The EMBO Journal (2005) 24, 1842–1851 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
www.embojournal.org

THE EMBO JOURNAL

A 'gain of function' mutation in a protein mediates production of novel modified nucleosides

Peng Chen[1], Pamela F Crain[2], S Joakim Näsvall[1], Steven C Pomerantz[2] and Glenn R Björk[1,*]

[1]Department of Molecular Biology, Umeå University, Umeå, Sweden and [2]Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT, USA

The mutation *sufY204* mediates suppression of a +1 frameshift mutation in the histidine operon of *Salmonella enterica* serovar Typhimurium and synthesis of two novel modified nucleosides in tRNA. The *sufY204* mutation, which results in an amino-acid substitution in a protein, is, surprisingly, dominant over its wild-type allele and thus it is a 'gain of function' mutation. One of the new nucleosides is 5-methylaminomethyl-2-thiouridine (mnm^5s^2U34) modified by addition of a $C_{10}H_{17}$ side chain of unknown structure. Increased amounts of both nucleosides in tRNA are correlated to gene dosage of the *sufY204* allele, to an increased efficiency of frameshift suppression, and to a decreased amount of the wobble nucleoside mnm^5s^2U34 in tRNA. Purified $tRNA^{Gln}_{cmnm^5s^2UUG}$ in the mutant strain contains a modified nucleoside similar to the novel nucleosides and the level of aminoacylation of $tRNA^{Gln}_{cmnm^5s^2UUG}$ was reduced to 26% compared to that found in the wild type (86%). The results are discussed in relation to the mechanism of reading frame maintenance and the evolution of modified nucleosides in tRNA.

The EMBO Journal (2005) **24,** 1842–1851. doi:10.1038/sj.emboj.7600666; Published online 28 April 2005
Subject Categories: RNA; proteins
Keywords: evolution; frameshift; mnm^5s^2U; modified nucleosides; tRNA

Introduction

The translation apparatus has evolved to read long messages and make functional proteins required for life. Although this apparatus has the ability to decode genetic messages faithfully, errors occur with frequencies of 10^{-3}–10^{-4} per codon (Kurland *et al*, 1996; Farabaugh and Björk, 1999). Most missense errors are not harmful, since many amino acids can be substituted without affecting the activity of the protein, whereas, in contrast, almost all frameshift errors are detrimental because a shift in the reading frame almost always results in a truncated, inactive peptide. Although the mechanism of how the ribosome maintains the reading frame is not known, we know that if the ribosome makes a pause by, for example, shortage of an aminoacyl-tRNA or a defective tRNA, the peptidyl-tRNA may slip and thereby cause a frameshift error (Farabaugh, 1996; Farabaugh and Björk, 1999). Structural changes of the tRNA, such as deficiency of modified nucleosides, which are derivatives of the four major nucleosides U, C, A and G, may affect the entry of the ternary complex into the ribosomal A site or the fit of the tRNA in the P site, and therefore influence frameshift frequency (Qian *et al*, 1998; Urbonavicius *et al*, 2001). (For details, see Supplementary Figure 1.)

More than 80 different modified nucleosides have, so far, been characterized in tRNA (Rozenski *et al*, 1999). Although they are present in many different positions of the molecule, position 34 (the wobble nucleoside) and position 37 (3′ of and next to the anticodon) are frequently modified (Sprinzl and Vassilenko, 2003) . Moreover, at these two positions a great variety of modified nucleosides are found, suggesting that the modified nucleosides in these two positions play a pivotal role in the decoding process. Transfer RNA from all organisms contains modified nucleosides and some of them are found in the same position and in the same subset of tRNAs from all organisms, suggesting that at least some of them were present in the organisms before the emergence of the three phylogenetic domains (Björk, 1986; Cermakian and Cedergren, 1998).

According to some theories, the early tRNA might have been a small hairpin-RNA (Hopfield, 1978) with an amino acid bound to it (Di Giulio, 1998). A relic of this primordial tRNA may be the presence of some modified nucleosides, which are today formed from the major RNA nucleosides (A, C, G, U) by the addition of an intact amino acid (e.g. N^6-threonyl-adenosine (t^6A), 2-lysyl-cytidine (k^2C), glutamate bound to Q of $tRNA^{Asp}$ (GluQ)) or parts of amino acids (methylated and thiolated nucleosides and acp^3U). Therefore, one way that modified nucleosides may have evolved is by altering the substrate recognition of an enzyme involved in intermediary metabolism to recognize tRNA as substrate. Alternatively, a protein already having an affinity for tRNA may evolve a new enzymatic activity. Such alterations are predicted to be dominant over their wild-type allele and thus result in a 'gain of function' phenotype. By performing a similar chemical reaction as earlier but now also using tRNA as substrate, a novel modified nucleoside may have evolved. If the presence of this novel modified nucleoside provides an evolutionary advantage, the alteration will be fixed and the enzyme may thereafter evolve into a sophisticated tRNA modifying enzyme. Results in this paper address this aspect of tRNA modification.

A +1 frameshift mutation results from addition of one base in a coding sequence and such mutations may be suppressed by various alterations in the translation apparatus (Farabaugh, 1996). The first suppressors characterized were dominant and they have gained the ability to suppress a +1 frameshift mutation. Such suppressors have an altered tRNA with an extra nucleoside in the anticodon loop (Roth, 1981). We have shown that a deficiency of some modified nucleo-

*Corresponding author. Department of Molecular Biology, Umeå University, S-901 87 Umeå, Sweden. Tel.: +46 90 785 6756; Fax: +46 90 77 2630; E-mail: glenn.bjork@molbiol.umu.se

Received: 15 November 2004; accepted: 8 April 2005; published online: 28 April 2005

 ©2005 European Molecular Biology Organization

sides in tRNA also induces the ability to suppress many different +1 frameshift mutations and, in fact, it seems to be a common function of many modified nucleosides to improve the reading frame maintenance (Urbonavicius *et al*, 2001). According to our frameshift model (Supplementary Figure 1) the aberrantly modified tRNA is either poorly aminoacylated or/and that the ternary complex containing such a defective tRNA *per se* enters the A-site inefficiently. Such events stall the ribosome and allow the peptidyl-tRNA to slip one nucleotide forward, which returns the ribosome to the zero frame. So far, mutations inducing aberrant tRNA modification are recessive and results in a deficiency of a modification ('loss of function mutations'). However, this paper describes, for the first time, a dominant 'gain of function' mutation that alters the amino-acid sequence of a protein, induces suppression of a frameshift mutation, and mediates an efficient synthesis of new modifications in the tRNA.

Results

The sufY204 mutation suppresses the +1 frameshift mutation hisC3737 and causes an amino-acid substitution in a protein

A *sufC10* mutant of *Salmonella enterica* serovar Typhimurium (Riddle and Roth, 1970) contains a mutation, *sufY204*, which suppresses the *hisC3737* mutation (Sroga *et al*, 1992). Following localization of the *sufY204* mutation on the chromosome of *S. enterica,* the DNA sequence of the *sufY*$^+$ and *sufY204* alleles revealed that the *sufY204* mutation was a G to A base substitution resulting in a Gly67→Glu67 (G67E) change of the SufY protein sequence. (For details, see Supplementary data.) Two other *sufY* mutants (*sufY205* and *sufY206*) isolated earlier (Riddle and Roth, 1970) were also sequenced and both had a substitution of the same amino acid of the SufY protein: *sufY205* had the same amino-acid substitution as *sufY204* (G67E), while *sufY206* had a G67R substitution.

The mutation in hisC3737 is an insertion of C

We noticed that the *sufY204* mutation suppressed only the *hisC3737* and the *hisC3734* mutations among many other frameshift mutations in the *his* operon available to us (Supplementary Table I and Björk *et al*, 1989). Sequencing revealed that mutations *hisC3737* and *hisC3734* are identical, both being C insertions that give a run of five Cs resulting in a proline codon CCC, a glutamine codon CAA, and a stop codon UAA in the zero frame (Supplementary Table I). Since the *sufA6* and *sufB2* frameshift suppressors, which encode an altered $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{GGG}$, respectively, also suppress the *hisC3737* and *hisC3734* mutations and are known to induce frameshifts at runs of Cs (Supplementary Table I), we suspected that one of the two proline CCC codons present within the '+1 frameshifting window' in the zero frame might be the site of frameshifting.

The *hisD3749* mutation is a frameshifting site containing a CCC codon followed by a stop codon in the zero frame (Supplementary Table I). The suppression of *hisD3749* by the *sufB2* mutation, which alters $tRNA^{Pro}_{GGG}$, is not mediated by this tRNA but by the near cognate $tRNA^{Pro}_{cmo^5UGG}$ (Qian *et al*, 1998). The mutated cognate $tRNA^{Pro}_{GGG}$ is out-competed in the selection at the ribosomal A-site by the near-cognate $tRNA^{Pro}_{cmo^5UGG}$ at the CCC codon in the zero frame. Following a normal three-nucleotide translocation, the $tRNA^{Pro}_{cmo^5UGG}$ is in the P site. Provided that the ribosome pauses at the next codon, the peptidyl-$tRNA^{Pro}_{cmo^5UGG}$ slips forward one nucleotide causing the ribosome to return to the +1 frame (cf. sequence for *hisD3749* in Supplementary Table I). This frameshifting is abolished in an *aroD* mutant, since the *aroD* mutation blocks the synthesis of cmo^{5}U34 (Björk, 1980), which promotes $tRNA^{Pro}_{cmo^5UGG}$ to out-compete the mutated cognate *sufB2* $tRNA^{Pro}_{GGG}$ at a low frequency in reading the CCC codon. Since the *sufY204*-mediated suppression was inhibited by the presence of an *aroD* mutation (data not shown), the *sufY204*-induced suppression is dependent on a cmo^{5}U34-containing tRNA. We therefore propose that at the two possible frameshifting sites, -CCC-GCG- or -CCC-CAA- present within the frameshifting window in a *hisC3737* mutant (Supplementary Table I), the $tRNA^{Pro}_{cmo^5UGG}$ may slip in the P-site into the +1 frame provided that a slow entry of the next tRNA occurs, which should stall the ribosome. At the first possible site within the frameshifting window, CCC-GCG, the pausing should be induced by a slow entry of Ala-$tRNA^{Ala}_{cmo^5UGC}$, which decodes GCG. At the second site, the slow entry should be caused by the Gln-$tRNA^{Gln}_{cmnm^5s^2UUG}$, which reads CAA and to a lesser extent CAG (Yokoyama *et al*, 1985). Which of these two sites is the frameshifting site is addressed in the next section.

Translational frameshifting occurs at the CCC-CAA-UAA sequence

To identify the frameshifting site in *hisC3737*, we used a series of plasmids containing a +1 frameshift mutation placed in the beginning of the *lacZ* gene (Figure 1). The frameshifting window is small, with only four codons

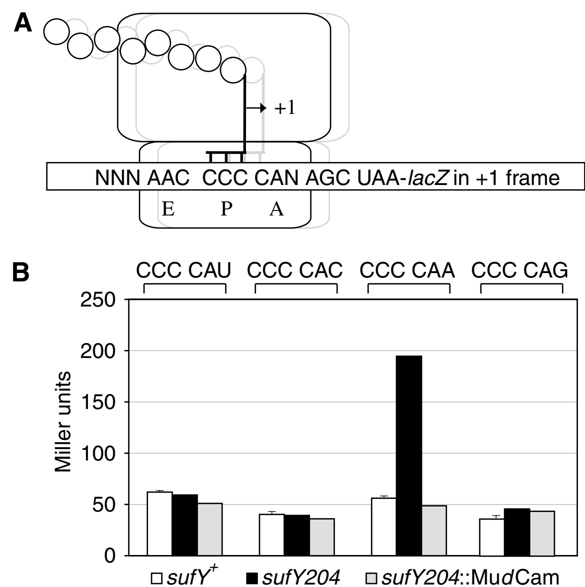

Figure 1 (**A**) pTHF plasmid constructs used to measure +1 frameshifting (Hagervall *et al*, 1993). The RNA sequence is shown in triplets to indicate the reading frame. (**B**) +1 frameshifting (measured by β-galactosidase activity) on pTHF40, pTHF41, pTHF42, pTHF43 plasmid in *sufY*$^+$ (GT5633), *sufY204* (GT5424) and *sufY*::Mu*d*Cam (GT5520) strains.

©2005 European Molecular Biology Organization

between the upstream stop codon in the +1 frame and the downstream stop codon in the zero frame. The reporter *lacZ* gene is placed in the +1 frame and thus β-galactosidase activity reflects the level of +1 frameshifting. When CCC-GCG was tested, we did not observe any change in the level of +1 frameshifting between $sufY^+$ and *sufY204* strains (data not shown). Frameshifting sites were constructed by placing a CCC codon in the beginning of the *lacZ* gene followed by any of the four CAN codons to test whether the second site CCC-CAA in the *hisC3737* frameshifting window was the frameshifting site. We introduced these plasmids into strains GT5633 ($sufY^+$) and GT5424 (*sufY204*). No increased frameshifting compared to the wild-type strain was observed when the CCC codon was followed by CAU, CAC or CAG codons. However, a four-fold increase in +1 frameshifting in the *sufY204* strain compared to the $sufY^+$ strain was observed, when plasmid (pTHF42), which contained the sequence CCC-CAA, was present in the cells (Figure 1). Although the surrounding codon context is different in plasmid pTHF42 compared to that around the *hisC3737* mutation, frameshifting occurs in both assay systems, indicating that CCC-CAA is the minimal requirement for this +1 frameshifting to occur. According to our frameshifting model (Farabaugh and Björk, 1999; Urbonavicius *et al*, 2001) (Supplementary Figure 1), a defective $tRNA^{Gln}_{cmnm^5s^2UUG}$, which reads CAA codon and less well CAG, may cause a slow entry to the ribosomal A site, resulting in a pause that allows the P-site proline $tRNA^{Pro}_{cmo^5UGG}$ decoding the CCC codon to slip one nucleotide forward and thus shift frame. We therefore expect that the *sufY204* mutation in some way alters the structure of the $tRNA^{Gln}_{cmnm^5s^2UUG}$ resulting in poor coding capacity of $tRNA^{Gln}_{cmnm^5s^2UUG}$ and/or poor charging of it, which both should stall the ribosome. The CAG codon is also decoded by $tRNA^{Gln}_{CUG}$, which only reads the CAG codon, consistent with our observation that no increased frameshifting occurred when the synonymous glutamine codon CAG was next to and 5′ of CCC (Figure 1). To further support our suggestion that it was a proline tRNA that shifted frame, we determined the amino-acid sequence of the frameshift product. The frameshift sequence -CCC-CAA-UAA was inserted between the genes *gst* and *malE* encoding the glutathione-*S*-transferase (GST) and the maltose-binding protein, respectively. The *malE* gene is in the +1 frame relative to the *gst* gene (Herr *et al*, 2001; Hansen *et al*, 2003). The complete fusion protein was purified from a strain containing the *sufY204* mutation and a plasmid (pUST290) harboring the above-mentioned 'GST-CCC-CAA-UAA-MalE' construct. To liberate the slippage junction fused to MalE, the frameshift product was treated with PreScission Protease. After digestion, the N-terminal sequence of the slippage junction was determined. The first 12 amino acids of the peptide were GPLGILN**P**KANN, where **P** was the last amino acid inserted in the zero frame, suggesting that the frameshifting tRNA at the frameshift site CCC-CAA-UAA was a proline tRNA (Supplementary data).

Taken together, these results suggest that the frameshift occurs in the P-site by slippage of peptidyl-$tRNA^{Pro}_{cmo^5UGG}$. According to our frameshifting model (Supplementary Figure 1), such slippage requires that the ribosome stalls at the A-site, suggesting that the $tRNA^{Gln}_{cmnm^5s^2UUG}$ is in some way defective in the *sufY204* mutant causing such a ribosomal stalling.

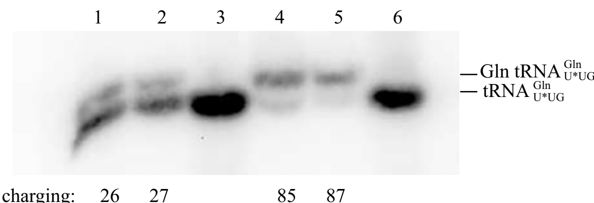

Figure 2 Charging level of $tRNA^{Gln}_{U*UG}$ in *sufY204* or $sufY^+$ strains. tRNA was prepared from strains GT5633 ($sufY^+$) and GT5424 (*sufY204*) as described (Materials and methods). The charging level is calculated by dividing the amount of Gln-$tRNA^{Gln}_{cmnm^5s^2UUG}$ by those of Gln-$tRNA^{Gln}_{cmnm^5s^2UUG}$ and $tRNA^{Gln}_{cmnm^5s^2UUG}$. Two clones were used for each strain, and a deacylated sample was loaded as a control. Lane 1: GT5424a (*sufY204*), 2: GT5424b (*sufY204*), 3: GT5424 (*sufY204*), deacylated tRNA, 4: GT5633a ($sufY^+$), 5: GT5633b ($sufY^+$), 6: GT5633 ($sufY^+$), deacylated tRNA.

Aminoacylation of $tRNA^{Gln}_{cmnm^5s^2UUG}$ is reduced in the sufY204 mutant

A defective $tRNA^{Gln}_{cmnm^5s^2UUG}$ may result in inefficient charging and thereby stalling the ribosome that would induce a slippage of the peptidyl-tRNA. We therefore prepared tRNA from *sufY204* and $sufY^+$ strains under acidic condition in order to preserve the amino acid charged on the tRNA molecules. By running an acidic denaturing polyacrylamide gel, Gln-$tRNA^{Gln}_{cmnm^5s^2UUG}$ and deacylated $tRNA^{Gln}_{cmnm^5s^2UUG}$ can be separated and detected by Northern blot (Figure 2). The charging level in the $sufY^+$ strain was 86% and in the *sufY204* mutant the charging level was reduced to 26%, which is more than a three-fold reduction. According to our frameshifting model, the low level of charged $tRNA^{Gln}_{cmnm^5s^2UUG}$ would at least partly explain the suppression of the *hisC3737* mutation.

The sufY204 mutation is dominant and results in a 'gain of function' phenotype

During the mapping of the *sufY204* mutation, we obtained a Mu*d*Cam insertion that abolished the ability to suppress the *hisC3737* mutation (phenotypically His^-) as well as the +1 frameshifting monitored as β-galactosidase activity (Figure 1). This *sufY2522*::Mu*d*Cam insertion was located at the end of *sufY204* coding sequence resulting in a *sufY* gene having both the *sufY204* mutation and the Mu*d*Cam insertion (Supplementary data). Since the transposon insertion abolished the suppressor activity, the *sufY204* mutation is a 'gain of function mutation' and consequently should be dominant over its wild-type allele. To test this hypothesis, we introduced a plasmid carrying the wild-type allele of *sufY* ($sufY^+$) into strain GT6026 (*hisC3737*, *sufY204*) resulting in a strain having the $sufY^+$ allele on the plasmid and the *sufY204* allele on the chromosome. Such a strain is still His^+ demonstrating that the *sufY204* mutation is dominant over its wild-type allele (Table I). Introduction of a plasmid containing the *sufY204* allele into a wild-type strain induced a His^+ phenotype stronger than that induced by a single copy of the *sufY204* mutation residing on the chromosome. Strong suppressor activity was observed irrespectively if the strain possessed the $sufY^+$ or the *sufY204* allele on the chromosome (Table I). Thus, the *sufY204* mutation is dominant, consistent with the suggestion that it mediates a gain of function. We further noticed that multiple copies of the *sufY204* allele resulted in a stronger suppression than that observed by the

©2005 European Molecular Biology Organization

Table I Levels of (c)mnm^{5}s^{2}U, UK1 and UK2 and suppression of the +1 frameshift mutation *hisC3737*

Strain	Relevant genotype		(c)mnm^{5}s^{2}U/t^{6}A[a]	UK1/t^{6}A[b]	UK2/t^{6}A[b]	*hisC3737* suppression
	Chromosome	Plasmid				
GT6028	*sufY204*	pCL1921 (vector)	0.12	0.10	0.32	+
GT6031	*sufY*$^+$	pCL1921 (vector)	0.24	0.01	0.01	–
GT6026	*sufY204*	pUST210 (*sufY*$^+$)	0.10	0.09	0.33	+
GT6029	*sufY*$^+$	pUST210 (*sufY*$^+$)	0.32	0.04	0.04	–
GT6027	*sufY204*	pUST211 (*sufY204*)	0.08	0.17	0.45	+ +
GT6030	*sufY*$^+$	pUST211 (*sufY204*)	0.07	0.14	0.39	+ +

[a]The numbers are calculated as the absorbance of each modified nucleoside relative to the absorbance of t^{6}A at 254 nm.
[b]The relative retention time of UK1 and UK2 to ms^{2}i^{6}A were 0.890 (UK1) and 0.921 (UK2).

presence of only one copy (cf. strains GT6028, GT6027 and GT6030).

The sufY204 mutation induces the appearance of novel modified nucleosides in bulk tRNA and in purified $tRNA^{Gln}_{cmnm^5s^2UUG}$

Possible explanations for the *sufY204*-mediated frameshifting might be a changed structure of the $tRNA^{Gln}_{cmnm^5s^2UUG}$ *per se* or a reduced level of Gln-$tRNA^{Gln}_{cmnm^5s^2UUG}$ (Figure 2) caused by the structural alteration. Transfer RNA from a *sufY204* mutant strain possessed two prominent 'UnKnown' novel compounds (denoted UK1 and UK2, Figure 3). Note also that compounds UK1 and UK2 are present in the wild-type tRNA, although at very low levels. Since the *sufY2522*::Mu*d*Cam strain is His$^-$, we expected that these compounds would be absent, which was the case (Figure 3). Interestingly, if the *sufY204* allele was present both on the chromosome and on a plasmid, the amount of these UKs increased, suggesting that their synthesis was sensitive to gene dosage. Moreover, the amounts of these two unknown compounds positively correlated with the efficiency of frameshift suppression (Table I).

Not only did we observe increased amounts of UKs by the action of the *sufY204* allele, but also a decreased synthesis of (c)mnm^{5}s^{2}U34 (Table I). These modified nucleosides, which are present in the wobble position of $tRNA^{Gln}_{cmnm^5s^2UUG}$, $tRNA^{Lys}_{mnm^5s^2UUU}$, and $tRNA^{Lys}_{mnm^5s^2UUU}$, were reduced to about 50% in the *sufY204* strain compared to the levels found in a *sufY*$^+$ strain (Table I, cf. strains GT6028 to GT6031). This reduction was more pronounced (33% of the wild-type level) in a strain containing the *sufY204* allele both on the chromosome and on the plasmid. Thus, there is an inverse relationship between the amounts of UKs and (c)mnm^{5}s^{2}U, suggesting that the appearance of the novel nucleosides occurs at the expense of (c)mnm^{5}s^{2}U. Accordingly, when ^{35}S-labeled tRNA was analyzed, UK2 contained radioactivity consistent with the suggestion that it is a derivative of mnm^{5}s^{2}U (data not shown).

To verify the model of how *sufY204* induced frameshift suppression, we purified $tRNA^{Gln}_{cmnm^5s^2UUG}$ from wild-type and *sufY204* mutant cells and analyzed the nucleoside modification pattern (Table II). An unknown modified nucleoside (UK in Table II) was found in purified $tRNA^{Gln}_{cmnm^5s^2UUG}$ from the *sufY204* mutant, but was absent in $tRNA^{Gln}_{cmnm^5s^2UUG}$ from the *sufY*$^+$ strain. The sample of $tRNA^{Gln}_{cmnm^5s^2UUG}$ was pure, since other modified nucleosides, which should be present in $tRNA^{Gln}_{cmnm^5s^2UUG}$ (ψ, m^{5}U, m^{2}A, Gm, Um, s^{4}U, cmnm^{5}s^{2}U) were observed, but not any other modified nucleosides that may have originated from contaminating tRNA species. The UK, which had a UV spectrum similar to UK1 and UK2 found in bulk tRNA, had a relative retention time that was not identical neither to UK1, nor to UK2, and nor to UK3 (see below). The reason that the UK found in purified $tRNA^{Gln}_{cmnm^5s^2UUG}$ did not migrate as any of UK1, UK2 or UK3 may be caused by an alteration of the structure caused by the extensive purification of $tRNA^{Gln}_{cmnm^5s^2UUG}$. Still, the UV spectrum is identical to that of UK1–UK3 and the level of cmnm^{5}s^{2}U is reduced in the $tRNA^{Gln}_{cmnm^5s^2UUG}$ from the *sufY204* mutant.

Structure studies of UK2 by electrospray ionization mass spectrometry (ESI-MS)

By LC/MS we determined the M_r of UK2 to be 745, which is greater than that of any modified nucleoside hitherto characterized (Rozenski *et al*, 1999). However, nuclease P1, which we routinely use to degrade the tRNA, is sensitive to bulky modifications (Gehrke and Kuo, 1989; Crain, 1990) and leaves as the end product a dinucleotide in which the hypermodified nucleoside is the 5′ member. Given the U*UG anticodon of $tRNA^{Gln}$ (U* denotes an uncharacterized derivative of uridine), we predict that UK2 may be a dinucleotide, U*pU, whose degradation is resistant to nuclease P1. Indeed, when UK2 was degraded to uridine by snake venom diesterase, an endonuclease not as sensitive to bulky substituents as P1 (Crain, 1990), the 5′ modified nucleoside component of UK2 could not be identified (data not shown). In view of the probable structure of this nucleoside (discussed below), it is likely that it was not eluted from the high-performance liquid chromatography (HPLC) column under the conditions used (Gehrke and Kuo, 1989).

If UK2 is a dinucleotide consisting of a normal U and the modified U*, the indicated M_r of U* is 439, which does not correspond to that of any known nucleoside (Rozenski *et al*, 1999). Therefore, UK2 was purified and its structural features were examined following collision-induced dissociation (CID) of the protonated molecular ion (MH$^+$), m/z 746 (Figure 4A). From studies of CID of dinucleotides (Phillips and McCloskey, 1993), ions corresponding to protonated free base (BH_2^+) ions resulting from cleavage of the glycosidic bond with proton transfer from ribose to the base (Pomerantz and McCloskey, 1990) can be expected for both U ($B=111$) and U* ($B=306$) (Figure 4B); they occur at m/z 113 and 308, respectively. An unusual fragmentation pathway is manifested in the loss of 136 Da from both MH$^+$ and BH_2^+ ions of U*, giving m/z 610 and m/z 172, respectively. The m/z 172 ion is consistent with the value expected for the BH_2^+ ion of mnm^{5}s^{2}U, and m/z 610 is consistent with an assignment as the MH$^+$ ion of the dinucleotide mnm^{5}s^{2}UpU. The difference in mass between UK2 (U*pU) and mnm^{5}s^{2}UpU is 136,

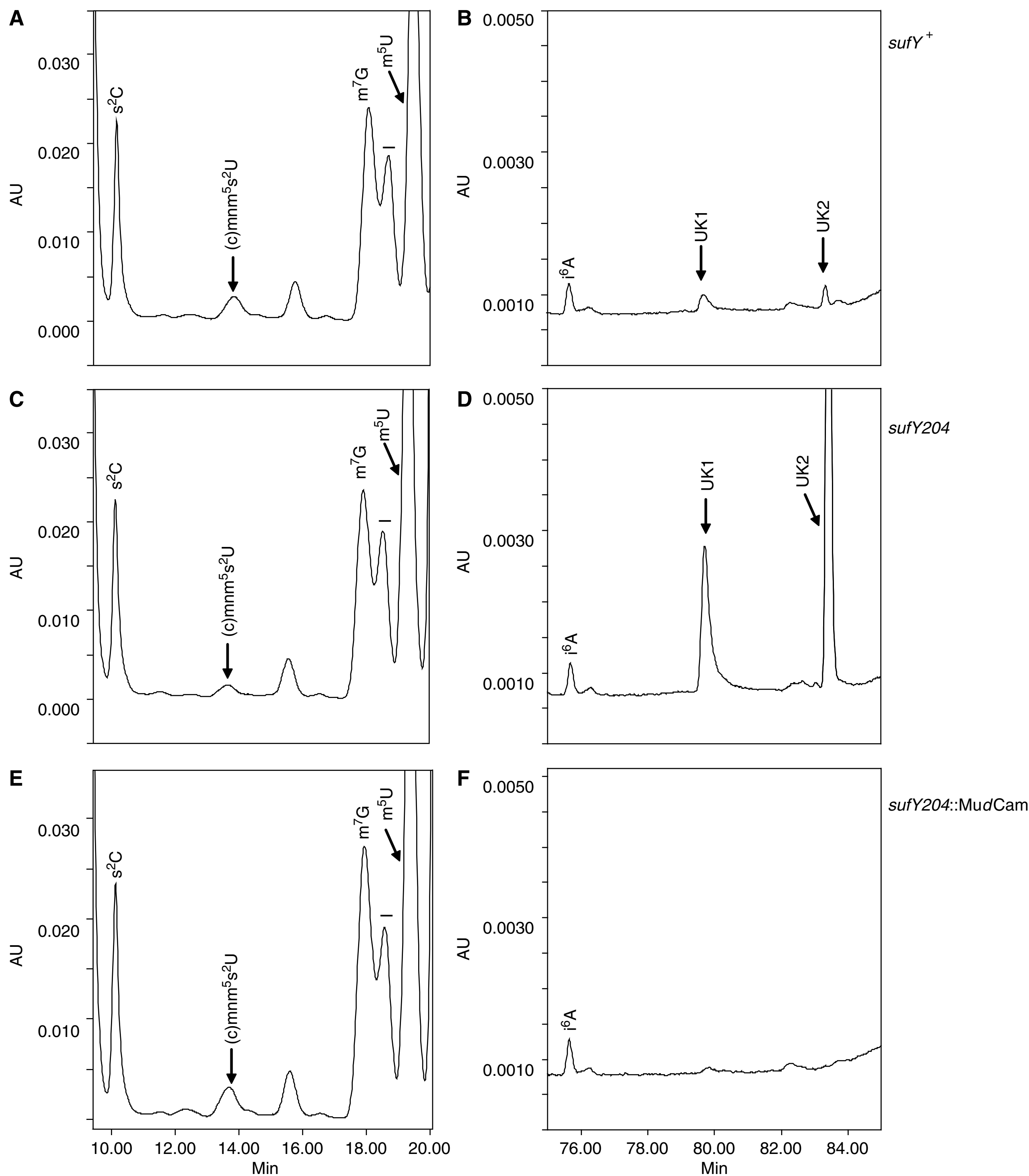

Figure 3 HPLC chromatograms of tRNA hydrolysates. The (**A, C, E**) panels show the chromatograms from 9.6 to 20 min and (**B, D, F**) panels show the chromatograms from 75.2 to 84.8 min. Note the different scales on the *Y*-axes between panels A, C and E versus panels B, D and F. The arrows indicate the elution positions of (c)mnm^{5}s^{2}U, UK1 or UK2 (relative retention time to ms^{2}i^{6}A: UK1 = 0.890, UK2 = 0.921). The (c)mnm^{5}s^{2}U denotes the combined areas of the cmnm^{5}s^{2}U and mnm^{5}s^{2}U, which migrates very close to each other. See quantification of various modified nucleosides in Table I. A, B: tRNA hydrolysate from strain GT6031 (pCL1921/*hisO1242, hisC3737, sufY*$^+$, *zbb2523*::Tn*10*); C, D: from strain GT6028 (pCL1921/*hisO1242, hisC3737, sufY204, zbb2523*::Tn*10*); E, F: from strain GT5520 (*hisO1242, hisC3737, sufY2522*::Mu*d*Cam).

suggesting that UK2 may consist of a dinucleotide of mnm^{5}s^{2}UpU plus an additional 137 Da substituent (net addition of 136 Da) that is present on the mnm^{5}s^{2}U base moiety. Apparently, the presence of this additional 137 Da substituent is inhibiting the P1 nuclease activity, thereby generating the U*pU dinucleotide of M_r 745.

The 137 Da fragment that has been added to mnm^{5}s^{2}U forms a stable ion, (m/z 137, Figure 4), and LC/MS analysis

©2005 European Molecular Biology Organization

Table II Relative levels of modified nucleosides in $tRNA^{Gln}_{cmnm^5s^2UUG}$ from *sufY*$^+$ and *sufY204* mutant

Strain		Levels of modified nucleosides relative to m^2A[a]							
		ψ	$cmnm^5s^2U$[b]	m^5U	s^4U	Um	Gm	m^2A	UK[c]
GT5633	*sufY*$^+$	1.39	0.65	0.58	0.10	0.29	0.90	1.0	⩽0.03 (⩽2.9%)
GT5424	*sufY204*	1.41 (101%)	**0.19 (29%)**	0.57 (98%)	0.09 (89%)	0.22 (75%)	0.66 (73%)	1.0	**1.13**

[a]The numbers are calculated as the level of each modified nucleoside relative to the level of m^2A. Using another internal standard, the levels of m^2A were the same in these two strains. For pair wise comparison between *sufY*$^+$ and *sufY204* mutants, the number in the *sufY*$^+$ is set to 100%, then a relative value is presented within parenthesis for the *sufY204* mutant. Bold numbers indicate significant differences compared to the level in the wild type.
[b]Using chemically synthesized mnm^5s^2U and $cmnm^5s^2U$ as standards, the relative retention time to A (RT-A) was for $mnm^5s^2U = 0.369$ and for $cmnm^5s^2U = 0.345$. We therefore tentatively suggest that the wobble nucleoside in gln1 tRNA from the *sufY*$^+$ strain is $cmnm^5s^2U$ (see also Material and methods).
[c]UK observed in this experiment with purified gln1 tRNA had similar UV spectrum as UK1 and UK2, but its retention time relative to m^2A (1.619) was between that of UK1 (1.563) and UK2 (1.636).

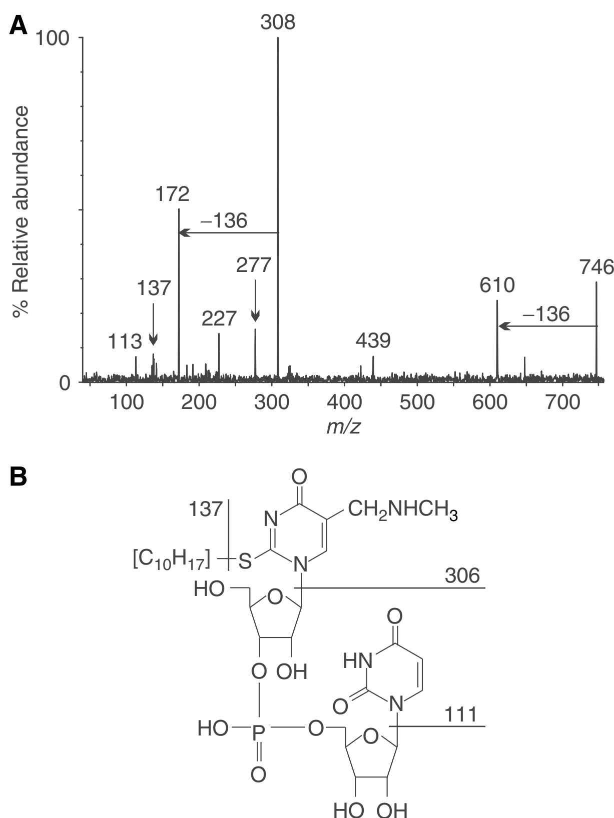

Figure 4 LC/ESI-MS analysis of UK2 isolated from a digest of total tRNA from strain GT6027 (*psufY204/hisO1242, hisC3737, sufY204*). (**A**) Product ion mass spectrum from collision-induced dissociation of the MH^+ ion of UK2. (**B**) Partial structure of UK2 deduced from mass spectral data.

of UK2 using deuteriated solvents (Edmonds *et al*, 1988) was carried out to determine the number of exchangeable H atoms in this ion, and to confirm the other ion assignments proposed above. This measurement was conducted at high resolution to permit an accurate mass to be determined for *m/z* 137 at the same time. The results indicated that there are no exchangeable hydrogens in the 137 Da fragment, its composition is $C_{10}H_{17}$, and it contains two double bonds. The spectrum and its interpretation are presented in Supplementary Figure 2. The data do not permit determination of the nature of the $C_{10}H_{17}$ substituent, but its composition is compatible with that of a geranyl group (or an isomer of it).

The appearance of the novel modifications is related to the degree of modification of mnm^5s^2U

As stated above, the UKs are related to the wobble nucleoside mnm^5s^2U. If so, mutations that result in accumulation of intermediates in the synthesis of this modified nucleoside should influence the structure of the UKs in a way predicted from the structure of the intermediates. The proposed biosynthetic pathway of mnm^5s^2U is shown in Figure 5 (Björk and Hagervall, 2005). Mutations in *mnmA* result in a deficiency of the s^2-group (Sullivan *et al*, 1985), whereas mutations in *mnmE* result in a deficiency of the mnm^5-side chain (Elseviers *et al*, 1984). The *mnmC1* mutation results in accumulation of $cmnm^5s^2U$, whereas mutation *mnmC2* results in the accumulation of nm^5s^2U (Hagervall *et al*, 1987). In the *mnmA* mutant no detectable UKs were observed, and in the *mnmE* mutant the level of UK2 was reduced to about 1.1% of that found in the *mnmE*$^+$ strain (Figure 5). Thus, the s^2- and mnm^5-groups are required for the synthesis of the UKs. In the *mnmC1* mutant, which accumulates $cmnm^5s^2U$, UK1 becomes the major unknown, whereas in the *mnmC2* mutant, which accumulates nm^5s^2U, UK3 becomes the major unknown compound (Figure 5). Note that UK3 was not induced by the *sufY204* mutation in the wild-type background, in which only UK2 and UK1 were synthesized. Clearly, the various UKs are related to the mnm^5s^2U derivatives found in the mutants suggesting that UK1 is associated with $cmnm^5s^2U$, UK2 with mnm^5s^2U and UK3 with nm^5s^2U. Accordingly, the M_rs of UK1, UK2 and UK3 should be related to each other in the same manner as those of $cmnm^5s^2U$ (M_r 347), mnm^5s^2U (M_r 303) and mnm^5s^2U (M_r 289), which indeed they are (Figure 5). Since the M_rs of the three UKs were related to the 5-side chain in the intermediates, the derivatization of these derivatives does not occur on this side chain but rather on some other part of the nucleoside. These results strengthen the suggestion that the altered SufY protein modifies the wobble nucleoside mnm^5s^2U34.

Selenation is not involved in the synthesis of UKs

Recently, the YbbB protein of *Escherichia coli* was shown to catalyze the selenation of tRNA and it was suggested that YbbB is the selenouridine synthase (Wolfe *et al*, 2004). This enzyme, which contains a rhodanese domain (Figure 6),

©2005 European Molecular Biology Organization

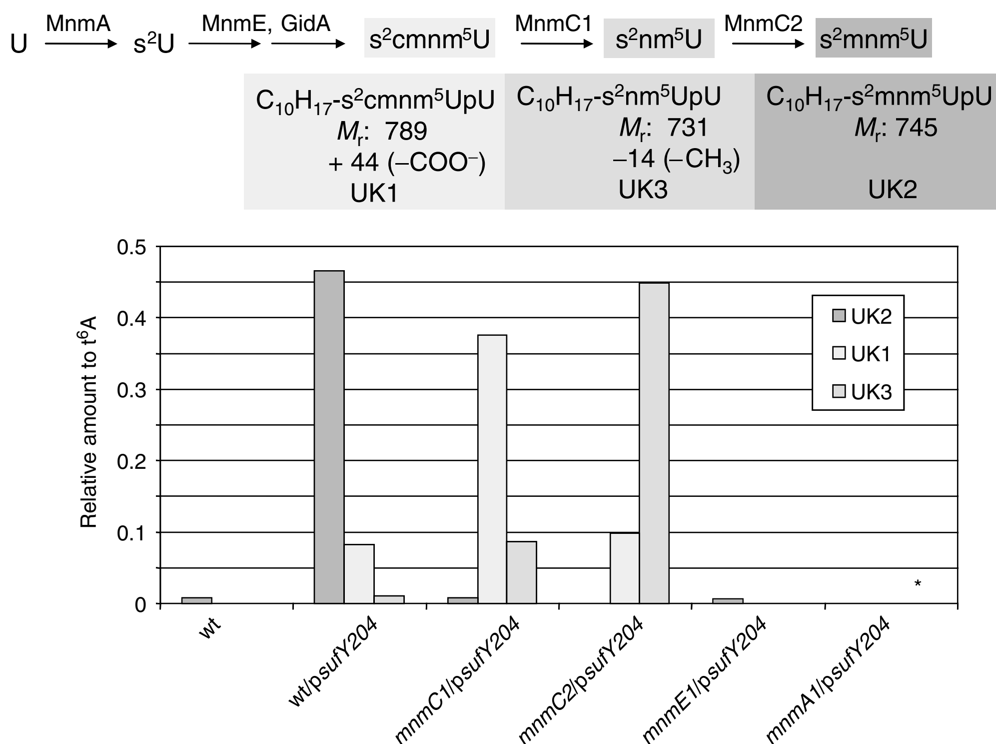

Figure 5 Levels of UK1, UK2 and UK3 in *E. coli* mutants *mnmA*, *mnmE*, *mnmC1*,and *mnmC2* containing a plasmid (pUST211, *sufY204*) harboring the *Salmonella sufY204* allele. tRNAs were prepared and analyzed by HPLC. Amounts of UK1, UK2 and UK3 are presented relative to the amount of t^6A. The retention times for UKs relative to ms^2i^6A are: UK1 (0.890), UK3 (0.917) and UK2 (0.921). *: Not detected (less than 0.003 relative to t^6A).

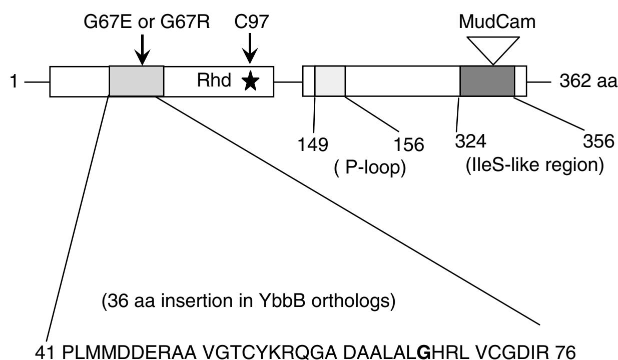

Figure 6 Overview of the SufY protein in *S. enterica* (modified from Wolfe *et al*, 2004). The Rhodanese domain (Rhd: aa 3–130) and the catalytic Cys97 (star in the Rhd domain with arrow above) are indicated. The 36 amino acid insertion (aa 41–76) within the Rhd domain in nine YbbB orthologs according to Wolfe *et al* (2004) is shown in gray, the sequence of which is shown below. P-loop (ATP binding motif) is present between amino acids 149–156. IleS-like region (aa 324–356) is shown in dark gray according to (Wolfe *et al*, 2004). The MudCam transposon insertion is shown with a triangle at D342 in the *S. enterica* sequence. The amino–acid substitutions in *sufY204* (G67E) or *sufY206* (G67R) mutants are shown with arrows.

exchanges the sulfur of mnm^5s^2U34 for selenium. The selenium donor is selenophosphate, which is produced by the *selD* gene product (Veres *et al*, 1994). Indeed, deletion of the *selD* gene results in inability to incorporate selenium into proteins and tRNAs (Kramer and Ames, 1988; Stadtman *et al*, 1989). It was further shown that Cys97, which is conserved in the sulfurtransferases with a rhodanese domain, is required for the incorporation of Se. Since the *sufY* gene is the *Salmonella* homologue to the *E. coli ybbB* gene, we wanted to know if selenation is involved in the synthesis of the UKs in *S. enterica*.

Since selenation requires the selenium donor $SePO_3$, the formation of which is catalyzed by the SelD protein, we constructed a *selD* deletion mutant in *Salmonella*. Introduction of a *sufY204* plasmid revealed that UK1 and UK2 were still made in the *selD* mutant, demonstrating that selenation is not required for the formation of these unknown nucleotides (Figure 7). Compared to a plasmid containing the *sufY204* mutation, a plasmid containing the *sufY204* mutation in conjunction with a C97A substitution reduced the level of UK1 and UK2 to 36 and 13%, respectively. Thus, the Cys97 is important but not absolutely required for the formation of UKs.

Discussion

In this paper we report, for the first time, a dominant mutation ('a gain of function mutation'), which alters a protein and influences the modification of tRNA. Apparently, the wild-type form of the protein has an intrinsic ability to modify tRNA, although at such a low efficiency that this modification hitherto has not been observed. Although the structure of the modification is not established, it is clear that it is a derivative of the wobble nucleoside $(c)mnm^5s^2U34$, which is present in tRNAs specific for Gln, Lys and Glu.

The *sufY204* mutation was discovered as a suppressor of a +1 frameshift mutation. According to the model how tRNA modification influences reading frame maintenance (Urbonavicius *et al*, 2001), we predict that the *sufY204*

 ©2005 European Molecular Biology Organization

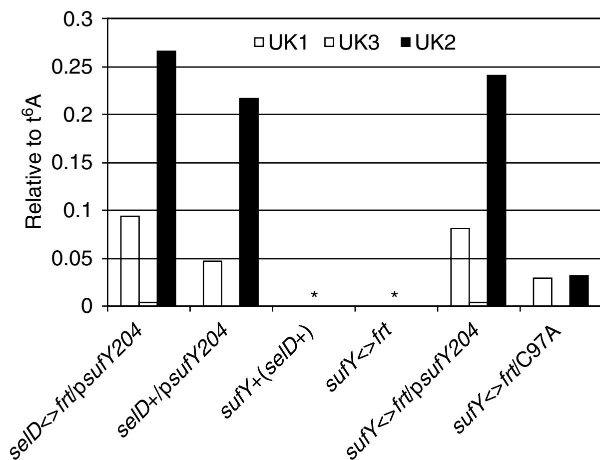

Figure 7 Levels of UK1, UK3, and UK2 in *sufY〈〉frt* or *selD〈〉frt* mutants containing plasmid pUST211 (*sufY204*) or plasmid pUST273 (with C97A and G67E alterations in the SufY/YbbB). tRNAs were prepared and analyzed by HPLC (Materials and methods). Amounts of UK1, UK3 and UK2 are presented relative to the amount of t^6A. *: not detected (less than 0.003 AU relative to t^6A).

mutation should alter the $tRNA^{Gln}_{cmnm^5s^2UUG}$, since it acted at the sequence CCC-CAA-UAA (Figure 1). If the unknown modification were located at or in the vicinity of the wobble nucleoside $cmnm^5s^2U34$ of $tRNA^{Gln}_{cmnm^5s^2UUG}$, we expect that it might influence the aminoacylation of this tRNA, since the wobble nucleoside is part of the recognition determinant for the GlnRS (Ibba *et al*, 1996). Indeed, this was the case (Figure 2) and therefore the induced frameshifting can be explained as a low level of ternary complex that in turn causes the ribosome to stall and allow the peptidyl-$tRNA^{Pro}_{cmo^5UGG}$ to slip into the +1 frame. The $C_{10}H_{17}$ fragment, which we suggest is bound to the s^2-group of mnm^5s^2U, can of course, directly influence the anticodon–codon interaction, provided that a ternary complex is formed containing the $C_{10}H_{17}$-modified tRNA. The s^2-group of mnm^5s^2U34 negates the bifurcated hydrogen bonding to a G as third nucleoside in the codon. It further improves the stacking with U35 of the anticodon, which results in an efficient reading of the A-ending codon (Murphy *et al*, 2004). One can envision that a large bulky substituent, such as the $C_{10}H_{17}$-fragment, at the wobble nucleoside may reduce severely the entry of such a tRNA to the A-site. In fact, it is questionable if such a $C_{10}H_{17}$ containing tRNA ever enters the A-site. Alternatively, the $C_{10}H_{17}$-modified tRNA may never be charged resulting in a low level of aminoacylation, which causes stalling of the ribosome. Irrespective of what actually is the dominating cause of the induction of a +1 frameshift, both these alternatives are consistent with the model how altered modification of tRNA influences reading frame maintenance (Urbonavicius *et al*, 2001).

The SufY (YbbB) protein has in its C-terminal a helical region, which may contribute to binding of the anticodon of tRNA and a rhodanese domain in the N-terminal part (Figure 6). Moreover, a P loop (Walker motif), which is found in proteins that bind ATP or GTP, is present just downstream of the rhodanese domain. The YbbB homologs contain an insertion of about 36 amino acids that is present between the CH2A rhodanese motif and the active site of a rhodanese (Cys97) (Wolfe *et al*, 2004). This insertion is conserved among the YbbB homologs, and Gly67, which is substituted by glutamate in the *sufY204* mutant inducing frameshift and the synthesis of two not characterized modified nucleosides, is conserved within this region in nine YbbB orthologs. Since the wild-type form of SufY can interact with tRNA (Wolfe *et al*, 2004) and it contains a possible tRNA binding domain (Figure 6), it suggests that the G67E alteration influences a potential enzymatic activity of SufY rather than the tRNA binding activity. The fact that SufY may contain a tRNA binding motif and uses tRNA as substrate in a tRNA modification reaction (Wolfe *et al*, 2004), suggests that SufY is a tRNA modifying enzyme rather than a regulatory protein influencing the synthesis of an uncharacterized tRNA modifying enzyme.

The emergence of various modifications has most likely occurred via different routes. One would be that an enzyme, which catalyzes the synthesis of an intermediate in central or intermediary metabolism, is altered to have an affinity for a tRNA. The enzyme will still catalyze the same or similar enzymatic reaction(s) but, in addition, will now also be able to use a base in the tRNA as substrate. Alternatively, the enzyme may already have an affinity for tRNA, which SufY apparently has (Wolfe *et al*, 2004), the alteration may change the efficiency of a low intrinsic catalytic activity. Interestingly, the wild-type form of SufY is able to catalyze the formation of UKs, although at a very low efficiency (Figure 3). Perhaps, the glycine at position 67 (or the entire 36 amino-acid insertion found in all YbbB orthologs, Figure 6) dampens an inherent enzymatic activity, which is catalyzing the formation of $C_{10}H_{17}$-mnm^5s^2U34. An appropriate amino-acid change at this position would relieve this negative effect, allowing the protein to regain its ability to form $C_{10}H_{17}$-mnm^5s^2U34. This suggests that the observed novel modification may have been present earlier in the evolution of *S. enterica* and *E. coli*. Perhaps the advantage of $C_{10}H_{17}$-mnm^5s^2U34 in tRNA at this early time was overriding the disadvantage of its ability to induce frameshifting as shown by us. However, if, in some way, this evolutionary advantage was lost, any negative element that abolished the formation of $C_{10}H_{17}$-mnm^5s^2U34, thereby reducing errors in reading frame maintenance to a level acceptable for the organism, would be selected.

Materials and methods

Bacteria and growth conditions

All bacteria are derivatives of *S. enterica* serovar Typhimurium strain LT2 or *E. coli* (Supplementary Table II). Complex medium NAA was prepared from Difco nutrient broth (0.8%, Difco Laboratories, Detroit, MI) supplied with 0.5% NaCl, adenine, tryptophan, tyrosine, phenylalanine, and the three aromatic vitamins *p*-hydroxybenzoate, 2,3-dihydroxybenzoate and *p*-aminobenzoate. All supplements were added at concentrations recommended (Davis *et al*, 1980). For solid medium, TYS agar (10 g of Tryptone, 5 g of yeast extract, 15 g of NaCl and 15 g of agar per liter) was used. Minimal medium was prepared either from medium E (Vogel and Bonner, 1956) or MOPS (morpholinepropanesulfonic acid) medium (Neidhardt *et al*, 1974) supplemented with 0.4% carbon source. Rich MOPS medium was prepared as described before (Neidhardt *et al*, 1977). Antibiotics were used at the following concentrations: carbenicilin 100 μg/ml, chloramphenicol 12.5 μg/ml, tetracyclin 15 μg/ml, spectinomycin 800 μg/ml.

Genetic procedures

Transductions were performed using a derivative of phage P22 containing the mutations HT105/I (Schmieger, 1972) and *int*-201

©2005 European Molecular Biology Organization

(Scott *et al*, 1975). Transduction was performed as described (Davis *et al*, 1980).

Molecular cloning techniques

Standard procedures of molecular cloning were used. Chromosomal DNA prepared from strain GT5520 (*hisO1242, hisC3737, sufY204*::MudCam, *zbb-2523*::Tn*10dTet)* and from the vector plasmid pBR322 were digested with *Pst*I and *Sal*I and then ligated using T4 DNA ligase (Boehringer Mannheim). *E. coli* strain DH5α (*supE44, ΔlacU169 (ϕ80lacZΔM15) hsdR17, recA1, endA1, gyrA96, thi-1, relA1*) was used in transformations. Plasmid preparation kit was from Qiagen, and to PCR amplify a gene of interest, puReTaq-Ready-to-go PCR beads (Amersham Pharmacia, Piscataway, NJ) were used. PCR fragments were purified using PCR Kleen Spin columns (Bio-Rad) for direct recovery of the PCR product. Alternatively, the Wizard DNA clean-up Resin (Promega, Madison, WI) was used for purifying PCR fragments from agarose gels. Automatic sequencing was performed using DYEnamic ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech Inc., Cleveland, OH).

Determination of β-galactosidase activity

Strains were grown at 37°C in NAA medium supplemented with 50 μg/ml carbenicilin overnight for selection of pTHF plasmids. Strains were subcultured to mid-log phase, β-galactosidase activity was measured as described by Miller (1972). ONPG was from Sigma (St Louis, MO). For each strain to be tested, three independent cultures were grown.

HPLC analysis of modified nucleosides

Strains were grown in LB medium at 37°C from 5 Klett units to 100 Klett units (approx. 4×10^8 cells/ml). Total tRNA was prepared by phenol-extraction in acidic conditions and purified through a Nucleobond column (Clontech) as described elsewhere (Urbonavicius *et al*, 2001; Björk and Nilsson, 2003). Total tRNA (100 μg) was digested with nuclease P1 (Boehringer Mannheim) followed by alkaline phosphatase (Sigma) treatment as described (Gehrke *et al*, 1982). The hydrolysates (and authentic cmnm^{5}s^{2}U) were analyzed by HPLC using a Supelcosil LC18 column (Supelco) with a Waters HPLC system. The gradient used was as described by Gehrke and Kuo (1989).

Tandem mass spectrometry (MS/MS) of dinucleotide UK2

Electrospray ionization LC/MS/MS was used for structure studies of the isolated dinucleotide. The data shown in Figure 4 were acquired on a Quattro II (Micromass) triple quadrupole mass spectrometer interfaced with an HP 1090 liquid chromatograph. The HPLC gradient was essentially as described (Pomerantz and McCloskey, 1990) except that the ammonium acetate concentration and solvent flow rate were reduced to 5 mM and 200 μl/min, respectively. The collision energy was 20 eV. The ion source and desolvation temperatures were 140 and 280°C, respectively.

Isolation of $tRNA^{Gln}_{cmnm^5s^2UUG}$

To isolate single tRNA species ($tRNA^{Gln}_{cmnm^5s^2UUG}$), Dynabead (Dynal, Oslo, Norway) was used. A biotinylated DNA oligo complementary to nucleotides 39–73 (35 nucleotides) of $tRNA^{Gln}_{cmnm^5s^2UUG}$ was bound to the Dynabeads and $tRNA^{Gln}_{cmnm^5s^2UUG}$ was hybridized to the Dynabeads in 2.4 M tetraethylammonium chloride (Tsurui *et al*, 1994). Purified $tRNA^{Gln}_{cmnm^5s^2UUG}$ was desalted and concentrated using Centricon YM-3 cartridges (Millipore, Bedford, MA) three times with 2 ml MilliQ water. In all, 5–10 μg specific tRNA was digested with nuclease P1 and bacterial alkaline phosphatase, and modified nucleosides were analyzed by HPLC as described above.

Tentative identification of the wobble nucleoside in $tRNA^{Gln}_{cmnm^5s^2UUG}$ *as cmnm*^{5}*s*^{2}*U*

The wobble nucleoside in gln1 tRNA, which reads CAA and CAG, is a derivative of mnm^{5}s^{2}U34. To investigate whether it may be the cmnm^{5}s^{2}U derivative, we first determined the relative retention time of a synthetic cmnm^{5}s^{2}U to A (0.345) and to U (1.387) as compared to mnm^{5}s^{2}U, which migrated slightly different relative to A (0.369) and U (1.481). In purified gln1 tRNA, a compound with the same UV spectrum as mnm^{5}s^{2}U migrated similarly as synthetic cmnm^{5}s^{2}U relative to A and U. A minor peak (20 % of the compound suggested to be cmnm^{5}s^{2}U) was also observed with migration properties similar to mnm^{5}s^{2}U. Thus, we tentatively conclude that under the conditions used to purify gln1 tRNA, 80% of the wobble nucleoside in this tRNA is cmnm^{5}s^{2}U and about 20% is mnm^{5}s^{2}U. Although this analysis suggests that the wobble nucleoside is cmnm^{5}s^{2}U in gln1 tRNA, we did not spike the purified gln1 tRNA with the marker, nor have we determined its relative molecular mass (M_r). Therefore, it cannot be ruled out that the compound in gln1 tRNA is not cmnm^{5}s^{2}U but a compound with the same spectrum and the same relative retention time as cmnm^{5}s^{2}U. Nonetheless, we use the nomenclature $tRNA^{Gln}_{cmnm^5s^2UUG}$ to indicate the gln1 tRNA.

Analysis of the aminoacylation level of $tRNA^{Gln}_{cmnm^5s^2UUG}$

Cells were grown at 37°C to a cell density of 2×10^8 cells/ml in 100 ml LB medium. All subsequent steps were carried out in the cold. Total RNA was prepared under acidic conditions and electrophoresis of RNA was as described (Varshney *et al*, 1991) *except* that 8% denaturing polyacrylamide gel was used. The portion of the gel containing tRNAs of interest (which migrate at the same position as xylene cyanol under this condition) was electroblotted using a semidry electroblot apparatus (10 V for 30 min, 1 × TBE as transfer buffer) onto a Zeta-Probe membrane (Bio-Rad). After transfer, the membrane was rinsed briefly in 2 × SSC, and tRNA was crosslinked to the membrane using a UV Stratalinker (Stratagene). The oligonucleotide used to detect $tRNA^{Gln}_{cmnm^5s^2UUG}$ was complementary to nucleotides 25–50 in the $tRNA^{Gln}_{cmnm^5s^2UUG}$. The oligonucleotide was labeled using adenosine 5′[γ-^{32}P]-triphosphate (5000 Ci/mmol, Amersham) and polynucleotide kinase (Roche Molecular Biochemicals). The membrane was prehybridized in Church buffer (0.5 M Na_2HPO_4, 1 mM EDTA, 1% BSA, 7% SDS, pH 7.2) at 70°C for 30 min, hybridized with ^{32}P-labeled oligo at 70°C for 30 min, and cooled slowly to room temperature. The membrane was washed three times with 6 × SSC, 0.1% SDS at 42°C for 5 min, and visualized and quantified by Phosphor-Imager analysis using ImageQuant software (Molecular Dynamics).

Deletion of the sufY and the selD genes by linear transformation

The method used has been described earlier (Datsenko and Wanner, 2000). Plasmid pKD4 was used as template plasmid. The 1.6 kb PCR fragment carrying the Kan^R cassette was electroporated into strain GT6315 (pKD46/LT2) and Kan^R clones were selected. The Kan^R cassette was then removed by recombination at FRT sites present on both sides of the cassette, thus the resulting strain carried a ‘scar’ replacing the *sufY* or *selD* genes. Such replacements are denoted *sufY*⟨⟩*frt* and *selD*⟨⟩*frt*, respectively. The primers for replacing *sufY* gene were designed to create a replacement of the *sufY* gene from 25nt upstream of the start codon to 2nt downstream of the stop codon. The primers for replacing *selD* were designed to create a replacement of the *selD* gene from 46nt upstream of the start codon to 23nt downstream of the stop codon (Supplementary data).

Site-directed mutagenesis of sufY

A QuickChange^{TM} Site-Directed Mutagenesis Kit (Stratagene) was used to change Cys97 to Ala97; plasmid pUST211 (carrying *sufY204* allele) was used as template.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Kerstin Jacobsson for HPLC analysis and for purification of UKs for mass spectrometry analyses. We are grateful for the generous gift of the strain containing the original *sufC* mutation from John Roth, University of California at Davis, California, USA and to Tord Hagervall for the plasmids pTHF40–43. Norma M Wills and John F Atkins are acknowledged for providing us with the vector pGHM57 and with the information on the assay system. The synthetic markers cmnm^{5}s^{2}U and mnm^{5}s^{2}U were kind gifts from Andrzej Malkiewicz, Technical University, Poland. We thank Tord Hagervall and Anders Byström (Umeå) and James McCloskey (Utah) for the critical reading of the manuscript. This work was supported by grants from the Swedish Cancer Foundation (Project 680) and Swedish Science Research council (Project BU-2930), and by NIH Grant GM29812.

©2005 European Molecular Biology Organization

References

Björk GR (1980) A novel link between the biosynthesis of aromatic amino acids and transfer RNA modification in *Escherichia coli*. *J Mol Biol* **140:** 391–410

Björk GR (1986) Transfer RNA modification in different organisms. *Chemica Scripta* **26B:** 91–95

Björk GR, Hagervall TG (2005) Transfer RNA modification. In *Escherichia coli and Salmonella Cellular and Molecular Biology*, Curtiss III R, Ingraham JL, Kaper JB, Maloy S, Neidhardt FC, Riley MM, Squires CL, Wanner BL (eds). Washington, DC: American Society for Microbiology. http://www.ecosal.org/ecosal/modules/index.jsp

Björk GR, Nilsson K (2003) 1-Methylguanosine-deficient tRNA of *Salmonella enterica* serovar Typhimurium affects thiamine metabolism. *J Bacteriol* **185:** 750–759

Björk GR, Wikström PM, Byström AS (1989) Prevention of translational frameshifting by the modified nucleoside 1-methylguanosine. *Science* **244:** 986–989

Cermakian N, Cedergren R (1998) Modified nucleosides always were: an evolutionary model. In *Modification and Editing of RNA*, Grosjean H, Benne R (eds), pp 535–541. Washington DC: ASM Press

Crain PF (1990) Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry. *Methods Enzymol* **193:** 782–790

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645

Davis W, Botstein D, Roth JR (1980) *A Manual for Genetic Engineering: Advanced Bacterial Genetics*. New York: Cold Spring Harbor Laboratory

Di Giulio M (1998) Reflections on the origin of the genetic code: a hypothesis. *J Theor Biol* **191:** 191–196

Edmonds CG, Pomerantz SC, Hsu FF, McCloskey JA (1988) Thermospray liquid chromatography/mass spectrometry in deuterium oxide. *Anal Chem* **60:** 2314–2317

Elseviers D, Petrullo LA, Gallagher PJ (1984) Novel *E. coli* mutants deficient in biosynthesis of 5-methylaminomethyl-2-thiouridine. *Nucleic Acids Res* **12:** 3521–3534

Farabaugh PJ (1996) Programmed translational frameshifting [review]. *Microbiol Rev* **60:** 103–134

Farabaugh PJ, Björk GR (1999) How translational accuracy influences reading frame maintenance. *EMBO J* **18:** 1427–1434

Gehrke CW, Kuo KC (1989) Ribonucleoside analysis by reversed-phase high-performance liquid chromatography. *J Chromatogr* **471:** 3–36

Gehrke CW, Kuo KC, McCune RA, Gerhardt KO, Agris PF (1982) Quantitative enzymatic hydrolysis of tRNAs: reversed-phase high-performance liquid chromatography of tRNA nucleosides. *J Chromatogr* **230:** 297–308

Hagervall TG, Edmonds CG, McCloskey JA, Björk GR (1987) Transfer RNA(5-methylaminomethyl-2-thiouridine)-methyltransferase from *Escherichia coli* K-12 has two enzymatic activities. *J Biol Chem* **262:** 8488–8495

Hagervall TG, Tuohy TM, Atkins JF, Björk GR (1993) Deficiency of 1-methylguanosine in tRNA from *Salmonella typhimurium* induces frameshifting by quadruplet translocation. *J Mol Biol* **232:** 756–765

Hansen TM, Baranov PV, Ivanov IP, Gesteland RF, Atkins JF (2003) Maintenance of the correct open reading frame by the ribosome. *EMBO Rep* **4:** 499–504

Herr AJ, Nelson CC, Wills NM, Gesteland RF, Atkins JF (2001) Analysis of the roles of tRNA structure, ribosomal protein L9, and the bacteriophage T4 gene 60 bypassing signals during ribosome slippage on mRNA. *J Mol Biol* **309:** 1029–1048

Hopfield JJ (1978) Origin of the genetic code: a testable hypothesis based on tRNA structure, sequence, and kinetic proofreading. *Proc Natl Acad Sci USA* **75:** 4334–4338

Ibba M, Hong K-W, Soll D (1996) Glutaminyl-tRNA synthetase: from genetics to molecular recognition. *Genes Cells* **1:** 421–427

Kramer GF, Ames BN (1988) Isolation and characterization of a selenium metabolism mutant of *Salmonella typhimurium*. *J Bacteriol* **170:** 736–743

Kurland CG, Hughes D, Ehrenberg M (1996) Limitations of translation accuracy. In *Escherichia coli and Salmonella. Cellular and Molecular Biology*, Neidhardt FC, Curtiss III R, Ingraham JL, Lin ECC, Brooks Low K, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (eds), pp 979–1004. Washington, DC: American Society for Microbiology

Miller JH (1972) *Experiments in Molecular Genetics*. New York: Cold Spring Harbor Laboratory

Murphy FV, Ramakrishnan V, Malkiewicz A, Agris PF (2004) The role of modifications in codon discrimination by tRNA(Lys)(UUU). *Nat Struct Biol* **11:** 1186–1191

Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. *J Bacteriol* **119:** 736–747

Neidhardt FC, Bloch PL, Pedersen S, Reeh S (1977) Chemical measurement of steady-state levels of ten aminoacyl-transfer ribonucleic acid synthetases in *Escherichia coli*. *J Bacteriol* **129:** 378–387

Phillips DR, McCloskey JA (1993) A comprehensive study of the low energy collision-induced dissociation of dinucleoside monophosphates. *Int J Mass Spectrom Ion Processes* **128:** 61–82

Pomerantz SC, McCloskey JA (1990) Analysis of RNA hydrolyzates by liquid chromatography-mass spectrometry. *Methods Enzymol* **193:** 796–824

Qian Q, Li JN, Zhao H, Hagervall TG, Farabaugh PJ, Björk GR (1998) A new model for phenotypic suppression of frameshift mutations by mutant tRNAs. *Mol Cell* **1:** 471–482

Riddle DL, Roth JR (1970) Suppressors of frameshift mutations in *Salmonella typhimurium*. *J Mol Biol* **54:** 131–144

Roth JR (1981) Frameshift suppression. *Cell* **24:** 601–602

Rozenski J, Crain PF, McCloskey JA (1999) The RNA modification database: 1999 update. *Nucleic Acids Res* **27:** 196–197

Schmieger H (1972) Phage *P22*-mutants with increased or decreased transduction abilities. *Mol Gen Genet* **119:** 75–88

Scott JF, Roth JR, Artz SW (1975) Regulation of histidine operon does not require hisG enzyme. *Proc Natl Acad Sci USA* **72:** 5021–5025

Sprinzl M, Vassilenko KS (2003) tRNA sequences and tRNA genes database http://www.uni-bayreuth.de/departments/biochimie/sprinzl/trna/

Sroga GE, Nemoto F, Kuchino Y, Björk GR (1992) Insertion (*sufB*) in the anticodon loop or base substitution (*sufC*) in the anticodon stem of $tRNA^{Pro2}$ from *Salmonella typhimurium* induces suppression of frameshift mutations. *Nucleic Acids Res* **20:** 3463–3469

Stadtman TC, Davis JN, Zehelein E, Böck A (1989) Biochemical and genetic analysis of *Salmonella typhimurium* and *Escherichia coli* mutants defective in specific incorporation of selenium into formate dehydrogenase and tRNAs. *Biofactors* **2:** 35–44

Sullivan MA, Cannon JF, Webb FH, Bock RM (1985) Antisuppressor mutation in *Escherichia coli* defective in biosynthesis of 5-methylaminomethyl-2-thiouridine. *J Bacteriol* **161:** 368–376

Tsurui H, Kumazawa Y, Sanokawa R, Watanabe Y, Kuroda T, Wada A, Watanabe K, Shirai T (1994) Batchwise purification of specific tRNAs by a solid-phase DNA probe. *Anal Biochem* **221:** 166–172

Urbonavicius J, Qian Q, Durand JM, Hagervall TG, Björk GR (2001) Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J* **20:** 4863–4873

Varshney U, Lee CP, RajBhandary UL (1991) Direct analysis of aminoacylation levels of tRNAs *in vivo*. Application to studying recognition of *Escherichia coli* initiator tRNA mutants by glutaminyl-tRNA synthetase. *J Biol Chem* **266:** 24712–24718

Veres Z, Kim IY, Scholz TD, Stadtman TC (1994) Selenophosphate synthetase—enzyme properties and catalytic reaction. *J Biol Chem* **269:** 10597–10603

Vogel HJ, Bonner DM (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J Biol Chem* **218:** 97–106

Wolfe MD, Ahmed F, Lacourciere GM, Lauhon CT, Stadtman TC, Larson TJ (2004) Functional diversity of the rhodanese homology domain: the *Escherichia coli* ybbB gene encodes a selenophosphate-dependent tRNA 2-selenouridine synthase. *J Biol Chem* **279:** 1801–1809

Yokoyama S, Watanabe T, Murao K, Ishikura H, Yamaizumi Z, Nishimura S, Miyazawa T (1985) Molecular mechanism of codon recognition by tRNA species with modified uridine in the first position of the anticodon. *Proc Natl Acad Sci USA* **82:** 4905–4909

©2005 European Molecular Biology Organization