC184 was used (Wikström et al. 1992). After introducing this plasmid into the His⁺ strain GT5148 (*hisO1242 hisC3072 argU2343*), all cells became His⁻, supporting the argument that the *sufF44* mutation was in the *argU* (sometimes called *fimU*) tRNA$_{mnm^5UCU}^{Arg}$ gene.

New mutants defective in tRNA$_{mnm^5UCU}^{Arg}$ were isolated

By using mutagens hydroxylamine or nitrosoguanidine, 10 new *purE*-linked frameshifting mutants were isolated that could suppress the *hisC3072* mutation. The sequence of the *argU* gene from all mutants revealed that five different tRNA$_{mnm^5UCU}^{Arg}$ mutations had been created (Fig. 2) and one mutation was located in the upstream region of the *argU*
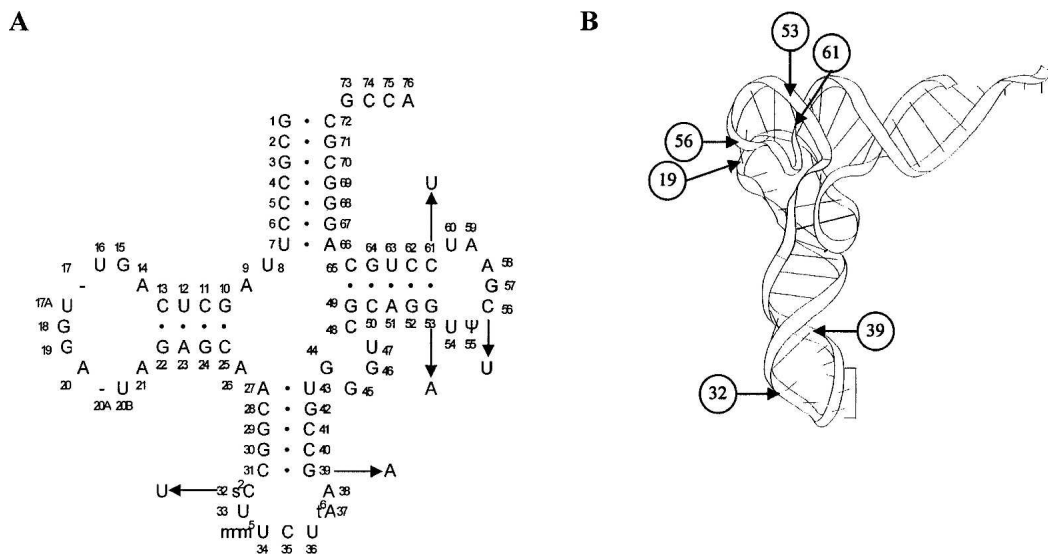

FIGURE 2. (*A*) Cloverleaf structure of tRNA$_{mnm^5UCU}^{Arg}$ with the mutations discussed in this paper indicated. The C61U mutation was isolated in five independent cases, and one of them is the original mutant *sufF44*; all the other mutations were obtained once. (*B*) Tertiary structure of tRNA^{Phe} from yeast (see Kim et al. 1974). The arrows are pointing to the positions of mutated nucleotides.

gene (position −10 according to the transcriptional start) (Saxena and Walker 1992).

The mutations C61U and G53A in the TΨC stem and G39A in the anticodon stem disrupted base-pair interactions in the tRNA$_{mnm^5UCU}^{Arg}$ cloverleaf structure. Mutation C56U disrupted the G19–C56 pair, which locks the D and TΨC loops together. Mutation C32U changed a residue in the anticodon loop, which also eliminated the possibility to form the 2-thiocytidine (s^{2}C) modification at position 32 of tRNA. Each of those changes can cause defects in the tRNA structure and influence the function of tRNA. Mutation T-10C was a change in the *argU* promoter −10 consensus sequence altering the conserved sequence TATAAT to TACAAT, which could influence expression levels of tRNA$_{mnm^5UCU}^{Arg}$ (Youderian et al. 1982; Hawley and McClure 1983).

The thio-group of s^{2}C32 does not influence frameshifting in the *hisC3072* mutant

Mutation C32U prevented s^{2}C modification formation at tRNA position 32. Lack of s^{2}C32 decreases the rate of A-site selection at the AGG codon, but not at the AGA codon when the frameshifting site is CCC-AGA/G-UAA (Jäger et al. 2004). Since the frameshifting site in the *hisC3072* mutant was different (-GGG-GAA-AGA-N$_{87}$-UGA), we tested whether tRNA$_{mnm^5UCU}^{Arg}$ lacking the s^2-group of s^{2}C32 would be able to suppress the *hisC3072* mutation. Therefore, a deletion of the gene *ttcA*, which codes for one of the steps in the formation of s^{2}C (Jäger et al. 2004), was introduced into strain GT885 (*hisO1242 hisC3072*), creating strain GT6863 (*hisO1242 hisC3072 ttcA5<>kan*). However, this double mutant was still His$^-$, indicating that lack of the sulfur modification s^{2}C32 in tRNA$_{mnm^5UCU}^{Arg}$ was not a cause of frameshifting (data not shown), a result consistent with earlier results (Jäger et al. 2004).

Several of the *argU* mutants have reduced levels of tRNA$_{mnm^5UCU}^{Arg}$

As the *argU2342* (T-10C) mutation was located in the promoter region of the *argU* gene, it might alter the level of tRNA$_{mnm^5UCU}^{Arg}$. Therefore, we compared the ratios between the yields of tRNA$_{mnm^5UCU}^{Arg}$ and tRNA$_{mnm^5s^2UUG}^{Gln}$ or tRNA$_{cmnm^5UmAA}^{Leu}$ in total RNA isolated from the *argU2342* (T-10C) mutant as well as from the wild type. Northern blot analysis of tRNAs fractionated on a polyacrylamide gel was performed by probing with different oligonucleotides homologous to tRNA$_{mnm^5UCU}^{Arg}$, tRNA$_{mnm^5s^2UUG}^{Gln}$, or to tRNA$_{cmnm^5UmAA}^{Leu}$ (Fig. 3). Bands corresponding to tRNA from different hybridization experiments were quantified, and their relative ratios were calculated. The results revealed that the *argU2342* (T-10C) mutant had only 33% of the amount of tRNA$_{mnm^5UCU}^{Arg}$ compared to wild-type cells, in-
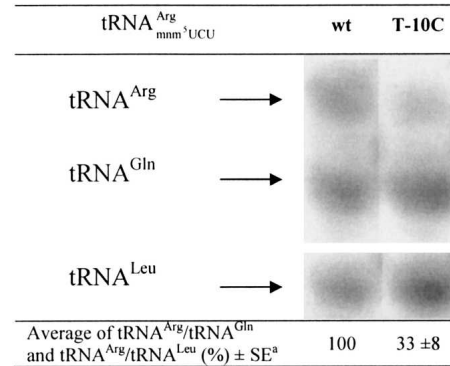

tRNA$_{mnm^5UCU}^{Arg}$	wt	T-10C
tRNA^{Arg} →		
tRNA^{Gln} →		
tRNA^{Leu} →		
Average of tRNA^{Arg}/tRNA^{Gln} and tRNA^{Arg}/tRNA^{Leu} (%) ± SE[a]	100	33 ±8

FIGURE 3. Relative levels of tRNA$_{mnm^5UCU}^{Arg}$ in wild type and the promoter mutant *argU2342* (T-10C). [a]The bands specific for different deacylated tRNAs were quantified and the ratio between them calculated. The result is the percentage of the level observed in wild type ± standard error. The difference observed between the levels in mutant and in wild type is significant based on a *t*-test ($p = 0.003$).

dicating reduced expression of the *argU* gene as a result of the mutation in the −10 sequence of its promoter.

Since mutations influencing the secondary structure of tRNA$_{mnm^5UCU}^{Arg}$ might destabilize the tRNA, similar experiments were also performed with other mutants. The radioactive signal obtained from the probe binding to tRNA$_{mnm^5UCU}^{Arg}$ was normalized to the signals obtained from tRNA$_{mnm^5s^2UUG}^{Gln}$- or tRNA$_{cmnm^5UmAA}^{Leu}$-binding probes in each strain. The comparison of tRNA$_{mnm^5UCU}^{Arg}$ levels in the mutants versus the level in the wild type demonstrated that the *argU2344* (C61U), *argU2343* (G53A), and *argU2341* (C32U) mutations reduced tRNA$_{mnm^5UCU}^{Arg}$ concentrations more than twofold (Table 2). However, *argU2345* (G39A) and *argU2346* (C56U) had similar levels of tRNA$_{mnm^5UCU}^{Arg}$ compared to the wild type.

Except the C32U mutant, all mutants had a reduced arginylation of tRNA$_{mnm^5UCU}^{Arg}$

Changes in the tRNA structure could influence the efficiency of arginylation of tRNA$_{mnm^5UCU}^{Arg}$. We therefore compared the levels of charged tRNA$_{mnm^5UCU}^{Arg}$ versus uncharged tRNA$_{mnm^5UCU}^{Arg}$ in all of the mutants. To prevent deacylation of bound amino acids, tRNAs were isolated under acidic conditions. An aliquot of tRNAs from each preparation was deacylated by alkaline hydrolysis. Both deacylated and untreated aliquots were run on an acidic polyacrylamide gel, on which charged and uncharged tRNAs were separated. This gel was later used in Northern blot analysis and hybridized to a radioactively labeled probe specific for tRNA$_{mnm^5UCU}^{Arg}$ (Fig. 4). Both charged and uncharged tRNA$_{mnm^5UCU}^{Arg}$ forms isolated from *argU2341* (C32U) migrated faster on the gel, which might be caused by lack of the s^{2}C32 modification. In the wild type, most of the tRNA$_{mnm^5UCU}^{Arg}$ was in the charged form, comprising 75% of total tRNA$_{mnm^5UCU}^{Arg}$. Previous works describe similar levels of aminoacylation of different tRNA species in wild-type

TABLE 2. Levels of $tRNA_{mnm^5UCU}^{Arg}$

$tRNA_{mnm^5UCU}^{Arg}$	wt	C32U	G53A	C61U	C56U	G39A
Relative level of $tRNA_{mnm^5UCU}^{Arg}$ [a]	100	48 ± 10 [b]	32 ± 3 [b]	39 ± 6 [b]	126 ± 11	141 ± 31

[a]Results are the percentage of deacylated $tRNA_{mnm^5UCU}^{Arg}$ levels compared to the wild-type level ± standard error.
[b]The difference observed between the levels in the mutant and in wild type is significant based on a *t*-test ($p < 0.01$).

cells under steady-state growth (Varshney et al. 1991; Kruger and Sorensen 1998; McClain et al. 1999). Visual analysis of the bands in *argU2346* (C56U), *argU2344* (C61U), *argU2343* (G53A), and *argU2345* (G39A) mutants clearly indicated that in those samples the amount of uncharged $tRNA_{mnm^5UCU}^{Arg}$ form increased. To facilitate the comparison, we quantified the intensity of different bands. Due to blurriness, it was hard to determine the precise boundaries of each band; therefore, the whole experiment was repeated three times, and the results are presented in Figure 4. The analysis showed that the aminoacylation level in the *argU2346* (C56U) decreased the most (44% of the $tRNA_{mnm^5UCU}^{Arg}$ was charged). In *argU2344* (C61U), *argU2343* (G53A), and *argU2345* (G39A), 50%–55% of the $tRNA_{mnm^5UCU}^{Arg}$ was aminoacylated. Mutation C32U did not change the charging level of $tRNA_{mnm^5UCU}^{Arg}$. The quantification of charging in the *argU2342* (T-10C) was uncertain because of the diminished amounts of $tRNA_{mnm^5UCU}^{Arg}$, although visual estimation indicated unaffected arginylation levels of $tRNA_{mnm^5UCU}^{Arg}$ in this mutant. Such a result was the most expected, since the T-10C mutation did not change the structure of $tRNA_{mnm^5UCU}^{Arg}$.

Wild-type $tRNA_{mnm^5s^2UUC}^{Glu}$ and not the altered $tRNA_{mnm^5UCU}^{Arg}$ is the frameshifting tRNA

One mutant, *argU2343* (G53A), was chosen for analysis to clarify the nature of the slippage event at the frameshift

sequence of *hisC3072*. For that purpose, plasmid pUST274 was constructed (Fig. 5A). The frameshift sequence was inserted between the genes encoding the glutathione-S-transferase (GST) and the maltose-binding protein (MBP) with six histidine residues (6×His) at the carboxy terminus in the *gst-malE* fusion gene. *malE* is in the +1 frame relative to *gst*, explaining why the complete GST-MBP-6×His fusion protein is only synthesized when +1 frameshifting occurs. If +1 frameshifting does not occur, translation terminates at the UAA stop codon found downstream of the *gst* gene, and only GST is produced (Figs. 5A, 6). The complete fusion protein was purified from strain GT5164 [*argU2343* (G53A)/pUST274] using the GST and 6×His affinity tags. To liberate the slippage junction fused to the MBP-6×His, the frameshift product was treated with PreScission Protease. This protease cuts the protein at the specific protease site between the GST moiety and the rest of the peptide. After digestion the sample was analyzed by mass spectrometry, and the observed values were compared to the predicted masses of peptides having different possible amino acid composition (Fig. 6). The major peak observed in a mass spectrum in the 41500–46000 Da range was at 43374.50 Da (data not shown). This correlated best with the expected 43374.15-Da mass of a peptide putatively produced when the frameshifting tRNA was glutamate tRNA. To verify this hypothesis further, the N terminus of the slippage junction fused to MBP-6×His was sequenced. The first 12 amino acids of the peptide were determined as
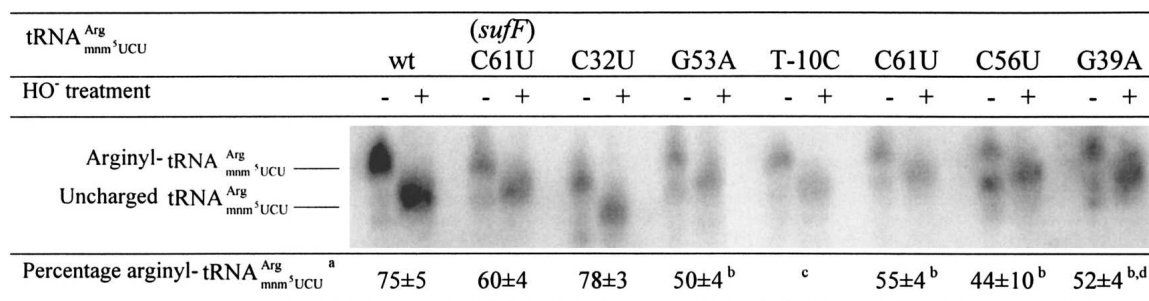

FIGURE 4. Charging levels of different $tRNA_{mnm^5UCU}^{Arg}$ mutants. Bands corresponding to arginyl- $tRNA_{mnm^5UCU}^{Arg}$ and uncharged $tRNA_{mnm^5UCU}^{Arg}$ are indicated. [a]The percentage and standard error of arginyl-$tRNA_{mnm^5UCU}^{Arg}$ [100× arginyl-$tRNA_{mnm^5UCU}^{Arg}$/(arginyl-$tRNA_{mnm^5UCU}^{Arg}$ + uncharged $tRNA_{mnm^5UCU}^{Arg}$)] are the average of three independent experiments. [b]The difference in charging observed between the mutant and the wild type is significant based on a *t*-test ($p \leq 0.03$). [c]The quantification was unreliable because of too high background and diminished amounts of $tRNA_{mnm^5UCU}^{Arg}$ in the T-10C mutant (Fig. 3). [d]The double bands of uncharged tRNA in G39A lanes are due to artifacts in this particular gel. The bands migrated as uniform bands in the other two experiments.
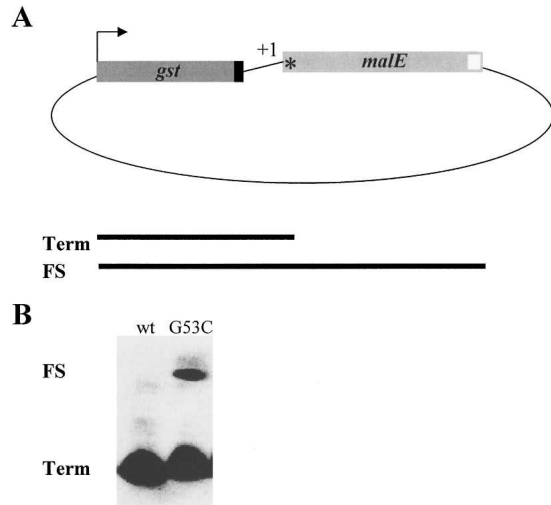
A

B

FIGURE 5. Plasmid pUST274 construct used for measuring +1 frameshifting. (*A*) The *malE* gene is in the +1 frame relative to the *gst* gene. In the 3′-terminus of *gst*, the sequence coding for a PreScission protease recognition site is indicated as a black box; the first stop codon encountered in the 0 frame in *malE* is marked with an *; 6×His, the six-histidine tag is indicated as a white box. (*B*) Frameshifting (FS) and termination (Term) products from the wild type and *argU2343* (G53C) mutant separated on an SDS gel and were detected by anti-GST antibodies in Western blot analysis.

GPLGIPGE**DAIL**, where **E** (glutamate) was the last amino acid inserted in 0 frame, suggesting that the frameshifting tRNA at the frameshift site GGG-GAA-AGA in *hisC3072* was wild-type tRNA$^{\text{Glu}}_{\text{mnm}^5\text{s}^2\text{UUC}}$ and not the mutated tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$.

Efficiency of frameshifting

Our data suggested a correlation between the frameshifting, which induces His⁺ phenotype, and the decrease in arginyl-tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ levels. To receive a quantitative estimate for the frequency of frameshifting in tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ mutants, we used the above-mentioned plasmid pUST274 containing the GGG-GAA-AGA sequence. This plasmid was introduced into the wild type and *argU2343* (G53A) mutant, which had the lowest level of arginyl-tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$. Total protein extracts from GT5164/pUST274 and LT2/pUST274 strains were fractionated by gel electrophoreses. The termination product and complete fusion proteins were detected by Western blot analysis using anti-GST antibody (Fig. 5B). The extract prepared from the mutant strain contained two clear bands (26 kDa and 70 kDa), while the wild type had only the termination product. Comparison of the frameshifting and termination peptide abundances indicated that for tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ with alteration in position 53, the frequency of frameshifting at the GGG-GAA-AGA sequence was ~5%. We expect a lower frequency of frameshifting induced by the other mutants, since they reduce the level of charged tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ less (Table 2; Fig. 4).

DISCUSSION

tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ is a tRNA, found in low quantities in *E. coli* (Ikemura 1981a; Dong et al. 1996). We describe five different alterations in this tRNA or in the promoter for its structural gene (*argU*) that induce frameshifts at the sequence GGG-GAA-AGA-(NNN)$_{29}$-UAA in the *hisC3072* mutated gene. We suggest that the mutations decreased the concentration of charged tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ and thereby were allowing tRNA$^{\text{Glu}}_{\text{mnm}^5\text{s}^2\text{UUC}}$, which decodes GAA upstream of the arginine codon AGA, to frameshift. This suggestion is consistent with the frameshifting model presented in Figure 1.

Alterations influencing the structure of a tRNA can destabilize this tRNA and increase its degradation—why it could become less abundant. In addition, changed tRNA structure can disturb its aminoacylation by cognate aminoacyl-tRNA synthetase. Indeed, the *argU2343* (G53A) and *argU2344* (C61U) mutants exhibited decreased amounts and reduced charging of tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ (Table 2; Fig. 4). The concentration of arginyl-tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ in those mutants was only 21% and 28% of the wild-type level, respectively, considering both the level of arginylation and the total level of tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$. The G53A and C61U mutations altered bases in the TΨC stem and disrupted the conserved C61–G53 base pair, which presence is critical for the characteristic TΨC loop structure. Replacement of C61 by U61 induces destabilization of this structure, as revealed by changed reactivity of the phosphate 60, which makes a hydrogen bond with the amino group of C61 (Dirheimer et al. 1995). Interestingly, a single alteration of C to U at position 61 in another tRNA, namely, *Salmonella* tRNA$^{\text{Gly}}$ (*sufS617*), induces suppression of a −1 frameshift mutation (O'Mahony et al. 1989).

The *argU2341* (C32U) mutation lowered the abundance of tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ twofold (Table 2). The residue at position 32 forms a noncanonical bifurcated hydrogen bond with residue 38 at the stem/loop junction and shapes the anticodon loop (Auffinger and Westhof 1999). Phylogenetic data revealed that the presence of pyrimidines in position 32 was preferred in the majority of tRNAs; 56% of the cases containing C32 and 39% U32. The distribution of the 32–38 pair is biased toward C-A, since it is found in 42%, s²C-A in 1%, and U-A in 5% of all sequenced tRNAs. Although both C32–A38 and U32–A38 pairs adopt the same configuration in crystal structures, substitution of a C-A pair by U-A affects the discriminating ability of tRNA transcripts and their frameshifting efficiency (Lustig et al. 1993; O'Connor 1998; Auffinger and Westhof 1999). Our results also demonstrate a lowered tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$ stability by an s²C32-to-U32 change. We also show that this effect could not be attributed to the lack of the s²-group, but rather to the change of the base in position 32. The *argU2341* (C32U) mutation did not affect the aminoacylation of tRNA$^{\text{Arg}}_{\text{mnm}^5\text{UCU}}$, indicating a normal interaction with the arginyl-tRNA synthetase (ArgRS). Similar unchanged argi-
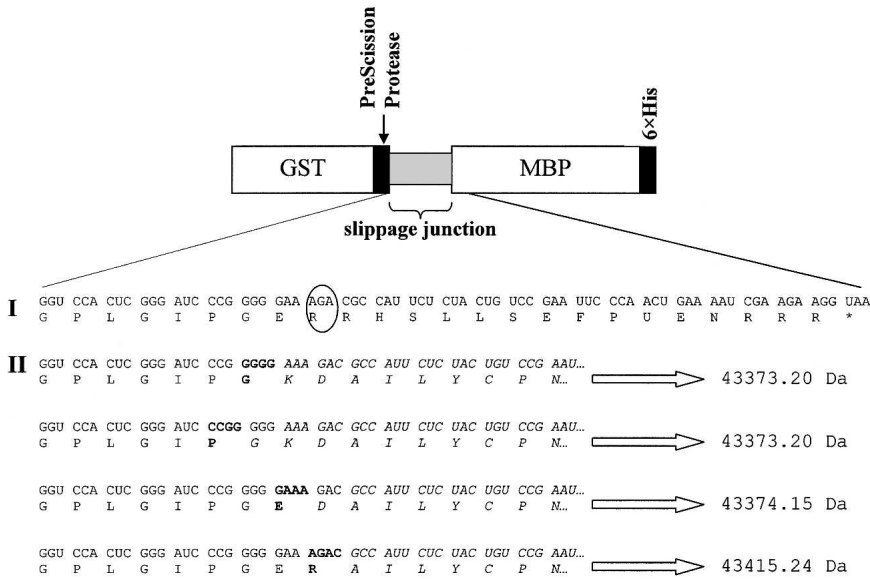
FIGURE 6. Various alternatives of fusion proteins produced by slippage by different tRNAs. After PreScission Protease digestion the GST part is cleaved away and the molecular mass of the resulting peptides is analyzed. (**I**) The peptide part liberated after the cleavage if no frameshifting occurred; the coding sequence is also presented. The critical AGA codon for R (arginine) is indicated. (**II**) Fragments of different peptides liberated after the cleavage if different tRNAs were slipping. The amino acid inserted by the frameshifting tRNA and the sequence where this tRNA frameshifts are in bold. The sequence translated and amino acids inserted in the +1 frame are in italic. On the *right* side are the expected masses of those hypothetical peptides.

nylation in vitro was reported for tRNA$_{ICG}^{Arg}$ lacking s^{2}C32 (Kruse et al. 1978). We conclude that the base and not the s^2-modification stabilizes tRNA structure, whereas neither of them interferes with tRNA$_{mnm^5UCU}^{Arg}$ and ArgRS interaction.

The C56U and G39A alterations did not affect the stability of tRNA$_{mnm^5UCU}^{Arg}$ (Table 2), but decreased its aminoacylation (Fig. 4). The *argU2346* (C56U) mutant had the most pronounced decrease (twofold) when compared to the wild type. Base C56 is a highly conserved nucleotide in tRNAs and is involved in forming the G19–C56 pair, which locks the D and TΨC loops together (Dirheimer et al. 1995). Changing the G19–C56 pair into a weaker G19–U56 pair most likely destabilizes the D–TΨC interaction, though probably not strongly enough to influence the stability of tRNA$_{mnm^5UCU}^{Arg}$ (Table 2). In contrast, the structural rigidity of the tRNA$_{mnm^5UCC}^{Gly}$ was drastically reduced by the C56A mutation that prevented base-pairing to G19 (Herr et al. 1999). Changed bonding between bases 19 and 56 in our C56U mutant could influence the interference with the adjacent nucleotide A20, which together with C35, U/G36, and A/G73 are the major positive recognition elements in tRNA^{Arg}:s for ArgRS (McClain and Foss 1988; McClain et al. 1990; Giegé et al. 1998). The A at position 20 is conserved in tRNA^{Arg}:s from all organisms except yeast. Alteration of this A to U affects both the suppression efficiency and the acceptor identity of suppressor tRNA^{Arg} (McClain

and Foss 1988). Therefore, our observation that the C56U alteration induced less arginylation is consistent with other observations (McClain and Foss 1988; McClain et al. 1990; Delagoutte et al. 2000) and implicates this region of the tRNA being, indeed, important for an optimal tRNA–ArgRS interaction.

The T-10C base substitution in the promoter region of the *argU2342* mutant greatly reduced the abundance of tRNA$_{mnm^5UCU}^{Arg}$ (Fig. 3). The promoter of *argU* encoding tRNA$_{mnm^5UCU}^{Arg}$ has similar features to strong rRNA promoters such as an upstream activator sequence, a −35 sequence very similar to the δ^{70} consensus sequence, and a −10 TATAAT consensus sequence (Lisser and Margalit 1993). However, *argU* expression is severely inhibited by sequences downstream of the transcription start point, rendering tRNA$_{mnm^5UCU}^{Arg}$ a minor tRNA (Saxena and Walker 1992). The conserved promoter elements are devoted to stabilizing the RNA polymerase at the promoter, directing the holoenzyme-dependent melting of the DNA, and allowing a productive initiation of transcription (Helmann and deHaseth 1999). The general rule concerning changes in the conserved −10 promoter region suggests that decreasing the homology to the consensus sequence will reduce gene expression (Hawley and McClure 1983). A single base substitution at the −10 position from T to C (TATAAT to TACAAT) as in the *argU2342* (T-10C) mutant has been previously isolated in the bacteriophage P22 *ant* gene promoter. That mutation caused a mild reduction of *ant* promoter activity (Youderian et al. 1982). A similar single substitution in the *rrnB* promoter P1 induced an ~10-fold decrease in its activity (Gaal et al. 1989). In the *argU2342* case, the T-10C mutation reduced threefold the synthesis of tRNA$_{mnm^5UCU}^{Arg}$.

In summary, the isolated mutants had either decreased amounts of tRNA$_{mnm^5UCU}^{Arg}$ and/or reduced charging of this tRNA due to compromised secondary or tertiary structure, and in one case due to impaired transcription of the *argU* gene. The A61U and G53A mutations in the T-stem alter the face of tRNA (consisting of T-stem and acceptor-stem helices), to which the elongation factor Tu (EF-Tu) · GTP binds (Nissen et al. 1995, 1999). This could affect the formation of the ternary complex (EF-Tu · GTP · aa-tRNA) required for tRNA selection to the A-site codon. The G1-to-A1 change in the acceptor-stem of the same tRNA$_{mnm^5UCU}^{Arg}$ was demonstrated to impair the EF-Tu · GTP binding (Sakamoto et al. 2004).

The earlier mentioned G1A mutant in $tRNA^{Arg}_{mnm^5UCU}$ [*argU10*(Ts)] and some other tRNA mutants, such as the *divE* having an A10G change in the major $tRNA^{Ser}_{cmo^5UGA}$ and *feeB* having a C77A change in the $tRNA^{Leu}_{U*AG}$, have one common phenotype—they all induce a cell division defect (Tamura et al. 1984; Garcia et al. 1986; Chen et al. 1991). When the *argU10*(Ts) mutant is shifted to nonpermissive temperature, it ceases to grow in ~1 h, although the rate of total protein synthesis, measured as TCA-precipitable material, is reduced only to two-thirds of the wild-type rate and continues for at least 2 h. In contrast, the synthesis of the λ cI repressor containing five AGA codons read by $tRNA^{Arg}_{mnm^5UCU}$ is severely reduced. This observation suggests that it is not the general protein synthesis but rather the synthesis of some specific protein(s) pivotal in controlling cell division that is affected (Chen et al. 1990). Note, however, that monitoring TCA-precipitable material does not reveal changes in the frequency of frameshifting. The *argU10*(Ts) mutant has a severely decreased amount of $tRNA^{Arg}_{mnm^5UCU}$, a reduced level of arginylation, and impaired binding of this tRNA to EF-Tu · GTP (Sakamoto et al. 2004). Therefore, it was inferred that decoding of the cognate AGA codon in this mutant is inefficient and inhibits the synthesis of specific protein(s) containing AGA codons in their mRNA. However, less efficient reading of a codon does not affect the overall expression of a protein (Kurland 1991).

Our data suggest an alternative mechanism eliciting the defects in cell division. Amino acid sequence analysis of the frameshift product in the *argU2343* (G53C) mutant revealed that while the mutated tRNA is $tRNA^{Arg}_{mnm^5UCU}$, the tRNA that frameshifts is $tRNA^{Glu}_{mnm^5s^2UUC}$. When the ribosome arrives at the -GGG-GAA-AGA- site and the AGA codon is in the ribosomal A-site, the $tRNA^{Glu}_{mnm^5s^2UUC}$ reading the GAA codon resides in the P-site (see Fig. 1). Due to decreased levels of charged $tRNA^{Arg}_{mnm^5UCU}$, the level of the ternary complex loaded with the arginyl-$tRNA^{Arg}_{mnm^5UCU}$ is reduced, which slows down the AGA reading and stalls the ribosome. Besides, ribosome stalling could be prolonged by inefficient A-site entry by the ternary complex containing altered $tRNA^{Arg}_{mnm^5UCU}$. Such ribosome pausing destabilizes cognate peptidyl-$tRNA^{Glu}_{mnm^5s^2UUC}$ in the P-site and facilitates its shift forward by 1 nt (Fig. 1; Qian et al. 1998; Farabaugh and Björk 1999; Gallant et al. 2000; Hansen et al. 2003). We also cannot exclude the alternative that ribosome pausing stimulates out-of-frame binding of an incoming aspartyl-tRNA cognate for the codon in the +1 frame (Stahl et al. 2002). The wild-type levels of $tRNA^{Arg}_{mnm^5UCU}$ are among the lowest in the cell (Dong et al. 1996). Observed frameshifting occurred when the arginyl-$tRNA^{Arg}_{mnm^5UCU}$ levels were decreased only by twofold. We assume that reduction down to "trace" levels of arginylated $tRNA^{Arg}_{mnm^5UCU}$, as in the *argU10*(Ts) mutant (Sakamoto et al. 2004), will elicit extensive frameshifting, at least at -GAA-AGA- sites. The peptidyl-$tRNA^{Glu}_{mnm^5s^2UUC}$, which frameshifts at the -GAA-

AGA- site, "takes off" from the GAA codon and lands at the AA-A codon. For frequent frameshifting, weak base-pairing at the "take-off" site is important (Gallant et al. 2004), whereas the ability to form Watson-Crick base pairs at the landing site is less critical, even though strong base-pairing is preferred (Herr et al. 2004). As a take-off site present on the 5′-side of the hungry codon, the GAA codon is moderately proficient, while some other codons, like UUU, are up to sevenfold more proficient (Gallant et al. 2004). Therefore, the frameshifting frequency would be probably higher at the UUU-AGA site then at the GAA-AGA site.

Based on such considerations and the results presented here, we suggest that cell division defects in various tRNA mutants are caused by frameshifting errors rather than inefficient translation in the zero frame of some specific mRNAs. A survey of the entire *E. coli* genome determined the frequency of different codon pairs (Boycheva et al. 2003). The arginine codon pairs AGA-AGA, AGG-AGG, AGA-AGG, and AGG-AGA were found in 45, 4, 17, and 14 locations, respectively. The shifty UUU-AGA and UUU-AGG codon pairs were found in 30 and nine locations, respectively. Even though the frequency of occurrence of those codon pairs is rather low, still it is quite possible that some of them are residing in the mRNAs coding for the proteins required for cell division. Frameshifting reduces the total number of ribosomes that traverse the whole mRNA and thereby decreases the synthesis of a mature protein(s). Such frameshifting events may be the main cause of many phenotypes associated with environmental and genetically induced changes in the level of aminoacylated tRNA. The *argU* mutants described in this paper did not induce any cell division defects at high temperature (data not shown). The *argU10*(Ts) mutant having only 5% of the wild-type amount of the arginyl- $tRNA^{Arg}_{mnm^5UCU}$ at permissive temperature has no growth defects then, but stops dividing at high temperature, when the amount of $tRNA^{Arg}_{mnm^5UCU}$ is reduced to trace level (Sakamoto et al. 2004). Among the mutants described in this paper, the most affected mutant [*argU2343* (G53A)] has 21% of its arginyl-$tRNA^{Arg}_{mnm^5UCU}$ left. Apparently, this amount is sufficient for normal cell division but low enough to induce frameshifting in *hisC3072* mRNA.

The supply of arginyl-tRNA also becomes exhausted under the induced translation of tandem AGG or AGA arginine codons (Spanjaard and van Duin 1988; Chen and Inouye 1990; Rosenberg et al. 1993). At AGG-AGG or AGA-AGA sites, up to 50% of the ribosomes frameshift into the +1 frame, and this effect can be suppressed by introducing extra copies of the gene coding for $tRNA^{Arg}_{mnm^5UCU}$ (Spanjaard and van Duin 1988; Spanjaard et al. 1990). Therefore, translation of genes having tandem AGG-AGG or AGA-AGA codons should be prone to trigger frameshifting. Several such genes exist in *S. enterica*, and one of them is the *trmD* gene, which codes for the tRNA (m^1G37) methyl-

transferase (TrmD) that modifies G37 to m^{1}G37 (Björk et al. 1989; Li and Björk 1999). Although mutants G53C and C61U of tRNA$^{Arg}_{mnm^5UCU}$ neither influenced the levels of TrmD peptide, nor the presence of m^{1}G in the tRNAs (data not shown), this does not rule out that the expression of other genes having such tandem codons may be affected by mutations in the *argU* gene.

In contrast to Riddle and Roth (1972b) and our presumption that *sufF* is involved in modification of tRNA$^{Gly}_{mnm^5UCC}$, we demonstrate that this gene encodes tRNA$^{Arg}_{mnm^5UCU}$. We also show that it is not the tRNA$^{Gly}_{mnm^5UCC}$ that frameshifts at the GGG-GAA-AGA site. Even so, Riddle and Roth's suggestion that *sufF* influences tRNA$^{Gly}_{mnm^5UCC}$ modification may still be correct as the effect could be indirect; e.g., the particular modification enzyme at sites prone to frameshift could contain the AGA codons and their translation by the mutated tRNA$^{Arg}_{mnm^5UCU}$ could become impaired. To test this possibility, we purified tRNA$^{Gly}_{mnm^5UCC}$ from the wild type and the *sufF44* mutant, but no differences in the modification patterns were observed (data not shown). Yet an influence on tRNA modification by *sufF* was not ruled out since we do not know the migration properties of mnm^{5}U in our HPLC system.

MATERIALS AND METHODS

Bacteria and growth conditions

The bacterial strains used were derivatives of *S. enterica* (Table 1). As rich media we used either Luria-Bertani (LB) (Bertani 1951) or the complex medium NAA (Difco nutrient broth [0.8%]; Difco Laboratories) supplemented with aromatic amino acids, aromatic vitamins, and adenine (Davis et al. 1980). The minimal medium was made from the basal medium E (Vogel and Bonner 1956) with 15 g of agar per liter and supplemented with 0.2% glucose and required amino acids or vitamins (Davis et al. 1980). TYS-agar (10 g of Trypticase Peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) was used as solid rich medium.

Genetic procedures

Transduction with phage P22 HT105/1 (*int-201*) (Schmieger 1972) was performed as previously described (Davis et al. 1980).

Plasmid pUST274 was constructed by cloning the DNA fragment containing the frameshift sequence into the BamHI and EcoRI sites of vector pGHM57 (Herr et al. 2001). The DNA fragment was made from the complementary oligonucleotides (5′-TTT*GGATCC*CGGGGGAAAGACGCCATTCTCTACTGTCCGAAT TCTTTT-3′ and 5′-AAAAG*AATTC*GGACAGTAGAGAATGGCGT CTTTCCCCCGGGATCCAAA-3′), and the ends were trimmed with BamHI and EcoRI endonucleases. Ligated plasmids were transformed into strain DH5α, analyzed by sequencing the insert, and retransformed by electroporation into different *S. enterica* strains. DNA sequencing was performed on chromosomal DNA, plasmids, or PCR products following the manual of the Applied Biosystems ABI PRISM Cycle Sequencing Ready Reaction Kit Big Dye.

Isolation of frameshift suppressors linked to purE

Phage P22 was grown on a culture of strain GT885 (*hisO1242 hisC3072*), and the phage stock was treated by hydroxylamine (Hong and Ames 1971). Alternatively, strain GT885 was treated with nitrosoguanidine, and phage P22 was grown on the mutagenized cells. Such phage stocks were used to infect strain GT5130 (*hisO1242 hisC3072 purE*::Tn*10*), and His$^+$ and Pur$^+$ transductants were selected on minimal medium. The obtained clone were tested for the linkage between His$^+$ and Pur$^+$ phenotypes by transductional analysis.

Cloning of the wild-type allele of the *argU2343* frameshift suppressor

The *S. enterica* gene bank used in this study was kindly provided by Tord Hagervall (Umeå, Sweden). It contains 20,000 clones harboring chromosomal DNA fragments produced by partial Sau3A digestion and inserted into plasmid pLG399 (Stoker et al. 1982). This gene bank was introduced into the histidine prototrophic (His$^+$) strain GT5164 (*hisO1242 hisC3072 argU2343 fimC2529*::Tn*10d*Tc), and kanamycin-resistant (Km^R) clones were screened for lost ability to grow without added histidine (His$^-$ phenotype).

Analysis of aminoacylation levels and cellular levels of tRNA$^{Arg}_{mnm^5UCU}$

Cells were grown at 37°C to a density of 4–6×10^8 cells/mL (100–150 Klett units) in LB medium. After harvesting by centrifugation, total RNA was prepared under acidic conditions as described by Varshney et al. (1991), and ~20 μg of the purified tRNA was used in further analysis. Half of it was deacylated in 0.5 M Tris-HCl (pH 9.0) at 37°C for 20 min, and both untreated and deacylated tRNA samples were separated on a 6.5% polyacrylamide gel containing 8 M urea. The tRNA was transferred to a Zeta-Probe GT membrane (BioRad) (Hagervall et al. 1998). Northern hybridization with tRNA-specific oligonucleotides was used for detection of different tRNA species. To avoid binding discrepancies induced by changed homologies due to mutations, the probe for tRNA$^{Arg}_{mnm^5UCU}$ was complementary to the sequence, which was identical in all *argU* mutants and wild-type tRNA. The tRNA-specific oligonucleotides were complementary to nucleotides 14–31 of tRNA$^{Arg}_{mnm^5UCU}$ (5′-CACGGCTTAGAAGGCCGTTG-3′), nucleotides 24–50 of tRNA$^{Gln}_{mnm^5s^2UUG}$ (5′-GGGAATGCCGGTATCAAAAACCGGTG-3′), and nucleotides 41–48 and binding to the Variable loop of tRNA$^{Leu}_{cmnm^5UmAA}$ (5′-ACAGCGCGAACGCCGAGG-3′). The oligonucleotides were 5′-labeled using [γ-^{32}P]-ATP (5000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase (USB). The membrane was prehybridized for 30 min and hybridied with the labeled oligonucleotide for another 30 min in hybridization buffer (1% crystalline BSA [fraction V], 1 mM EDTA, 7% SDS, 0.5 M sodium phosphate buffer at pH 7.2) at 70°C. After washing the radioactive bands were visualized and the radioactivity was quantified using a PhosphorImager and the ImageQuant analysis program.

Protein analysis

A previously described system was used to monitor frameshifting efficiency and to purify the frameshift product (Herr et al. 2001;

Hansen et al. 2003). It employs a fusion protein consisting of maltose-binding protein (MBP) fused to glutathione-S-transferase (GST) at its N terminus and having six histidine residues (6×His) at the carboxy terminus (GST-MBP-6×His). A frameshift mutation in plasmid pUST274 has been inserted at the fusion point between the *gst* and *malE* genes (encoding the GST and MBP proteins, respectively). The resulting proteins produced from the fusion gene either following termination after the *gst* gene, or following a +1 frameshift and translation of the *malE* gene were purified by passing the protein extract over glutathionine-Sepharose (Amersham Biosciences). The presence of the two forms of protein was confirmed by Western blot analysis using anti-GST-HRP conjugate and ECL Plus Detection reagents (Amersham Biosciences). Bands specific for termination (26 kDa) and frameshift product (~70 kDa) were quantified and their relative ratio calculated. The full-length GST-MBP-6×His fusion protein was further purified by passing over Ni-NTA-agarose (QIAGEN). To strip the GST part from the frameshift product, the purified GST-MBP-6×His was digested by PreScission Protease (Amersham Pharmacia Biotech). Final cleanups and mass measurements of digestion products were performed as previously described (Hansen et al. 2003). In addition, digestion products were fractionated by gel electrophoresis and the 43-kDa peptide, corresponding to the slippage junction fused to MBP-6×His, was subjected to N-terminal sequencing by Edman degradation.

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